# การศึกษาลักษณะทางพันธุกรรมของยีนเอสวันของไวรัสหลอดลมอักเสบติดต่อที่แยกได้จากไก่ในประเทศไทยระหว่างปีพ.ศ. 2557-2559

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# GENETIC CHARACTERIZATION OF S1 GENE OF INFECTIOUS BRONCHITIS VIRUS ISOLATED FROM CHICKENS IN THAILAND DURING 2014-2016

Miss Sirorat Munyahongse

A Thesis Submitted in Partial Fulfillment of the Requirements
for the Degree of Master of Science Program in Veterinary Pathobiology
Department of Veterinary Pathology
Faculty of Veterinary Science
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โรคหลอดลมอักเสบติดต่อ เกิดจากเชื้อไวรัสหลอดลมอักเสบติดต่อ เป็น โรคติดเชื้อไวรัสที่ระบบทางเดินหายใจที่สำคัญในไก่ โรคนี้จัดเป็นโรคที่ทำให้เกิดความสูญเสียทางเศรษฐกิจในอุตสาหกรรมการเลี้ยงไก่ทั่วโลก เนื่องจากเชื้อไวรัสหลอดลมอักเสบติดต่อสามารถเกิดการกลายพันธุ์ (mutation) และเกิดการแลกเปลี่ยนลำดับพันธุกรรม (recombination) ของสายพันธุกรรมได้อย่างรวดเร็ว และบ่อยครั้ง โดยเฉพาะอย่างยิ่งในส่วนยีน S1 มีผลทำให้เกิดการอุบัติของเชื้อไวรัสหลอดลมอักเสบติดต่อสายพันธุ์ใหม่ขึ้นมาอย่างต่อเนื่อง และเป็นจำนวนมาก ซึ่งมักส่งผลกระทบต่อประสิทธิภาพของวัคซีนป้องกันโรคหลอดลมอักเสบติดต่อที่ใช้อยู่ในปัจจุบัน ดังนั้นการศึกษาติดตามลักษณะทางพันธุกรรมของเชื้อไวรัสหลอดลมอักเสบติดต่อที่ระบาดอยู่ในปัจจุบันจึงมีความสำคัญเป็นอย่างยิ่ง การศึกษาวิจัยครั้งนี้เป็นการศึกษาลักษณะทางพันธุกรรมของเชื้อไวรัสหลอดลมอักเสบติดต่อที่ระบาดในไก่ในประเทศไทยระหว่างปี พ.ศ. 2557-2559 โดยวิเคราะห์จากยีน S1 จากผลการศึกษาพบตัวอย่างที่ให้ผลบวกต่อเชื้อไวรัสหลอดลมอักเสบติดต่อด้วยวิธี RT-PCR ที่จำเพาะต่อยีน S1 จำนวนทั้งหมด 629 ตัวอย่าง คิดเป็นร้อยละ 37.7 จากตัวอย่างที่เก็บจากทุกภูมิภาคที่มีการเลี้ยงไก่ในประเทศไทยจำนวนทั้งหมด 1,668 ตัวอย่าง ผลการศึกษานี้แสดงให้เห็นว่าเชื้อไวรัสหลอดลมอักเสบติดต่อมีการกระจายตัวเป็นวงกว้างในทุกภูมิภาคที่มีการเลี้ยงไก่ในประเทศไทย นอกจากนี้ได้ทำการสุ่มเลือกเชื้อไวรัสหลอดลมอักเสบติดต่อมาทำการหาลำดับพันธุกรรม และวิเคราะห์ลักษณะทางพันธุกรรมของยีน S1 จำนวน 150 ตัวอย่างผลการศึกษาลักษณะทางพันธุกรรมของยีน S1 แสดงให้เห็นว่าเชื้อไวรัสหลอดลมอักเสบติดต่อในประเทศไทยที่ระบาดระหว่างปี พ.ศ. 2557-2559 มีทั้งหมด 5 จีโนไทป์ ได้แก่ QX-like IBV (จำนวน 129 ตัวอย่าง), Massachusetts (จำนวน 12 ตัวอย่าง), 4/91 (จำนวน 5 ตัวอย่าง), Connecticut (จำนวน 2 ตัวอย่าง) และ novel IBV genotype (จำนวน 2 ตัวอย่าง) จากผลการศึกษานี้แสดงให้เห็นว่าเชื้อไวรัสหลอดลมอักเสบติดต่อสายพันธุ์ QX-like จัดเป็นจีโนไทป์หลักที่มีการระบาดในฝูงไก่ในประเทศไทยในปัจจุบัน นอกจากนี้จากผลการวิเคราะห์การแลกเปลี่ยนลำดับพันธุกรรมของเชื้อไวรัสหลอดลมอักเสบติดต่อที่มีการระบาดในระหว่างปี พ.ศ. 2557-2559 พบการอุบัติของเชื้อไวรัสหลอดลมอักเสบติดต่อชนิดรีคอมบิแนนท์ จีโนไทป์ใหม่ ที่เกิดมาจากการแลกเปลี่ยนลำดับพันธุกรรมระหว่างเชื้อไวรัสหลอดลมอักเสบติดต่อสายพันธุ์ QX-like ที่ระบาดในประเทศไทย และเชื้อไวรัสหลอดลมอักเสบติดต่อสายพันธุ์ 4/91 จากผลการศึกษานี้โดยรวมพบว่าลักษณะทางพันธุกรรมของเชื้อไวรัสหลอดลมอักเสบติดต่อที่ระบาดในไก่ในประเทศไทยมีการเปลี่ยนแปลงไปเมื่อเปรียบเทียบกับการศึกษาก่อนหน้าแสดงให้เห็นว่าเชื้อไวรัสหลอดลมอักเสบติดต่อที่ระบาดในไก่ในประเทศไทยมีการปรับตัวและวิวัฒนาการอย่างต่อเนื่อง ดังนั้นการศึกษานี้ชี้ให้เห็นถึงความสำคัญของการตรวจติดตามลักษณะทางพันธุกรรมของเชื้อไวรัสหลอดลมอักเสบติดต่ออย่างต่อเนื่อง เพื่อให้สามารถควบคุมและป้องกันการแพร่กระจายของเชื้อไวรัสหลอดลมอักเสบติดต่อได้อย่างรวดเร็ว และมีประสิทธิภาพ

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# # 5875322631 : MAJOR VETERINARY PATHOBIOLOGY

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SIRORAT MUNYAHONGSE: GENETIC CHARACTERIZATION OF S1 GENE OF INFECTIOUS BRONCHITIS VIRUS ISOLATED FROM CHICKENS IN THAILAND DURING 2014-2016. ADVISOR: ASST. PROF. AUNYARATANA THONTIRAVONG, D.V.M., M.Sc., Ph.D., CO-ADVISOR: PROF. JIROJ SASIPREEYAJAN, D.V.M., Ph.D., 69 pp.

Infectious bronchitis (IB), caused by infectious bronchitis virus (IBV), is a highly contagious respiratory disease in chickens, causing significant economic losses in poultry industry worldwide. IBV has a high frequency of genetic mutation and recombination in its genome, particularly in the S1 gene, resulting in the emergence of several new IBV variants. This potentially affects the effectiveness of currently used IBV vaccine. Therefore, the continuous monitoring of the currently circulating IBV strains is essential. In this study, the genetic characteristic of IBV strains circulating in Thai chicken flocks during 2014-2016 was investigated by analysis of the complete S1 gene. A total of 629 (37.7%) out of 1,668 samples, collected from all chicken-raising regions of Thailand, was positive for IBV by S1-specific RT-PCR. The results showed that IBV was widely distributed in all chicken-raising regions of Thailand. Among the IBV positive samples collected in this study, 150 IBVs were randomly selected for complete S1 gene sequencing and analysis. The results based on the phylogenetic analysis of the complete S1 gene revealed that the 2014-2016 Thai IBVs characterized in this study were clustered into five genotypes, including QX-like IBV (n=129), Massachusetts (n=12), 4/91 (n=5), Connecticut (n=2) and a novel IBV genotype (n=2). The results demonstrated that QX-like IBV has become the predominant genotype currently circulating in Thailand. Recombination analysis of the S1 gene of the 2014-2016 Thai IBVs showed the emergence of a novel recombinant IBV genotype originating from Thai QX-like IBV and 4/91 vaccine strain, which was first identified in this study. Overall, the results from this study indicate that the genetic characteristic of Thai IBVs has changed when compared to the characteristic described in previous reports, suggesting the continuing evolution of IBV in Thailand. Therefore, this study highlights the importance of continuous IBV surveillance in chickens for effective control and prevention of IB.

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# CHAPTER 1

## Introduction

Infectious bronchitis (IB), caused by infectious bronchitis virus (IBV), is a highly contagious respiratory disease in chickens, causing significant economic losses in poultry industry worldwide. The disease is characterized by upper respiratory signs, decrease in egg production and quality, and occasional nephritis (Valastro et al., 2016). IBV is transmitted among chickens though inhalation and ingestion of infected respiratory secretion or feces (De Wit et al., 1998). IBV primarily infects the epithelium of the upper respiratory tract, which is followed by deciliation of the ciliated epithelial cells in trachea. IBV infected chickens show respiratory signs, including gasping, coughing, tracheal rales, nasal and ocular discharge (Hodgson et al., 2004). Although, the primary target site of IBV infection is the upper respiratory tract, the virus can also replicate in the urogenital, reproductive, gastrointestinal tracts and the lymphoid organs of infected chickens (Abdel-Moneim et al., 2009; De Wit et al., 2011b). Some strains of IBV, nephropathogenic strains, can cause severe renal damage, resulting in high mortality rate in young chickens. Infected chickens usually showed signs of severe depression and wet dropping (Ignjatovic et al., 2002). In addition, the reproductive system of adult chickens can also be infected with some strains of IBV, causing egg production drop and decrease in egg quality, including watery albumin and thin or rough shell. When IBV infects the reproductive system of young chickens, the virus can cause permanent lesions in the reproductive organs, resulting in false layers (Hong et al., 2012; Awad et al., 2016). Morbidity rate of chickens infected with IBV is close to 100%, while the mortality rate varies depending on the age of chickens, IBV strains, co-infection with other pathogens and secondary bacterial infection (Cavanagh and Gelb, 2008).

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IBV is an enveloped, single-stranded, positive sense RNA virus in the genus *Gammacoronavirus* of the family *Coronaviridae*. IBV genome encodes four structural proteins, including nucleocapsid (N), membrane (M), envelope (E) and spike (S) proteins (Lai and Cavanagh, 1997). Spike (S) glycoprotein is a large surface protein and is responsible for host cell receptor binding and fusion of the viral envelope with the cellular membrane. Spike glycoprotein is cleaved into two subunits, which are the S1 subunit containing receptor binding site that is responsible for viral attachment to the host cell and the S2 subunit that is responsible for virus and host cell membrane fusion (Cavanagh, 1995; Jackwood et al., 2001). Among the IBV genes, the S1 gene has been shown to have the highest variability since it involves in host cell attachment and contains virus-neutralizing and serotype-specific epitopes (Kant et al., 1992). Genetic mutation and recombination frequently occur in the hypervariable regions of the S1 subunit, leading to the emergence of several new IBV variants (Fellahi et al., 2015; Xu et al., 2016). Therefore, the genotypes of IBV are usually classified based on the genetic variation of the S1 subunit (Lee et al., 2003; Valastro et al., 2016).

Currently, several new IBV variants have been continuously emerging throughout the world due to the high frequency of mutation and recombination in the S1 gene, causing vaccination breaks (Thor et al., 2011a). It is well documented that different IBV genotypes and vaccine strains mostly do not cross-neutralize, leading to outbreaks of IB in vaccinated chicken flocks and making this virus is very difficult to control (Jackwood et al., 2012). The most current effective IBV control is identification of virus genotypes causing disease in the particular area followed by vaccination with matching vaccine strains against the circulating genotypes (Zhao et al., 2015). Therefore, the continued monitoring and updating of IBV genotypes in chicken flocks worldwide are essential for the development of the effective control and prevention strategies of IB.

To date, IBV is extensively distributed worldwide, and different countries have their own IBV variants and common genotypes (Valastro et al., 2016). In Thailand, five

IBV genotypes: a unique Thai IBV (THA50151), THA001, QX-like IBV, Massachusetts and 4/91, were reported to circulate in chickens during 2008-2013 (Pohuang et al., 2011; Promkuntod et al., 2015). Despite extensive vaccination with various IBV vaccine strains in Thailand, IB outbreaks have continuously occurred in Thai chicken flocks, possibly due to the emergence of new IBV variants. However, the genetic characteristic of IBV strains currently circulating in Thai chicken flocks is unknown. Therefore, this study aims to investigate the occurrence and genetic characteristic of IBV strains circulating in chicken flocks in all chicken-raising regions of Thailand during 2014- 2016 by analysis of the complete S1 gene.

**Hypothesis**

Several IBV genotypes can be detected in chickens in Thailand during 2014-2016.

**Expected benefits**

1. To gain the information on the occurrence and genetic characteristic and diversity of infectious bronchitis viruses isolated from chickens in Thailand during 2014-2016.
2. To set up and update the genome database of Thai infectious bronchitis viruses.
3. To obtain the current status of infectious bronchitis virus infection in chickens in Thailand for effective planning of the control and prevention strategies of infectious bronchitis.

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## Conceptual framework

The conceptual framework of this study is shown in Figure 1.

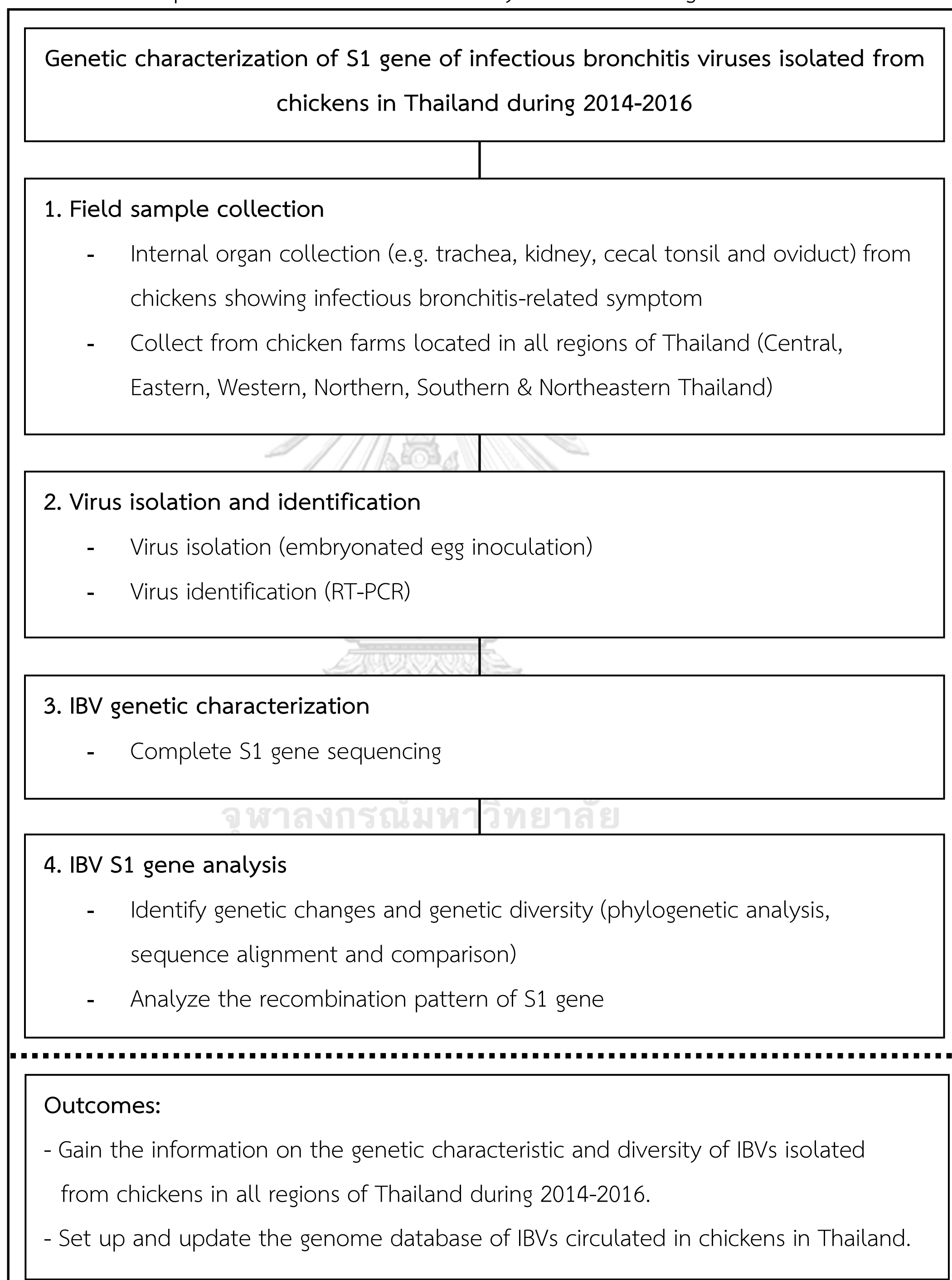


Figure 1 Conceptual framework of this study

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# CHAPTER 2

## Objective

To determine the occurrence and the genetic characteristic of IBVs isolated from chickens in Thailand during 2014-2016 by analysis of S1 gene.

# CHAPTER 3

# Literature review

Infectious bronchitis (IB)

Infectious bronchitis (IB), caused by infectious bronchitis virus (IBV), is considered to be one of the most significant respiratory diseases of chickens, causing major economic losses in poultry industry worldwide. The disease is characterized by upper respiratory signs, decrease in egg production and quality and occasional nephritis (Valastro et al., 2016). Clinical signs of IBV infected chickens include depressed, ruffled feathers, increased water intake, reduced weight gain and respiratory signs. In addition, chickens infected with nephropathogenic strains show signs of severe depression and wet dropping (Ignjatovic et al., 2002). Some strains of IBV can infect the reproductive system, resulting in egg production drop, decrease in egg quality and false layers due to damage of the reproductive organs (De Wit et al., 2011c; Hong et al., 2012). Morbidity rate of chickens infected with IBV is close to 100%, while the mortality rate varies depending on the age of chickens, IBV strains, co-infection with other pathogens and secondary bacterial infection (Cavanagh and Gelb, 2008).

**Infectious bronchitis virus (IBV)**

1. IBV classification and structure

IBV is an enveloped, single-stranded, positive sense RNA virus in the genus *Gammacoronavirus* of the family *Coronaviridae*, subfamily *Coronavirinae*, within the order Nidovirales (Table 1). Among four genera of subfamily *Coronavirinae*, the gammacoronaviruses mostly infect and cause diseases in avian species (Woo et al., 2012). IBV, a gammacoronavirus, causes a highly contagious respiratory disease with subsequent economic losses predominantly in chickens. However, IBV has also been

detected in a variety of avian species, including peafowl, partridge, blue-winged teal, pigeon, geese and ducks (Yao et al., 2016). IBV particle has a pleomorphic morphology, with diameter ranging from 70 to 120 nm and is surrounded by host-derived lipid envelope harboring spike club-shaped glycoproteins (Lai and Cavanagh, 1997). The core virus particle comprises the ribonucleocapsid, consisting of viral RNA and nucleocapsid (N) protein, which is encapsidated by the structural proteins, including envelope (E), membrane (M) and spike (S) proteins (Belouzard et al., 2012) (Figure 2).

Table 1 Coronavirus genera and species

(Woo et al., 2009; Ma et al., 2015)

| Genus | Species |
|---|---|
| **Alphacoronavirus** | Feline coronavirus (FCoV) |
| | Canine coronavirus (CCoV) |
| | Transmissible gastroenteritis virus (TGEV) |
| | Porcine Epidemic Diarrhea Coronavirus (PEDV) |
| **Betacoronavirus** | Bovine coronavirus (BCoV) |
| | Equine coronavirus (ECoV) |
| | Porcine haemagglutinating encephalomyelitis virus (PHEV) |
| | Canine respiratory coronavirus (CrCoV) |
| | Severe acute respiratory syndrome coronavirus (SARS-CoV) |
| **Gammacoronavirus** | Avian coronavirus : |
| | Infectious bronchitis virus (IBV) |
| | **Turkey enteric coronavirus (TCoV)** |
| | Beluga Whale coronavirus |
| **Delta-coronavirus** | Bulbul coronavirus |
| | Thrush coronavirus |
| | Munia coronavirus |
| | Porcine deltacoronavirus (PdCOV) |

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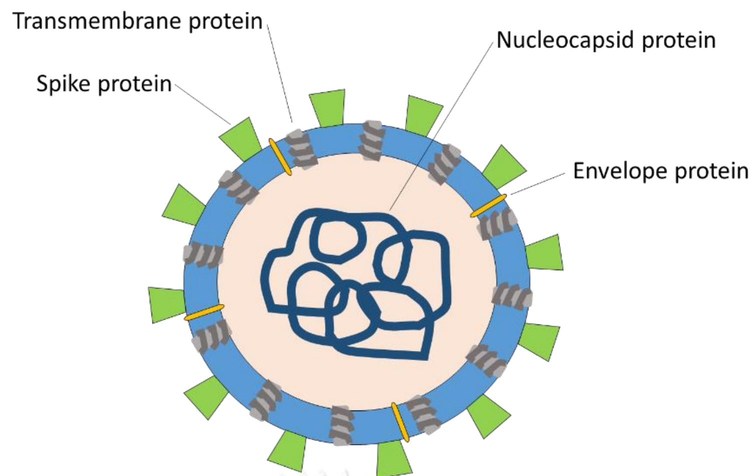


Figure 2 Diagram of coronavirus.

The virus particle consists of the viral envelope, where the spike glycoprotein is embedded, and the ribonucleocapsid (viral RNA and nucleocapsid protein) (Belouzard et al., 2012).

2. IBV genome component and function

IBV genome encodes 4 structural proteins serving as the vial membrane spike (S), integral membrane (M), small envelope (E) and nucleocapsid (N) proteins (Figure 2) (Lai and Cavanagh, 1997). The detailed functions of IBV proteins are given in Table 2. The size of IBV genome is approximately 27.6 kilobase in length (Montassier, 2010). Genome organization of IBV is shown in Figure 3. S protein is a large surface protein and is responsible for host cell receptor binding and the release of viral RNAs through membrane fusion. Moreover, S protein is the most highly variable protein of IBV as it contains virus-neutralizing and serotype-specific epitopes and is considered to be the major antigenic determinants of the host immune response (Cavanagh, 1995; Jackwood et al., 2001). M protein facilitates virus assembly through interaction with viral ribonucleocapsid and spike protein (Narayanan et al., 2000). E protein is associated with envelope formation, assembly, budding and ion channel activities (Wilson et al., 2006; Venkatagopalan et al., 2015). Like other coronaviruses, N protein, binding to the viral genome, is highly conserved among coronaviruses and play a critical role in virus replication, particularly in RNA replication, transcription and viral genome packaging

steps (McBride et al., 2014). Besides the structure proteins, IBV genome also encodes several non-structural and accessory proteins, which are mostly responsible for viral RNA replication and protein synthesis (Cavanagh, 2007).

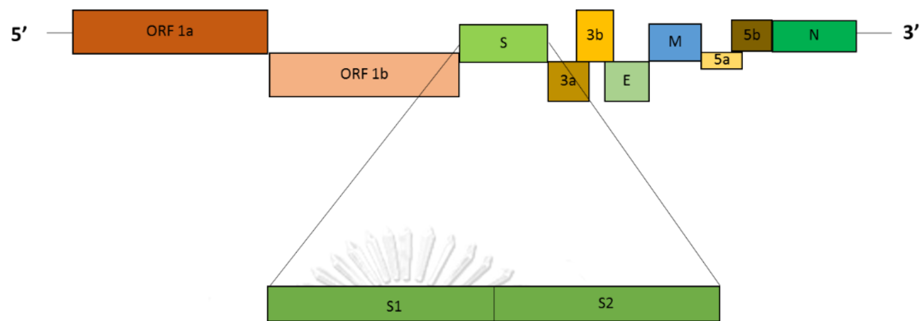


Figure 3 Genome organization of IBV
(Umar et al., 2016)

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Table 2 The functions of the IBV proteins
(Montassier, 2010; Umar et al., 2016; Xu et al., 2016)

| Gene | Size (nucleotides) | Protein | Function |
|---|---|---|---|
| **ORF 1 gene**<br>• ORF 1a<br>• ORF 1b | 19,904 | Nonstructural protein (nsp2-16) | The viral RNA-dependent RNA polymerase |
| **S gene** | 3,498 | S protein (spike glycoprotein) | Mediates host cell attachment, virus and cell membrane fusion and entry into the host cell |
| **ORF 3 gene**<br>• ORF 3a<br>• ORF 3b | <br>174<br>198 | Small nonstructural protein | Contribute to virus virulence |
| **E gene** | 327 | Small envelope protein | Associates with viral envelope formation, assembly, budding, ion channel activity and apoptosis |
| **M gene** | 678 | M protein (matrix protein) | Arranging and assembling the virus particles |
| **ORF 5 gene**<br>• ORF 5a<br>• ORF 5b | <br>198<br>249 | Small nonstructural protein | Contributes to virus virulence |
| **N gene** | 1,230 | N protein (nucleocapsid protein) | Viral transcription, replication, translation and packaging of viral genome during replication |

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3. Spike glycoprotein

S glycoprotein is the largest surface protein of coronaviruses with approximately 16-21 nm in length and comprising of 3,462 nucleotides (Shen et al., 2004). S protein is responsible for the fusion of the viral envelope with the host endosomal or cell membrane for subsequent release of virus RNA into the cytoplasm. This protein plays an important role in virus-host cell specificity and the pathogenicity of the virus (Caron, 2010). The ability of IBV to replicate in many organs has been reported to be related with the presence of N-acetylneuraminic acid (sialic acid) at the cell surface (Winter et al., 2006). It was reported that IBV attaches $\alpha$ 2,3-linked N-acetylneuraminic acid for starting the host cell infection (Promkuntod et al., 2014). S protein is cleaved by a furin-like host cell protease at cleavage recognition site, a highly basic pentapeptide motif RRFRR, into two subunits, which are the amino-terminal S1 subunit containing 535 amino acids and forming the bulb of S protein and the carboxy-terminal S2 subunit containing 627 amino acids and forming a narrow stalk anchoring to the viral envelope (Yamada and Liu, 2009) (Figure 4). The highly variable S1 protein, containing receptor-binding sites, is responsible for attachment to host cell receptor, while the conserved S2 protein is involved in the fusion of the viral envelope with the cellular membrane. Receptor binding domain (RBD) of IBV, is located on the N-terminal 253 amino acids of the spike and the critical amino acids for attachment are present within the N-terminal residues 19-69, which overlaps with a hypervariable region in the S1 gene (Promkuntod et al., 2014) (Figure 4). Although the S2 protein is not directly involved in host cell receptor binding, S1 and S2 might work together for the avidity and specificity of virus attachment (De Haan et al., 2006).

Among all of the IBV proteins, S1 subunit is the most important immunogenic component of the virus as it contains epitopes inducing neutralizing antibodies and plays a major role in virus-host cell specificity due to receptor binding domain composition (Promkuntod et al., 2014). As it contains several neutralizing epitopes, S1 protein shows a very high degree of genetic variation. Compared to conserved S2

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protein showing more than 90% amino acid identity among IBV serotypes, the amino acid variation in highly variable S1 protein among serotypes ranges from 2-3% to 50%, with an average of 20%-25% (Cavanagh, 2007). S1 protein contains 3 different hypervariable regions (HVRs), comprising of amino acid residue 38-67, 91-141 and 274-387 of the S1 gene (Moore et al., 1997) (Figure 4). The high genetic diversity of the S1 gene is considered as a result of genetic mutation and recombination, which frequently occur in the S1 subunit (Kant et al., 1992). Therefore, the genotypes of IBVs are usually classified based on the genetic variation of the S1 subunit (Lee et al., 2003; Valastro et al., 2016).

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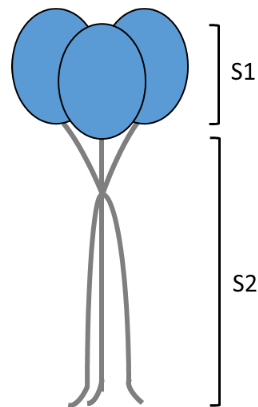


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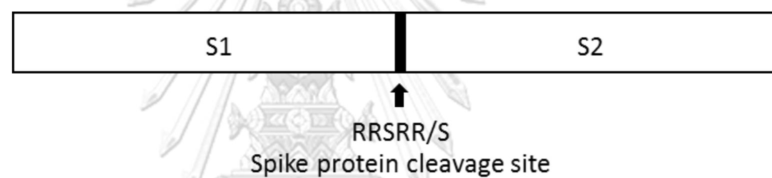


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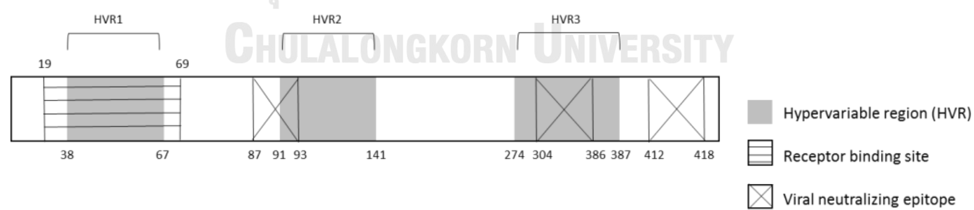


Figure 4 Diagram of the spike protein of IBV.

The spike (S) protein consists of two subunits, which are S1 and S2 subunits (A and B). S1 gene contains a receptor binding site, three different hypervariable regions and several neutralizing epitopes (C) (Belouzard et al., 2012) (Lee et al., 2001; Promkuntod et al., 2014; Umar et al., 2016).

4. Virus replication

After binding to the specific receptors on the host cell surface through the spike glycoprotein, IBV is internalized into the cytoplasm of infected host cells by receptor-mediated endocytosis. Virus uncoating step then occurs, resulting in the release of viral genomic RNA into the cytoplasm. Subsequently, a positive-strand genomic RNA is translated to a large polyprotein, which is cleaved into RNA-dependent RNA polymerase. A negative-strand RNA is generated and transcribed into subgenomic mRNAs by newly synthesized RNA-dependent RNA polymerease. Subgenomic mRNAs are then translated into several structural viral proteins. A negative-strand RNA also serves as a template for amplification of viral RNA (vRNA), leading to additional copies of vRNA. Newly synthesized viral RNAs and proteins are assembled into progeny virions at the endoplasmic reticulum-Golgi intermediate compartment (ERGIC), where the S proteins are inserted. The release of progeny virions occurs by fusion of virion-containing vesicles with the plasma membrane (Figure 5).

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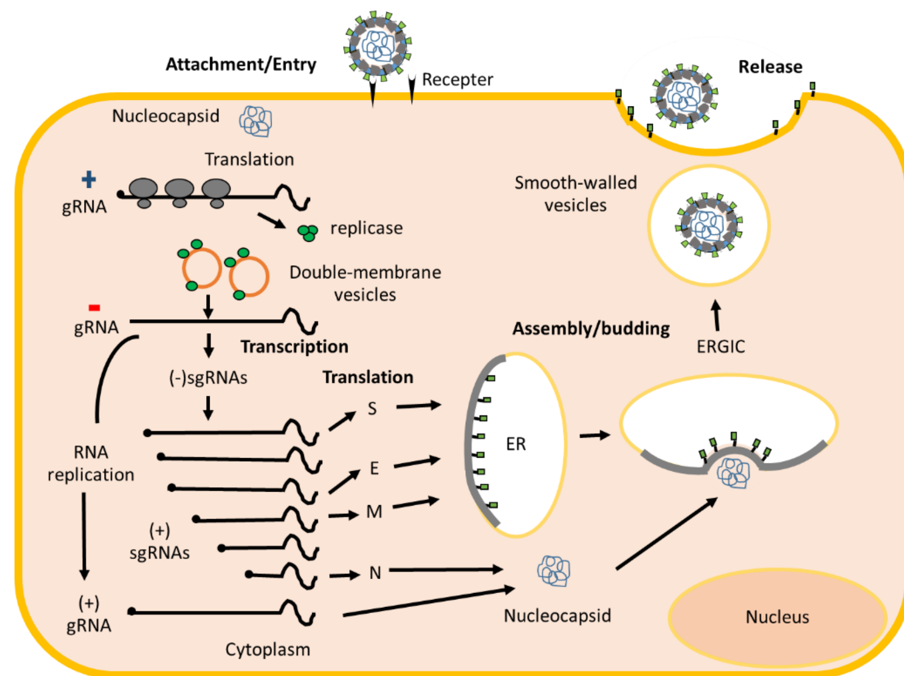


Figure 5 Schematic diagram of the IBV replication cycle

(Weiss and Navas-Martin, 2005; Masters, 2006)

5. Molecular mechanisms of IBV evolution

In contrast to a limited number of serotypes existing in other coronaviruses, there are multiple subtypes and genotypes of IBV. Moreover, several new IBV genotypes with little or no immunological cross protection have been continuously emerging worldwide due to rapid virus replication and evolution (Jackwood et al., 2012). The rapid evolution of IBV occurs through two mechanisms, including mutation and recombination (Lim et al., 2011; Selim K. et al., 2014). Mutations are the result from the amino acid substitutions, insertions and deletions frequently observed in the S1 proteins caused by the infidelity of the virus RNA polymerase (Yan et al., 2011; Jain et al., 2016). Recombination, resulting from RNA template switching occurred during RNA replication, has been frequently detected in multiple structural genes, particularly in the S1 gene (Ammayappan et al., 2008). Recombination hot spots have been reported in IBV, which are mainly located in the upstream of the S gene and in some nonstructural genes (Thor et al., 2011a). It is well documented that IBV has a very high

evolutionary rate as shown by a higher mean substitution rate ($1.2x10^{-3}$ substitutions/site/year) when compared to those from other avian viruses, including Newcastle disease virus ($3.2x10^{-4}$ substitutions/site/year) (Jenkins et al., 2002; Hanada et al., 2004). This finding partly explains the frequent emergence of new IBV variants with little or no cross protection.

## Pathogenesis of IBV

IBV is transmitted among chickens though inhalation and ingestion of infected respiratory secretion or feces (Alexander and Gough, 1977). Incubation period ranges from 18 to 36 hours depending on the route of infection (Jackwood and De Wit, 2013). The pathogenesis of IBV is shown in Figure 6. The primary target site of IBV infection is the epithelial cells of the upper respiratory, urogenital and reproductive tracts of chickens (Kameka et al., 2014). IBV infection is initiated via the respiratory tract regardless of the tissue tropism of the virus strains (respiratory, kidney and gonad). The virus can replicate and produce lesions in many types of epithelial cells in the respiratory tract (nasal turbinates, Harderian gland, trachea, lungs, and air sacs), kidney and gonads (oviduct and testes) (Alvarado et al., 2006). In the respiratory tract, IBV can replicate in many tissues, including trachea, lungs and air sac, resulting in loss of ciliary activity in trachea, tissue hemorrhage and inflammation. Respiratory signs of IBV infected chickens include gasping, coughing, tracheal rales, periorbital edema, nasal and ocular discharges (Khataby et al., 2016). Loss of ciliary activity is considered as a predisposing factor to secondary opportunistic bacterial infections, leading to bacterial septicemia, pericarditis, perihepatitis and airsaculitis (Dwars et al., 2009). Respiratory tissue damage caused by IBV usually recovers within 2-3 weeks after infection (Dwars et al., 2009). After initial infection in the respiratory tract, the virus can spread to other tissues via viremia. Many IBV strains can infect the epithelium in kidney tubules and nephron, causing nephritis with urate deposition and leading to kidney failure (Feng et al., 2012). Infected chickens usually show signs of severe depression and wet dropping (Ignjatovic et al., 2002). Moreover, some strains of IBV can also cause reproductive disorders in males and females by infecting the oviduct, the magnum and the uterus

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of laying chickens, resulting in egg production drop and poor egg quality, including shell-less, fragile or roughened shell eggs (uterus lesion) with watery albumin (magnum lesion) (Nii et al., 2014). In addition, IBV infection can interfere oviduct formation and causes cystic forming in IBV infected young chickens, resulting in false layers or silent layers. IBVs occasionally infect tissues in the gastrointestinal tract without causing any particular diseases (Cavanagh, 2003). Morbidity rate of chickens infected with IBV is close to 100%, while the mortality rate is approximately 25-30% (ranging from 0 to 82%) depending on host age, immune status, viral strains, co-infection with other pathogens and secondary bacterial infection (Cavanagh and Gelb, 2008; Jackwood and De Wit, 2013).

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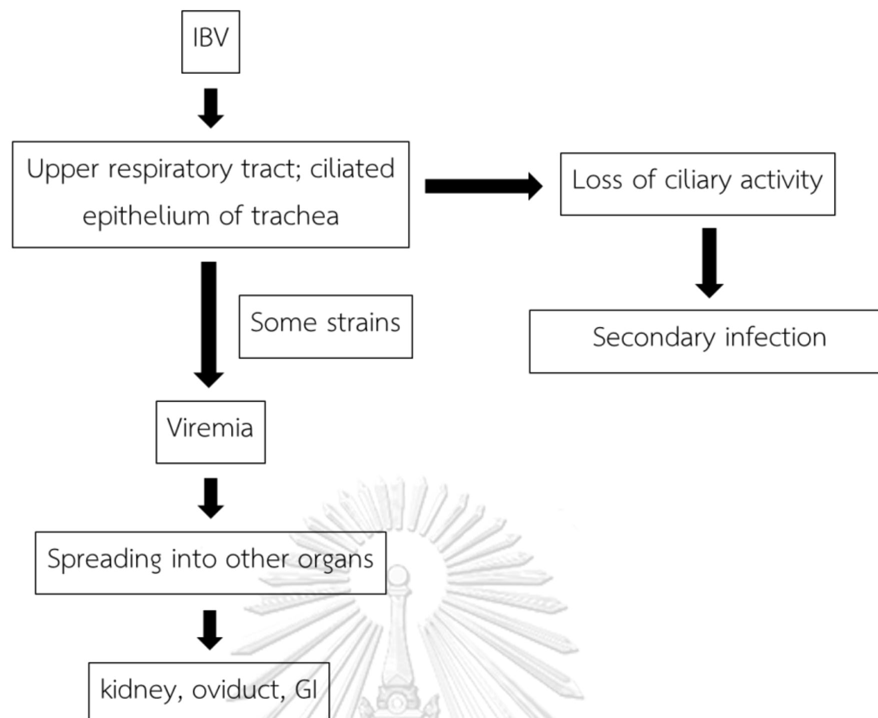


Figure 6 The pathogenesis of IBV in infected chickens.

**IBV strain classification**

Since various new IBV serotypes and genotypes have been continuously emerging in chicken populations worldwide, it is necessary to identify the circulating virus strains in order to choose the appropriate vaccine strains capable of inducing protective immunity. Serotype and genotype are commonly used for classification of IBV (Li et al., 2012). A conventional method for IBV strain classification is virus neutralization test (VN) conducted in embryonated eggs; however this method is time consuming and many new IBV variants do not cross-react with few available reference IBV antisera, making this method is less appropriate to classify the IBV strains. Currently, genetic typing method conducted by reverse transcription polymerase chain reaction (RT-PCR) and subsequent sequence analysis of the whole or partial S1 gene, is widely used due to rapid, inexpensive and close correlation with the serotype classification (Valastro et al., 2016). IBV strains that have greater than 90% amino acid similarity in S1 gene mostly are serological related (serotype) (Cavanagh, 2005).

**Epidemiology of IBV**

IBV was first reported in the United States in the 1930s (Jackwood and De Wit, 2013). The classic subtype causing respiratory disease of IBV is a Massachusetts strain isolated from chickens in the United States in the 1941 (Jia et al., 2002). In addition to IBV strains causing respiratory disease, other virus strains causing diseases in the urogenital and reproductive systems were first described in 1950s (Dhama et al., 2014). After it was first discovered, a number of IBV variants spread worldwide in both farmed and backyard chickens. While some IBV genotypes circulate only in the specific geographic regions, including Arkansas and its variant, which are USA common isolate (Cook et al., 2012), many genotypes were widely distributed in many parts of the world, including 4/91, Massachusetts and LX4 or QX-like variants (Jackwood, 2012). Prototype strains of IBV circulated worldwide are shown in Table 3. Recently, several new IBV genotypes have been continuously identified in many regions around the world, including TW-type IBV identified in China and Taiwan (Tsai et al., 2016), CK/CH/LDL/97I identified in China and the Middle East (Ababneh et al., 2012; Zhao et al., 2014) and CK/CH/LSC/99I and SAIBK identified in China (Liu et al., 2006; Yang and Ma, 2013). It is well documented that some strains of IBV variants were classified as the recombinant IBV strains mostly generated from recombination process between field and vaccine strains, including Guandong/Xindadi (XDN) genotype originated from QXIBV and 793B vaccine strain (Moreno et al., 2017) and ck/CH/LHLJ/140906 emerged from 4/91 and H120 vaccine strain (Zhang et al., 2015). In addition, some mutated IBV vaccine strains have been reported to cause the outbreak of IB in chickens, including ZJ971, which was a mutated H120 strain (Zhang et al., 2010).

IBV has been described in Thailand since 1953 (Chindavanig, 1962). Subsequently, many IBV isolates have been continuously identified during 2008-20113 despite the extensive use of IBV vaccines in Thailand and were grouped into at least five genotypes, including a unique Thai IBV (THA50151), THA001, QX-like IBV, Massachusetts and 4/91 (Pohuang et al., 2011; Promkuntod et al., 2015). Among the 2008-2013 Thai IBVs, THA90151 originated from THA80151 and THA001 and THA80151 originated from QXIBV and JX/99/01 (Pohuang et al., 2011)

Table 3 Prototype strains of IBV circulated worldwide.

| Number | Strain name | Tissue tropism/strain | Origin | References |
|---|---|---|---|---|
| 1 | Beaudette | Respiratory | USA | (Wickramasinghe et al., 2011) |
| 2 | Holte | Nephropathogenic | USA | (Cook et al., 2012) |
| 3 | Gray | Nephropathogenic | USA | (Glahn et al., 1988) |
| 4 | ArkDPI | Respiratory | USA | (Nix et al., 2000) |
| 5 | M41 | Respiratory | USA | (Wickramasinghe et al., 2011) |
| 6 | H120 | Vaccine | UK | (Zhang et al., 2010) |
| 7 | Ma5 | Vaccine | USA | (Zhang et al., 2010) |
| 8 | Connecticut | Respiratory | USA | (Uenaka et al., 1998) |
| 9 | Australian T | Nephropathogenic | Australia | (Ignjatovic et al., 2002) |
| 10 | QXIBV | Nephropathogenic and reproductive | China | (Terregino et al., 2008; De Wit et al., 2011c) |
| 11 | LX4 | Nephropathogenic | China | (Ma et al., 2012) |
| 12 | THA001 | Nephropathogenic | Thailand | (Pohuang et al., 2009) |
| 13 | 4/91 | Respiratory and reproductive | UK | (Shimazaki et al., 2008) |
| 14 | Delaware | Respiratory | USA | (Gelb et al., 1997) |

This study is composed of 4 parts as follows; **part 1** field sample collection, **part 2** virus isolation and identification, **part 3** IBV genetic characterization and **part 4** IBV S1 gene analysis.

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# CHAPTER 4

# Materials and methods

## Part 1: Field sample collection

From January 2014 to December 2016, the pooled organ samples, including trachea, kidney, cecal tonsil and oviduct, were collected from IBV suspected chickens. To obtain the accurate representation of IBV field strains circulating in Thailand, the samples were collected from broiler breeder and layer chicken farms located in all of the chicken-raising regions of Thailand, including the northern, the central, the northeastern, the eastern, the western and the southern parts of Thailand. All of the samples were collected from chicken flocks suspected to IBV infection by showing upper respiratory, reproductive or urogenital disorders. All chicken flocks were vaccinated with commercial IBV vaccine strain H120 and Ma5. Some chicken flocks,including layer, breeder and some broiler chickens, were vaccinated with 4/91 vaccine strain. The samples were stored at -80°C until tested.

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## Part 2: Virus isolation and identification

The tissue samples collected from affected chickens were inoculated into 10-day-old embryonated specific pathogen free (SPF) chicken eggs for virus isolation. Briefly, the pooled organ samples were homogenized in sterile phosphate-buffered saline (PBS) to give a 10% suspension (w/v), clarified by centrifugation at 3000 x g for 15 min and then filtered through 0.45 μm membrane filters (Corning, USA). The tissue suspensions were inoculated into the allantoic cavity of 10-day-old embryonated SPF chicken eggs (Thai SPF Company, Thailand). Three eggs were used for each sample. Embryos were examined twice a day for 7 days and allantoic fluid was harvested at 7 days post inoculation or upon embryo death. Two or three blind passages were

performed for each sample. The harvested allantoic fluids were stored at -80°C until used for RT-PCR analysis.

RT-PCR was performed to determine the presence of IBV RNA using S1 gene specific primers as described previously with minor modifications (Kwon et al., 1993). In brief, viral RNA was extracted from allantoic fluids by using NucleoSpin Extract Viral RNA Kit (Macherey-Nagel, Düren, Germany) according to the manufacturer's recommendations. The RNA was initially converted to cDNA using random hexamers and an Improm-II™ reverse transcription system (Promega, Wisconsin, USA) following the manufacturer's instructions. Briefly, a mixture of 5 µL of RNA template and 5 µL of the random primer was first incubated at 70°C for 15 minutes and chilled at 4°C for 5 minutes. Subsequently, 4 µL of 5x Buffer, 2 µL of $MgCl_2$, 1 µL of ImProm-II™ reverse transcriptase, 1 µL of dNTP, 0.3 µL of recombinant ribonuclease inhibitor and RNA nuclease-free water were added in a final volume 12 µL and then incubated at 42°C for 60 minutes followed by 72°C for 15 minutes. PCR was performed using TopTaq® Master Mix Kit (Qiagen, Germany). cDNA was amplified by using S1 gene specific primers as shown in Table 4. The PCR reaction was consisted of 3 µL of cDNA sample, 15 µL of 2xToptaq, 3 µL of coral load, 1.2 µL of forward and reverse specific primers and RNase-free water in a final volume of 30 µL. The PCR reaction was performed at 94°C for 5 minutes, 35 cycles of 94°C for 1 minute, 45°C for 2 minutes, 74°C for 5 minutes followed by a final extension step at 74°C for 10 min. Ten µL of PCR product was analyzed on a 1.5% agarose gel (Research Organics, USA) at 100 Volts for 1 hour. After electrophoresis the DNA bands were stained with SYBR® Safe DNA gel stain (Invitrogen, USA) and visualized by UV transilluminator. The samples were also tested for common chicken viruses that may cause similar symptoms, including avian influenza virus (AIV) and Newcastle disease virus (NDV) (Liu et al., 2007; Suarez et al., 2007).

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Table 4 The sequences of S1 gene specific primers used in this study (Kwon et al., 1993).

| Primer name | Sequence (5'-3') | Product size (base pairs; bp) |
|---|---|---|
| S1OLIGO3' | CATAACTAACATAAGGGCAA | 1,720 |
| S1OLIGO5' | TGAAAACTGAACAAAAGACA | |

**Part 3: IBV genetic characterization**

IBV positive samples were randomly selected for the complete S1 gene sequencing as previously described with minor modifications (Pohuang et al., 2011). The RNA was initially converted to cDNA using random hexamers and an Improm-II$^{TM}$ reverse transcription system (Promega, Wisconsin, USA) following the manufacturer's instructions as described earlier. The cDNA was used as a template for amplification of the complete S1 gene by PCR using a TopTaq Master Mix Kit (QIAGEN, Hilden, Germany and two sets of S1 gene specific primers as shown in Table 5. The PCR reaction was consisted of 3 µL of cDNA sample, 15 µL of 2xToptaq, 3 µL of coral load, 1.2 µL of forward and reverse specific primers and RNase-free water in a final volume of 30 µL. The PCR reaction was conducted at 95°C for 3 minutes, 40 cycles of 95°C for 30 seconds, 50°C for 45 seconds and 72°C for 1.30 minutes followed by a final extension at 72°C for 7 minutes. The PCR products were then purified with NucleoSpin Extract II (Macherey-Nagel, Düren, Germany) according to the manufacturer's recommendations and directly sequenced with the amplification primers in both directions by 1$^{st}$ BASE company (1$^{st}$ Base Laboratories Sdn Bhd, Malaysia).

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Table 5 The sequences of 2 sets of S1 gene specific primers used for S1 gene sequencing

(Pohuang et al., 2011).

| Primer name | Sequence (5'-3') | Product size (bp) |
|---|---|---|
| IBV_F20235 | GCCAGTTGTTAATTTGAAAAC | 986 |
| IBV_R21221 | TAATAACCACTCTGAGCTGT | |
| IBV_F21016 | ACTGGCAATTTTTCAGATGG | 1,065 |
| IBV_ R22081 | AACTGTTAGGTATGAGCACA | |

**Part 4: IBV S1 gene analysis**

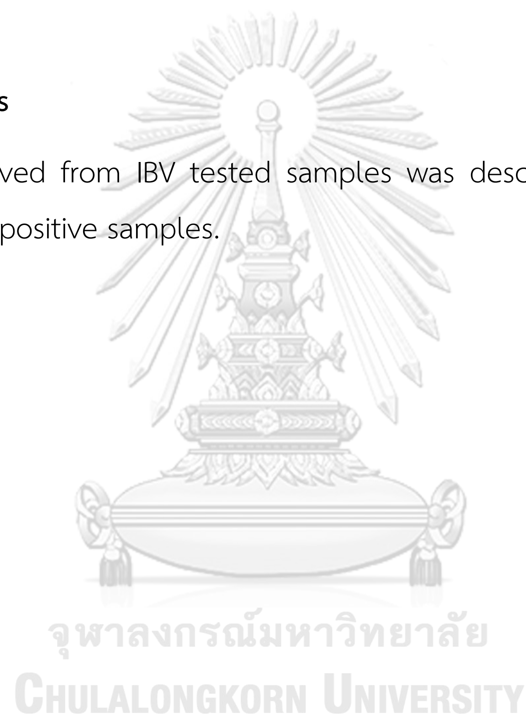
The nucleotide sequences of the S1 gene of IBVs isolated from this study were assembled and analyzed by using SeqMan software v.5.03 (DNASTAR Inc., Wisconsin, USA). The nucleotide sequences of Thai S1 gene characterized in this study were submitted to the GenBank database under the accession number MG190958-191077. Phylogenetic analysis was performed by comparing the complete S1 gene sequences of Thai IBVs with those of the selected reference IBVs available at the GenBank database. The nucleotide sequences were aligned in Muscle v.3.6 (Edgar, 2004). A phylogenetic tree of the nucleotide and amino acid sequences of IBVs were constructed in MEGA6 v.6.0 using neighbor-joining algorithm with the Kimura-2 parameter model applied to 1000 replications of bootstrap (Tamura et al, 2013). The nucleotide and amino acid identities among Thai IBVs obtained from this study, previously reported Thai IBVs, IBV vaccine strains commonly used in Thailand and the selected IBV reference strains available in the GenBank database were examined and compared using the MegAlign software v.5.03 (DNASTAR Inc., Wisconsin, USA).

To identify the recombinant events in the S1 gene sequences of Thai IBVs, the complete S1 gene sequences of all Thai IBVs were analyzed with the Recombinant Detection Program (RDP 4, version 4.83) (Martin et al., 2015). Seven detection methods in RDP4 version 4.83, including RDP, GENECONV, BootScan, MaxChi, Chimaera, SiScan and 3Seq, were used to identify the recombination events. A recombination event was

accepted only when it was detected by five or more methods implemented in the program, with a p-value lower than $10^{-14}$. Recombination events and breakpoints were further confirmed by SimPlot version 3.5.1 software (Reddy et al., 2015). Nucleotide identity was performed by using the Kimura (2-parameter) method with a transition-transversion ratio of 2. The window width and step size were 200 and 20 bp, respectively. In addition, the phylogenetic trees based on the different fragments of the S1 gene of the novel recombinant IBV variants were constructed to confirm the genetic recombination (Lim et al., 2011).

**Statistical analysis**

Data retrieved from IBV tested samples was descriptively presented in the percentage of IBV positive samples.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# CHAPTER 5

# Results

## Field sample collection

From 2014 to 2016, 1,668 suspected clinical samples of IBV infection were collected from broiler, breeder and layer chicken farms located in all chicken-raising regions of Thailand, including the central, the eastern, the northern, the northeastern, the western and the southern parts of Thailand. The detail of the samples collected in this study is showed in Table 6. Most chickens exhibited the clinical findings associated with the upper respiratory, reproductive and/or urogenital disorders. The mortality rates of affected chicken flocks ranged from 1.18%-19.33%, correlating with secondary bacterial infection.

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Table 6 The samples collected from IBV suspected chickens in Thailand during 2014-2016.

| Year | Province | No. of sample |
|---|---|---|
| **2014** | Ayutthaya | 5 |
| | Bangkok | 4 |
| | Chachoengsao | 29 |
| | Chaiyaphum | 7 |
| | Chanthaburi | 2 |
| | Chiangmai | 8 |
| | Chiangrai | 3 |
| | Chonburi | 46 |
| | Chumphon | 4 |
| | Kamphaengphet | 7 |
| | Kanchanaburi | 15 |
| | Khonkaen | 8 |
| | Lampang | 1 |
| | Lamphun | 6 |
| | Lopburi | 5 |
| | Nakhon Nayok | 2 |
| | Nakhon Pathom | 6 |
| | Nakhon Ratchasima | 64 |
| | Phetchabun | 4 |
| | Phetchaburi | 3 |
| | Pitsanulok | 23 |
| | Prachinburi | 32 |
| | Sakholnakhon | 1 |
| | Saraburi | 108 |
| | Songkhla | 4 |
| | Suratthani | 9 |
| | Tak | 1 |

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| Year | Province | No. of sample |
|---|---|---|
| **2014** | Ubon Ratchathani | 1 |
| | Udon Thani | 7 |
| | Uttaradit | 8 |
| | **Total** | **423** |
| **2015** | Ayutthaya | 8 |
| | Buriram | 2 |
| | Chachoengsao | 32 |
| | Chonburi | 153 |
| | Kanchanaburi | 11 |
| | Khonkaen | 6 |
| | Lampang | 3 |
| | Lamphun | 6 |
| | Loei | 2 |
| | Lopburi | 2 |
| | Nakhon nayok | 6 |
| | Nakhon Pathom | 3 |
| | Nakhon Ratchasima | 139 |
| | Nakhon Srithammarat | 2 |
| | Phetchaburi | 10 |
| | Pitsanulok | 30 |
| | Prachinburi | 34 |
| | Rayong | 4 |
| | Roi Et | 25 |
| | Saraburi | 110 |
| | Suratthani | 5 |
| | Tak | 2 |
| | Ubon Ratchathani | 2 |
| | Udon Thani | 2 |
| | Uttaradit | 6 |

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| Year | Province | No. of sample |
|---|---|---|
| **2015** | **Total** | **605** |
| **2016** | Ayutthaya | 16 |
| | Bangkok | 1 |
| | Chachoengsao | 27 |
| | chiangmai | 15 |
| | Chiangrai | 2 |
| | chonburi | 103 |
| | Khonkaen | 2 |
| | Lampang | 8 |
| | Lopburi | 2 |
| | Nakhon Nayok | 2 |
| | Nakhon Ratchasima | 116 |
| | Nakhon Pathom | 1 |
| | phetchabun | 49 |
| | Pitsanulok | 3 |
| | Prachinburi | 117 |
| | Rayong | 2 |
| | Sakholnakhon | 1 |
| | Saraburi | 147 |
| | Songkhla | 17 |
| | Tak | 3 |
| | Udon Thani | 5 |
| | Yasothon | 1 |
| | **Total** | **640** |
| | **Overall total** | **1,668** |

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**IBV isolation and identification**

Of 1,668 clinical samples collected, 629 (37.70%) tested positive for IBV by S1-specific RT-PCR. In addition, the chicken embryos inoculated IBV positive samples died within three to seven days post-inoculation with stunting and/or curling (Figure 7). All samples were negative for other common chicken viruses that may cause similar symptoms, including AIV and NDV (data not shown).

Most of the IBV positive samples (84.40%) derived from broilers, while the remaining samples obtained from layers and breeders. IBV positive samples were detected in farms located in 23, 25 and 21 provinces of Thailand in 2014, 2015 and 2016, respectively (Figure 8). These findings indicate that IBV infections were found throughout the country and were predominantly detected in the eastern of Thailand (Figure 8). The mean prevalence of IBV infection in Thailand in 2014, 2015 and 2016 were 48.93, 34.21 and 33.59%, respectively, while the overall mean prevalence of IBV infection in Thailand during 2014-2016 was 37.70%%.

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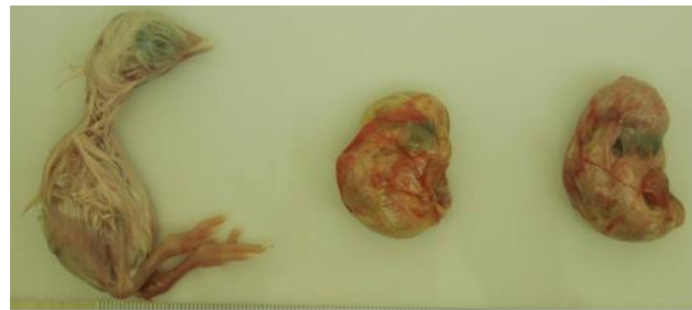


Figure 7 Chicken embryos infected with Thai IBVs.

Infected embryos died within 7 days post inoculation with stunting and curling (right) and a normal embryo is shown at left.

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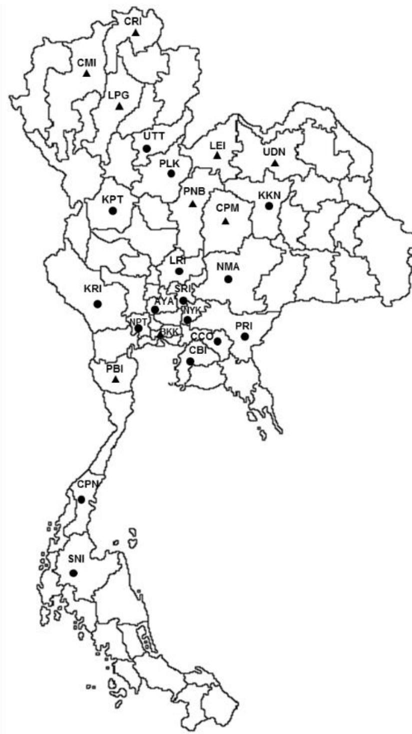
8A




8B

8C

8D

Figure 8 Geographic distribution of infectious bronchitis virus (IBV) in Thailand in 2014 (A), 2015 (B), 2016 (C) and during 2014-2016 (D).

Triangles represent the provinces where IBVs were detected. Circles represent provinces where IBVs were characterized. AYA: Ayutthaya ; BKK: Bangkok; BRM: Buriram; CCO: Chachoengsao; CBI: Chonburi; KPT: Kamphaeng Phet; KRI: Kanchanaburi; KKN: Khon Kaen; LPG: Lampang; LRI: Lopburi; NYK: Nakhon Nayok; NPT:Nakhon Pathom; NMA: Nakhon Ratchasima; NRT: Nakhon Sri Thammarat; PNB: Phetchabun; PBN: Phetchaburi; PLK: Phitsanulok; PRI: Prachinburi; RET: Roi Et; SRI: Saraburi; SKA: Songkhla; SPB: Suphan Buri; SNI: Suratthani; Tak: Tak; UDN: Udon Thani; UTT: Uttaradit and YST: Yasothon.

**Phylogenetic analysis of the S1 gene**

To characterize the genotypes of IBV circulating in Thailand during 2014-2016, 150 Thai IBV isolates were randomly selected for the complete S1 gene sequencing (Table 7). The phylogenetic analysis of the complete S1 genes demonstrated that Thai IBVs circulating in 2014 were divided into 4 genotypes, including QX-like IBV (n=42), Massachusetts (n=2), Connecticut (n=1) and a new genotype (n=1) (Figure 9A and B). Thai IBVs circulating in 2015 were divided into 3 genotypes, including QX-like IBV (n=47), Massachusetts (n=5) and 4/91 (n=2) (Figure 9C and D). In 2016, IBVs circulating in Thai chicken flocks were divided into 5 genotypes, including QX-like IBV (n=40), Massachusetts (n=5), 4/91 (n=3), Connecticut (n=1) and a new genotype (n=1) (Figure 9E and F). Overall, IBVs circulating in chickens in Thailand during 2014-2016 were divided into five distinct genotypes, including QX-like IBV (n=129), Massachusetts (n=12), 4/91 (n=5), Connecticut (n=2) and a novel IBV genotype (n=2) (Figure 9G and H). It is noted that a unique Thai IBV (THA50151) and THA001 reported previously in Thailand were not detected in this study. Overall, our result showed that the majority of Thai IBVs circulating during 2014-2016 (86%) were most closely related and grouped together with QXIBV (94.1% - 97.3% nucleotide identity and 90.7%-96.8% amino acid identity) (Table 8).

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Overall, the 2014-2016 Thai IBVs shared 95.5%-100% nucleotide identity and 89.3%-100% amino acid identity to each other, while they shared only 78.5%-79.7%, 79.1%-81.4%, 77.6%-78.6% nucleotide identity and 76.1%-78.3%, 77.6%-81.7%, 75.5%-77.2% amino acid identity with Massachusetts, 4/91 and Connecticut vaccine strains, respectively (Table 8). Interestingly, the phylogenetic analysis of the S1 genes also revealed that TH/IBV/2014/CU-179 and TH/IBV/2016/CU-92 identified in this study were grouped as a novel genotype of IBV, which was distinctly separated from the previously reported genotypes (Figure 9). These viruses shared only 88.5%-92.2%, 79.6%-79.9%, 87.9%-88% and 79.1%-79.3% nucleotide identity and 88.6%-93.8%, 76.6%-76.8%, 86.7%, 75.7%-75.9% amino acid identity with Thai QX-like IBVs, Massachusetts, 4/91 and Connecticut vaccine strains, respectively (Table 8).

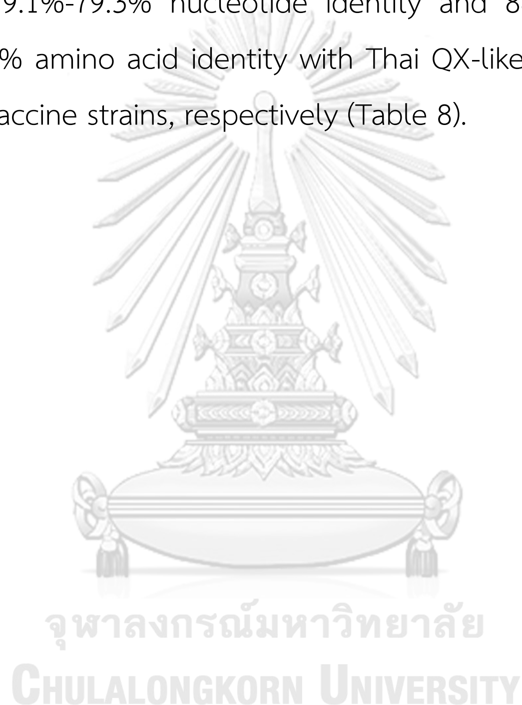
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Table 7 Details of IBVs characterized in this study

| Virus name | Type of raising | Age | Time of collection | Location | Accession No. |
|---|---|---|---|---|---|
| TH/IBV/2015/CU-1 | Broiler | 38 days | 2015/November | Saraburi | MG190958 |
| TH/IBV/2015/CU-2 | Broiler | 43 days | 2015/November | Saraburi | MG190959 |
| TH/IBV/2015/CU-3 | Broiler | 31 days | 2015/November | Prachinburi | MG190960 |
| TH/IBV/2015/CU-5 | Pullet | 17 weeks | 2015/November | Nakhon Nayok | MG190961 |
| TH/IBV/2015/CU-6 | Layer | 20 weeks | 2015/November | Saraburi | MG190962 |
| TH/IBV/2015/CU-7 | Broiler | 26 days | 2015/November | Chachoengsao | MG190963 |
| TH/IBV/2015/CU-8 | Broiler | 35 days | 2015/November | Saraburi | MG190964 |
| TH/IBV/2014/CU-9 | Broiler | 24 days | 2014/June | Ayutthaya | - |
| TH/IBV/2014/CU-10 | Broiler | 24 days | 2014/June | Saraburi | - |
| TH/IBV/2014/CU-11 | Layer | 31 weeks | 2014/December | Lopburi | MG190965 |
| TH/IBV/2014/CU-12 | Breeder | 25 weeks | 2014/September | Nakhon Ratchasima | MG190966 |
| TH/IBV/2014/CU-13 | Layer | 14 weeks | 2014/October | Nakhon Nayok | MG190967 |
| TH/IBV/2014/CU-14 | Broiler | 46 days | 2014/May | Nakhon Ratchasima | - |
| TH/IBV/2014/CU-15 | Broiler | 39 days | 2014/April | Kanchanaburi | - |
| TH/IBV/2014/CU-16 | Broiler | 24 days | 2014/April | Khon Khan | - |
| TH/IBV/2014/CU-17 | Layer | 31 weeks | 2014/December | Phitsanulok | MG190968 |
| TH/IBV/2014/CU-19 | Broiler | 34 days | 2014/June | Saraburi | - |
| TH/IBV/2015/CU-20 | Broiler | 30 days | 2015/January | Chachoengsao | MG190969 |
| TH/IBV/2015/CU-21 | Broiler | 41 days | 2015/February | Saraburi | MG190970 |
| TH/IBV/2015/CU-22 | Broiler | 38 days | 2015/January | Chonburi | MG190971 |
| TH/IBV/2015/CU-23 | Broiler | 20 days | 2015/January | Chonburi | MG190972 |
| TH/IBV/2015/CU-24 | Broiler | 34 days | 2015/January | Saraburi | MG190973 |
| TH/IBV/2015/CU-25 | Broiler | 34 days | 2015/February | Saraburi | MG190974 |
| TH/IBV/2015/CU-26 | Broiler | 36 days | 2015/February | Saraburi | MG190975 |
| TH/IBV/2015/CU-27 | Broiler | 44 days | 2015/February | Nakhon Ratchasima | MG190976 |
| TH/IBV/2015/CU-28 | Broiler | 27 days | 2015/February | Phetchaburi | MG190977 |
| TH/IBV/2015/CU-29 | Broiler | 27 days | 2015/February | Surat Thani | MG190978 |
| TH/IBV/2015/CU-30 | Broiler | 16 days | 2015/February | Nakhon Srithammarat | MG190979 |
| TH/IBV/2015/CU-31 | Broiler | 39 days | 2015/March | Saraburi | MG190980 |
| TH/IBV/2015/CU-32 | Broiler | 26 days | 2015/March | Chonburi | MG190981 |
| TH/IBV/2015/CU-33 | Layer | 52 weeks | 2015/March | Phitsanulok | MG190982 |
| TH/IBV/2015/CU-35 | Broiler | 38 days | 2015/March | Saraburi | MG190983 |
| TH/IBV/2015/CU-36 | Broiler | 35 days | 2015/March | Saraburi | MG190984 |
| TH/IBV/2015/CU-37 | Broiler | 37 days | 2015/May | Nakhon Ratchasima | MG190985 |
| TH/IBV/2015/CU-38 | Broiler | 45 days | 2015/June | Nakhon Ratchasima | MG190986 |
| TH/IBV/2015/CU-39 | Broiler | 34 days | 2015/December | Chachoengsao | MG190987 |
| TH/IBV/2015/CU-40 | Broiler | 20 days | 2015/July | Roi Et | MG190988 |

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| Virus name | Type of raising | Age | Time of collection | Location | Accession No. |
|---|---|---|---|---|---|
| TH/IBV/2015/CU-41 | Broiler | 36 days | 2015/August | Prachinburi | MG190989 |
| TH/IBV/2015/CU-42 | Broiler | 41 days | 2015/August | Nakhon Ratchasima | MG190990 |
| TH/IBV/2015/CU-43 | Broiler | 33 days | 2015/September | Saraburi | MG190991 |
| TH/IBV/2015/CU-44 | Broiler | 27 days | 2015/November | Saraburi | MG190992 |
| TH/IBV/2015/CU-45 | Broiler | 32 days | 2015/July | Saraburi | MG190993 |
| TH/IBV/2015/CU-47 | Broiler | 25 days | 2015/November | Saraburi | MG190994 |
| TH/IBV/2015/CU-48 | Broiler | 34 days | 2015/December | Chachoengsao | MG190995 |
| TH/IBV/2015/CU-49 | Broiler | 37 days | 2015/December | Chonburi | MG190996 |
| TH/IBV/2015/CU-50 | Broiler | 33 days | 2015/December | Saraburi | MG190997 |
| TH/IBV/2015/CU-51 | Broiler | 31 days | 2015/December | Saraburi | MG190998 |
| TH/IBV/2015/CU-52 | Broiler | 43 days | 2014/May | Saraburi | - |
| TH/IBV/2015/CU-53 | Broiler | 43 days | 2014/May | Saraburi | - |
| TH/IBV/2015/CU-54 | Broiler | 43 days | 2014/June | Saraburi | - |
| TH/IBV/2014/CU-56 | Broiler | 29 days | 2014/December | Prachinburi | MG190999 |
| TH/IBV/2014/CU-57 | Breeder | 30 weeks | 2014/November | Prachinburi | MG191000 |
| TH/IBV/2014/CU-58 | Broiler | 31 days | 2014/March | Saraburi | - |
| TH/IBV/2014/CU-59 | Broiler | 28 days | 2014/December | Saraburi | MG191001 |
| TH/IBV/2014/CU-60 | Layer | 46 weeks | 2014/December | Chonburi | MG191002 |
| TH/IBV/2014/CU-61 | Broiler | 41 days | 2014/December | Chonburi | MG191003 |
| TH/IBV/2014/CU-62 | Broiler | 50 days | 2014/December | Nakhon Ratchasima | MG191004 |
| TH/IBV/2014/CU-64 | Broiler | 30 days | 2014/June | Nakhon Pathom | - |
| TH/IBV/2014/CU-65 | Broiler | 26 days | 2014/October | Prachinburi | MG191005 |
| TH/IBV/2014/CU-66 | Broiler | 23 days | 2014/June | Khon Khan | - |
| TH/IBV/2014/CU-67 | Broiler | 31 days | 2014/April | Pitsanulok | - |
| TH/IBV/2014/CU-68 | Broiler | 40 days | 2014/January | Saraburi | - |
| TH/IBV/2014/CU-70 | Broiler | 30 days | 2014/April | Saraburi | - |
| TH/IBV/2014/CU-71 | Broiler | 19 days | 2014/June | Chumphon | - |
| TH/IBV/2014/CU-72 | Broiler | 32 days | 2014/March | Saraburi | - |
| TH/IBV/2014/CU-73 | Layer | 46 weeks | 2014/June | Saraburi | - |
| TH/IBV/2014/CU-76 | Broiler | 32 days | 2014/January | Nakhon Ratchasima | - |
| TH/IBV/2014/CU-77 | Layer | 30 days | 2014/September | Surat Thani | MG191006 |
| TH/IBV/2014/CU-78 | Broiler | 34 days | 2014/June | Uttaradit | - |
| TH/IBV/2014/CU-81 | Layer | 7 weeks | 2014/May | Prachinburi | - |
| TH/IBV/2014/CU-82 | Broiler | 42 days | 2014/June | Saraburi | - |
| TH/IBV/2014/CU-83 | Broiler | 21 days | 2014/March | Saraburi | - |
| TH/IBV/2016/CU-85 | Broiler | 28 days | 2015/June | Chonburi | MG191007 |
| TH/IBV/2016/CU-86 | Broiler | 26 days | 2016/March | Prachinburi | MG191008 |
| TH/IBV/2016/CU-87 | Broiler | 24 days | 2016/March | Songkhla | MG191009 |
| TH/IBV/2016/CU-89 | Broiler | 29 days | 2016/March | Saraburi | MG191010 |

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| Virus name | Type of raising | Age | Time of collection | Location | Accession No. |
|---|---|---|---|---|---|
| TH/IBV/2016/CU-90 | Broiler | 21 days | 2016/March | Saraburi | MG191011 |
| TH/IBV/2016/CU-91 | Broiler | 28 days | 2016/March | Chonburi | MG191012 |
| TH/IBV/2016/CU-92 | Broiler | 30 days | 2016/March | Lampang | MG191013 |
| TH/IBV/2016/CU-93 | Layer | 6 weeks | 2016/March | Prachinburi | MG191014 |
| TH/IBV/2016/CU-94 | Broiler | 31 days | 2016/March | Saraburi | MG191015 |
| TH/IBV/2016/CU-96 | Broiler | 38 days | 2016/February | Chonburi | MG191016 |
| TH/IBV/2016/CU-97 | Broiler | 19 days | 2016/February | Prachinburi | MG191017 |
| TH/IBV/2016/CU-99 | Breeder | 20 days | 2016/February | Phetchabun | MG191018 |
| TH/IBV/2016/CU-101 | Broiler | 20 days | 2016/April | Prachinburi | MG191019 |
| TH/IBV/2016/CU-102 | Broiler | 20 days | 2016/April | Prachinburi | MG191020 |
| TH/IBV/2016/CU-103 | Broiler | 19 days | 2016/April | Yasothon | MG191021 |
| TH/IBV/2016/CU-104 | Broiler | 26 days | 2016/April | Udon thani | MG191022 |
| TH/IBV/2016/CU-105 | Broiler | 40 days | 2016/April | Saraburi | MG191023 |
| TH/IBV/2016/CU-106 | Broiler | 20 days | 2016/January | Chonburi | MG191024 |
| TH/IBV/2016/CU-107 | Layer | 15 weeks | 2016/January | Saraburi | MG191025 |
| TH/IBV/2016/CU-108 | Broiler | 41 days | 2016/January | Saraburi | MG191026 |
| TH/IBV/2016/CU-109 | Broiler | 41 days | 2016/January | Saraburi | MG191027 |
| TH/IBV/2016/CU-110 | Broiler | 29 days | 2016/January | Saraburi | MG191028 |
| TH/IBV/2016/CU-111 | Broiler | 30 days | 2016/January | Nakhon Ratchasima | MG191029 |
| TH/IBV/2016/CU-112 | Broiler | 20 days | 2016/January | Chachoengsao | MG191030 |
| TH/IBV/2016/CU-113 | Broiler | 31 days | 2016/January | Tak | MG191031 |
| TH/IBV/2016/CU-114 | Broiler | 26 days | 2016/February | Chachoengsao | MG191032 |
| TH/IBV/2016/CU-115 | Layer | 26 weeks | 2016/February | Ayutthaya | MG191033 |
| TH/IBV/2016/CU-116 | Broiler | 29 days | 2016/February | Saraburi | MG191034 |
| TH/IBV/2016/CU-117 | Broiler | 29 days | 2016/February | Ayutthaya | MG191035 |
| TH/IBV/2016/CU-118 | Broiler | 23 days | 2016/April | Nakhon Ratchasima | MG191036 |
| TH/IBV/2016/CU-119 | Broiler | 23 days | 2016/April | Chachoengsao | MG191037 |
| TH/IBV/2016/CU-120 | Layer | 13 weeks | 2016/June | Chachoengsao | MG191038 |
| TH/IBV/2016/CU-123 | Layer | 18 weeks | 2016/May | Saraburi | MG191039 |
| TH/IBV/2016/CU-124 | Broiler | 40 days | 2016/May | Nakhon Ratchasima | MG191040 |
| TH/IBV/2016/CU-129 | Broiler | 21 days | 2016/September | Chonburi | MG191041 |
| TH/IBV/2016/CU-130 | Broiler | 31 days | 2016/September | Chonburi | MG191042 |
| TH/IBV/2016/CU-133 | Broiler | 30 days | 2016/August | Chonburi | MG191043 |
| TH/IBV/2016/CU-134 | Broiler | 30 days | 2016/August | Chonburi | MG191044 |
| TH/IBV/2016/CU-135 | Broiler | 30 days | 2016/August | Chonburi | MG191045 |
| TH/IBV/2016/CU-139 | Breeder | 42 weeks | 2016/October | Suphanburi | MG191046 |
| TH/IBV/2015/CU-140 | Broiler | 32 days | 2015/January | Chonburi | MG191047 |
| TH/IBV/2015/CU-141 | Broiler | 25 days | 2015/January | Chachoengsao | MG191048 |
| TH/IBV/2015/CU-142 | Broiler | 41 days | 2015/February | Buriram | MG191049 |

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| Virus name | Type of raising | Age | Time of collection | Location | Accession No. |
|---|---|---|---|---|---|
| TH/IBV/2015/CU-143 | Breeder | 28 days | 2015/February | Chonburi | MG191050 |
| TH/IBV/2015/CU-145 | Broiler | 30 days | 2015/February | Nakhon Ratchasima | MG191051 |
| TH/IBV/2015/CU-146 | Broiler | 30 days | 2015/February | Nakhon Ratchasima | MG191052 |
| TH/IBV/2015/CU-147 | Broiler | 35 days | 2015/March | Saraburi | MG191053 |
| TH/IBV/2015/CU-148 | Broiler | 36 days | 2015/March | Saraburi | MG191054 |
| TH/IBV/2015/CU-149 | Broiler | 38 days | 2015/May | Saraburi | MG191055 |
| TH/IBV/2015/CU-150 | Broiler | 34 days | 2015/May | Chachoengsao | MG191056 |
| TH/IBV/2016/CU-151 | Broiler | 36 days | 2016/September | Ayutthaya | MG191057 |
| TH/IBV/2016/CU-152 | Broiler | 28 days | 2016/July | Prachinburi | MG191058 |
| TH/IBV/2016/CU-153 | Broiler | 22 days | 2016/May | Nakhon Ratchasima | MG191059 |
| TH/IBV/2016/CU-155 | Layer | 25 weeks | 2016/August | Chonburi | MG191060 |
| TH/IBV/2016/CU-156 | Broiler | 29 days | 2016/September | Prachinburi | MG191061 |
| TH/IBV/2016/CU-157 | Broiler | 23 days | 2016/October | Prachinburi | MG191062 |
| TH/IBV/2016/CU-158 | Broiler | 34 days | 2016/October | Chonburi | MG191063 |
| TH/IBV/2016/CU-161 | Broiler | 18 days | 2016/September | Lampang | MG191064 |
| TH/IBV/2016/CU-162 | Layer | 14 weeks | 2016/November | Songkhla | MG191065 |
| TH/IBV/2016/CU-163 | Layer | 14 weeks | 2016/November | Songkhla | MG191066 |
| TH/IBV/2015/CU-165 | Broiler | 40 days | 2015/February | Chonburi | MG191067 |
| TH/IBV/2015/CU-167 | Broiler | 23 days | 2015/August | Nakhon Ratchasima | MG191068 |
| TH/IBV/2015/CU-168 | Broiler | 21 days | 2015/September | Uttaradit | MG191069 |
| TH/IBV/2015/CU-169 | Broiler | 27 days | 2015/November | Saraburi | MG191070 |
| TH/IBV/2014/CU-171 | Broiler | 31 days | 2014/January | Chonburi | - |
| TH/IBV/2014/CU-172 | Broiler | 26 days | 2014/March | Kamphaengphet | - |
| TH/IBV/2014/CU-173 | Broiler | 45 days | 2014/May | Nakhon Ratchasima | - |
| TH/IBV/2014/CU-175 | Broiler | 21 days | 2014/June | Nakhon Ratchasima | - |
| TH/IBV/2014/CU-176 | Broiler | 25 days | 2014/June | Chumphon | - |
| TH/IBV/2014/CU-177 | Broiler | 44 days | 2014/June | Nakhon Ratchasima | - |
| TH/IBV/2014/CU-178 | Broiler | 43 days | 2014/June | Nakhon Ratchasima | - |
| TH/IBV/2014/CU-179 | Broiler | 24 days | 2014/July | Ayutthaya | MG191071 |
| TH/IBV/2014/CU-182 | Broiler | 20 days | 2014/August | Khon Khan | MG191072 |
| TH/IBV/2014/CU-183 | Broiler | 36 days | 2014/August | Saraburi | MG191073 |
| TH/IBV/2014/CU-185 | Broiler | 21 days | 2014/August | Surat Thani | MG191074 |
| TH/IBV/2014/CU-186 | Broiler | 31 days | 2014/August | Kanchanaburi | MG191075 |
| TH/IBV/2014/CU-188 | Layer | 32 weeks | 2014/September | Pitsanulok | MG191076 |
| TH/IBV/2014/CU-189 | Broiler | 31 days | 2014/September | Chachoengsao | MG191077 |

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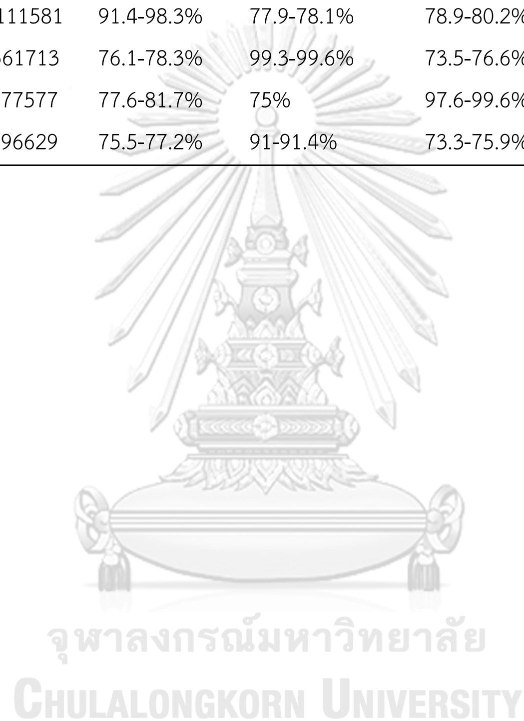
Table 8 Amino acid identity of the complete S1 of the 2014-2016 Thai IBVs with IBV reference strains and 2008-2013 Thai IBVs.

| Reference strains | Accession No. | 2014-2016 Thai IBVs | | | | |
|---|---|---|---|---|---|---|
| | | QX-like | Massachusetts | 4/91 | Connecticut | New genotype |
| QXIBV | AF193423 | 90.7-96.8% | 77.8-78.1% | 78.9-80.2% | 76.8-77% | 92% |
| LX4 | AY338732 | 89.7-96.1% | 77-77.2% | 78.5-79.4% | 76.3-76.4% | 91.2% |
| 2008-2013 Thai IBVs | | | | | | |
| - THA231251 | GQ885130 | 90.7-97.6% | 77-77.2% | 78.3-79.6% | 76.3-76.4% | 91.4-91.8% |
| - THA351052 | GU111581 | 91.4-98.3% | 77.9-78.1% | 78.9-80.2% | 77-77.2% | 92-92.3% |
| Massachusetts | AY561713 | 76.1-78.3% | 99.3-99.6% | 73.5-76.6% | 91.2-91.4% | 76.6-76.8% |
| 4/91 | KF377577 | 77.6-81.7% | 75% | 97.6-99.6% | 74.6-74.8% | 86.7% |
| Connecticut | KF696629 | 75.5-77.2% | 91-91.4% | 73.3-75.9% | 99.6-99.8% | 75.7-75.9% |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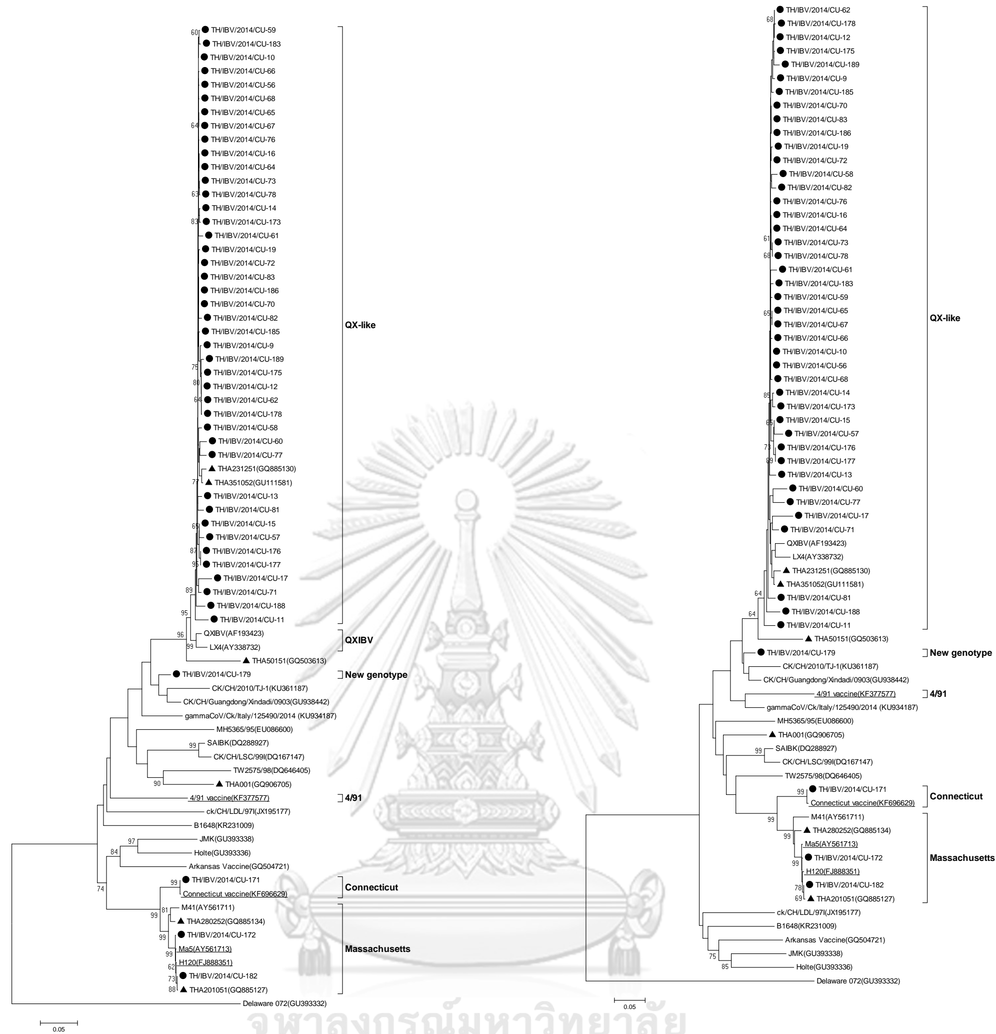
9A

9B

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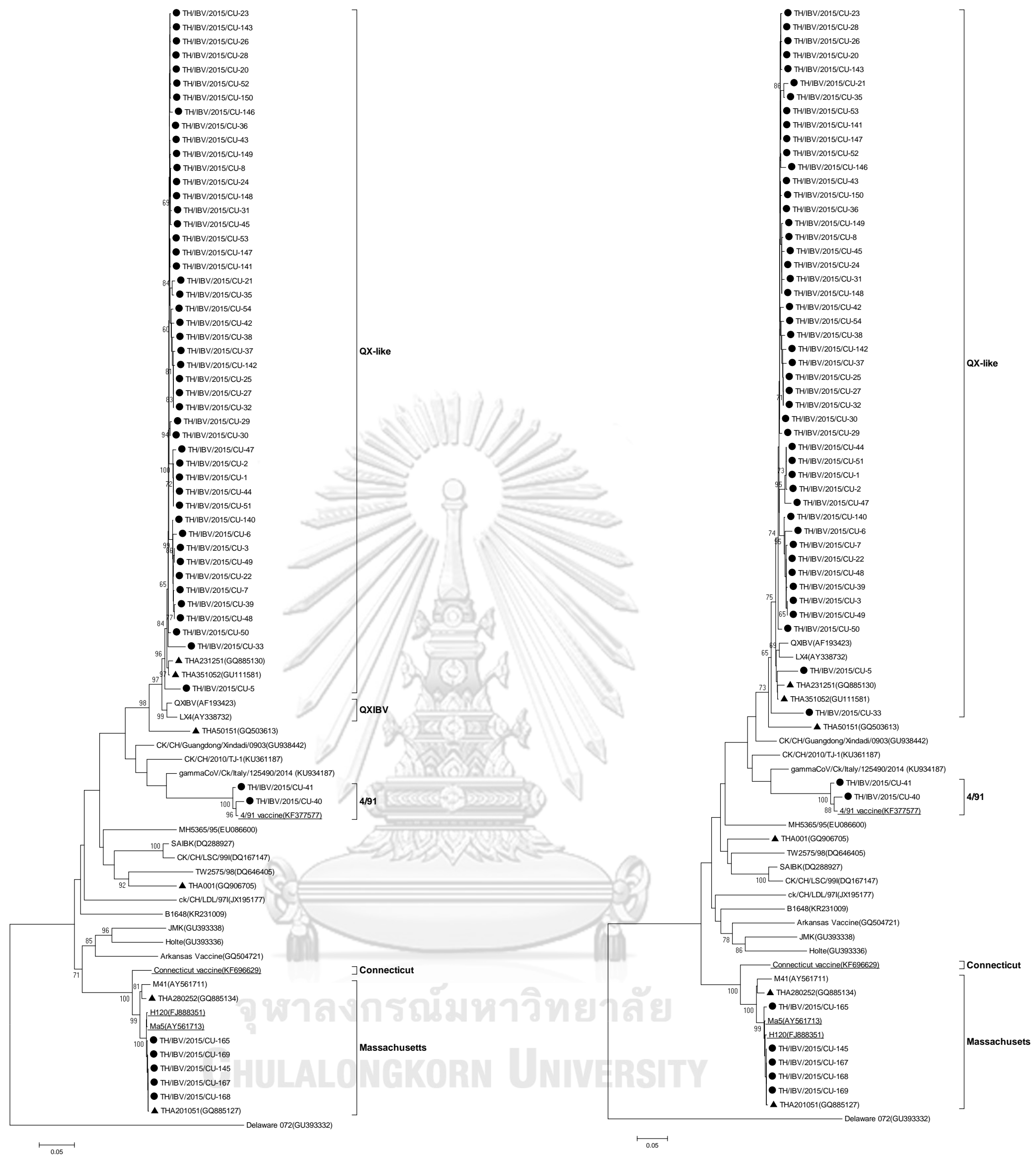
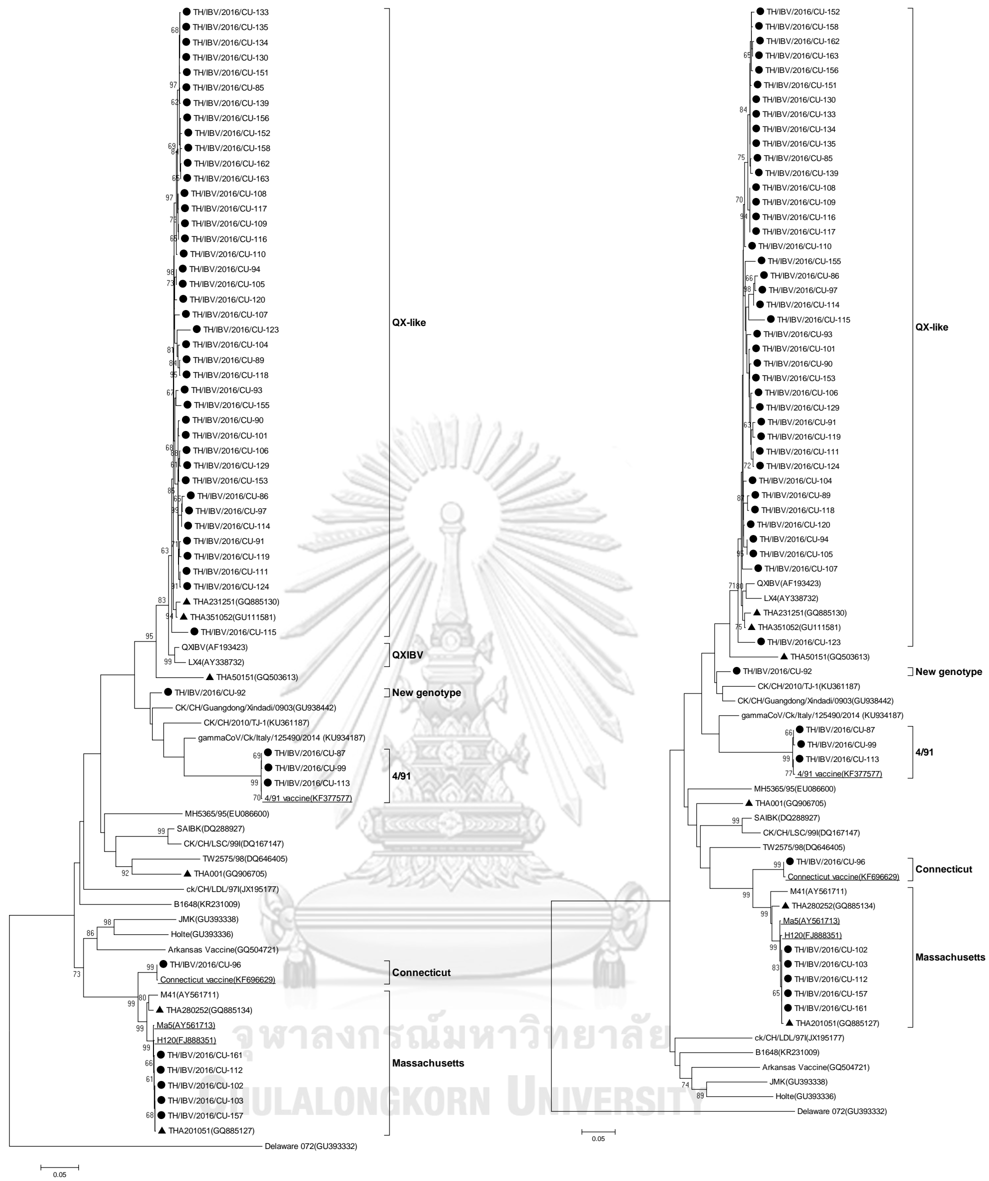


9E

9F

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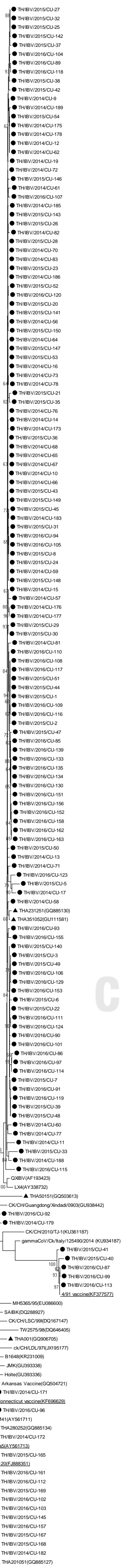
9G




9H

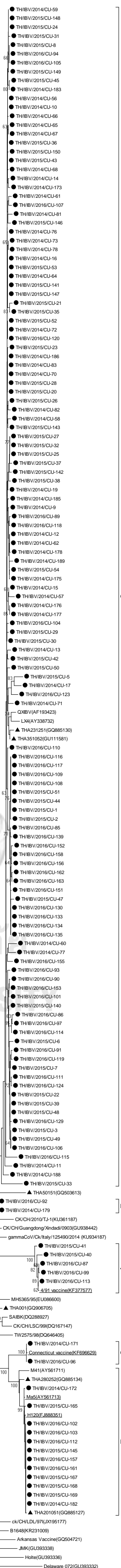


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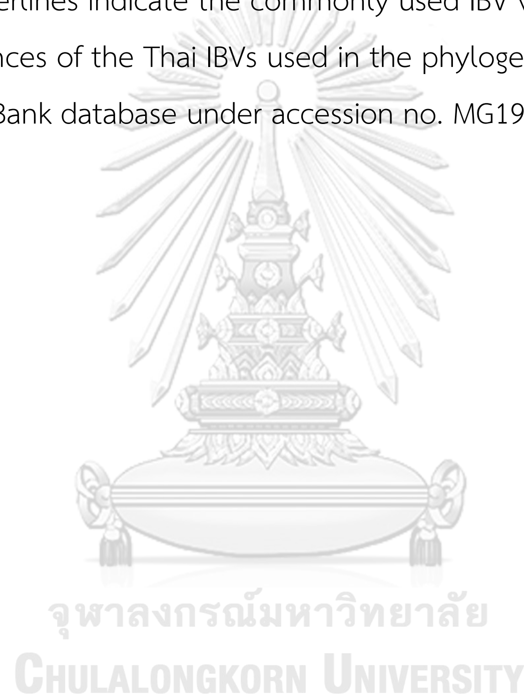
Figure 9 Phylogenetic tree of the complete S1 genes of Thai IBVs circulating in 2014 (A, B), 2015 (C, D), 2016 (E, F) and during 2014-2016 (G, H) characterized in this study and the selected reference IBVs.

The nucleotide (A, C, E and G) and amino acid (B, D, F and H) sequences were aligned using Muscle v.3.6 (Edgar, 2004). The phylogenetic trees were constructed in MEGA v.6.0 using the neighbor-joining algorithm with the Kimura-2 parameter model applied to 1,000 replications of bootstrap (Tamura et al., 2013). Circles indicate the 2014-2016 Thai IBVs identified in this study, triangles indicate the previously isolated Thai IBVs and underlines indicate the commonly used IBV vaccine strains. The nucleotide sequences of the Thai IBVs used in the phylogenetic analysis were submitted to GenBank database under accession no. MG190958-191077.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**S1 gene recombination analysis**

To examine the possible recombination events in the S1 genes of the 2014-2016 Thai IBVs, 150 Thai IBVs identified during 2014-2016 were analyzed using RDP4 and SimPlot software. Among the 150 Thai IBVs tested, a total of two novel recombinant variants (TH/IBV/2014/CU-179 and TH/IBV/2016/CU-92) were identified. Recombination analysis demonstrated that these two novel recombinant variants were originated from Thai QX-like IBV (TH/IBV/2014/CU-17) as a major parent and 4/91 vaccine strain as a minor parent (Table 9; Figure 10). The positions of the recombination breakpoint in TH/IBV/2014/CU-179 and TH/IBV/2016/CU-92 were located at nucleotide positions 870 and 858 in the S1 gene, respectively (Table 9; Figure 10). The recombination events were further confirmed by SimPlot software. Similar to the findings retrieved from RDP4 software, the two novel recombinant variants identified in this study were derived from Thai QX-like IBV (TH/IBV/2014/CU-17) and 4/91 vaccine strain (Figure 10). The positions of the recombination breakpoint in TH/IBV/2014/CU-179 and TH/IBV/2016/CU-92 were estimated at nucleotide positions 834 and 858 in the S1 gene, respectively (Figure 10).

To further confirm the origins of each part of the S1 gene of the novel recombinant variants, the S1 gene sequence of these variants were cut into two fragments according to the recombination analysis and conducted the phylogenetic analysis. The phylogenetic analysis based on each recombination region of the S1 gene of the novel recombinant variants demonstrated that the nucleotide positions at 1-834 of a TH/IBV/2014/CU-179 isolate was most closely related and clustered with Thai QX-like IBV (TH/IBV/2014/CU-17) (95.7% identity), while the nucleotide positions at 835-1656 shared high identity with 4/91 vaccine strain (100% identity) (Figure 10). For a TH/IBV/2016/CU-92 isolate, the nucleotide positions at 1-858 appeared very similar to Thai QX-like IBV (TH/IBV/2014/CU-17) (95.6% identity), while the nucleotide positions at 859-1651 was grouped together with 4/91 vaccine strain (99.9% identity) (Figure 10). Overall, the results collectively indicated that TH/IBV/2014/CU-179 and

TH/IBV/2016/CU-92 were the novel recombinant viruses, which possibly emerged from the recombination events between Thai QX-like IBV and 4/91 genotypes.

Table 9 Recombination events in the S1 gene of the novel recombinant Thai IBVs detected by RDP4 software.

| Virus name | Breakpoints | | Major parent[a] (similarity) | Minor parent[b] (similarity) | Detection methods (*p*-value)[c] |
|---|---|---|---|---|---|
| | Beginning | Ending | | | |
| TH/IBV/2014/CU-179 | 834 | 1656 | TH/IBV/2014/CU-17 (95.7%) | 4/91 (100%) | RDP, GENECONV, BootScan, MaxChi, Chimaera, SiScan, 3Seq ($6.120 \times 10^{-48}$, $4.268 \times 10^{-36}$, $8.791 \times 10^{-47}$, $5.589 \times 10^{-28}$, $2.676 \times 10^{-28}$, $9.925 \times 10^{-34}$, $7.524 \times 10^{-73}$) |
| TH/IBV/2016/CU-92 | 858 | 1651 | TH/IBV/2014/CU-17 (95.6%) | 4/91 (99.9%) | RDP, GENECONV, BootScan, MaxChi, Chimaera, SiScan, 3Seq ($6.120 \times 10^{-48}$, $4.268 \times 10^{-36}$, $8.791 \times 10^{-47}$, $5.589 \times 10^{-28}$, $2.676 \times 10^{-28}$, $9.925 \times 10^{-34}$, $7.524 \times 10^{-73}$) |

[a] Major parent is the gene sequence of parent providing the larger part of the recombinant virus's sequence.

[b] Minor parent is the gene sequence of parent providing the smaller part of the recombinant virus's sequence.

[c] All recombination events were detected by all seven detection methods and the recombination events were accepted only when they were detected by five or more methods implemented in the RDP4, with a *p*-value lower than $10^{-14}$

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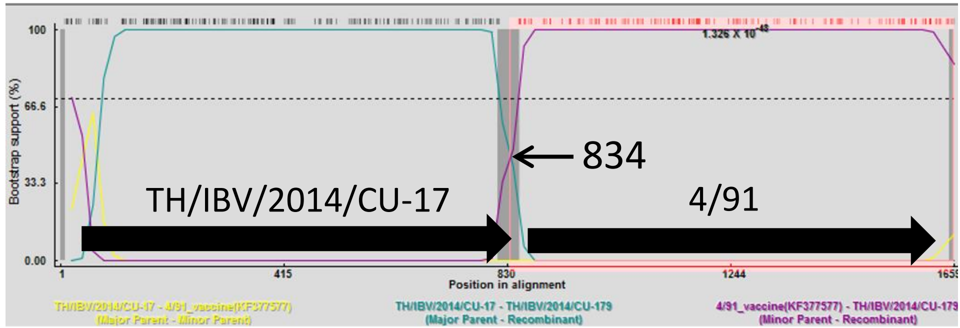
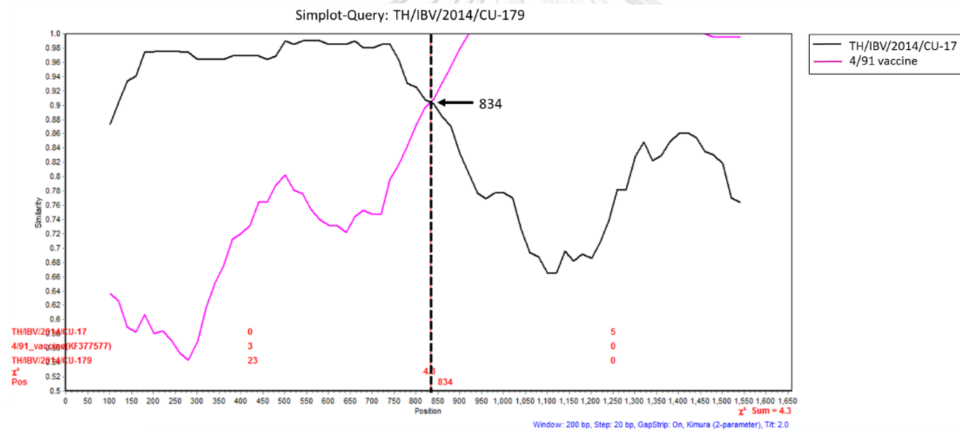
10A



10B

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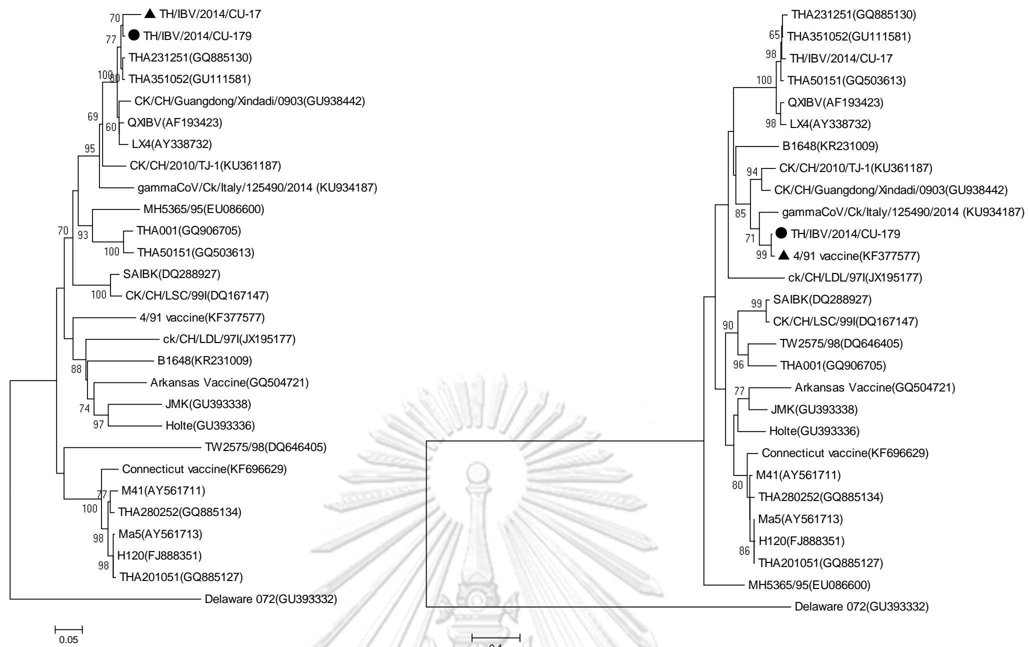
10C 1-834                                                    10D 835-1656

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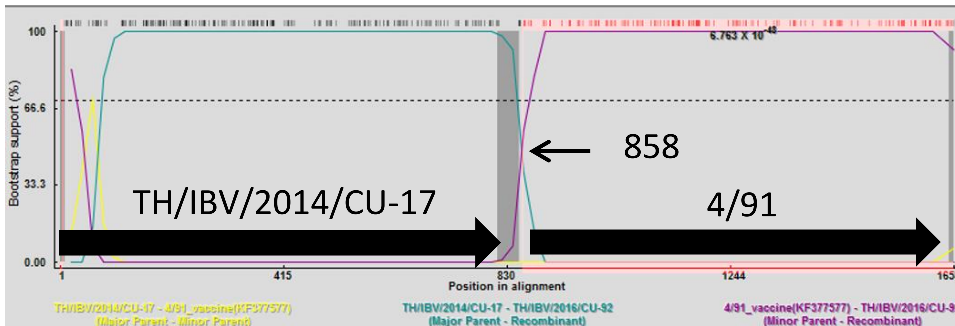
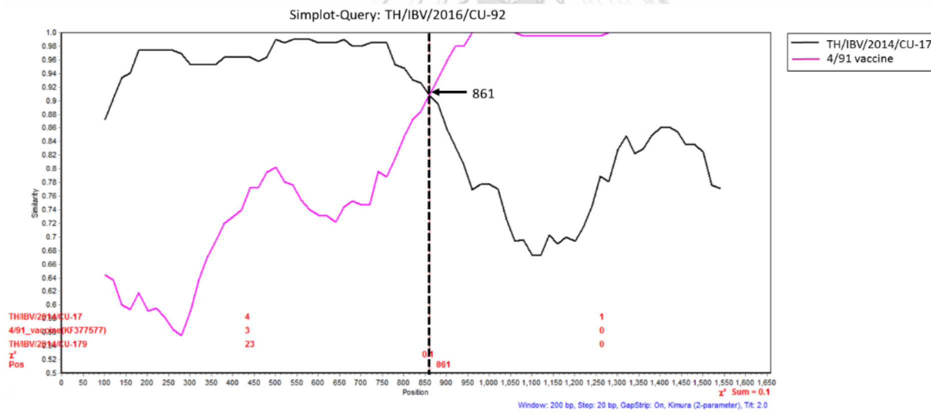


10F



**10G 1-858** **10H 859-1651**

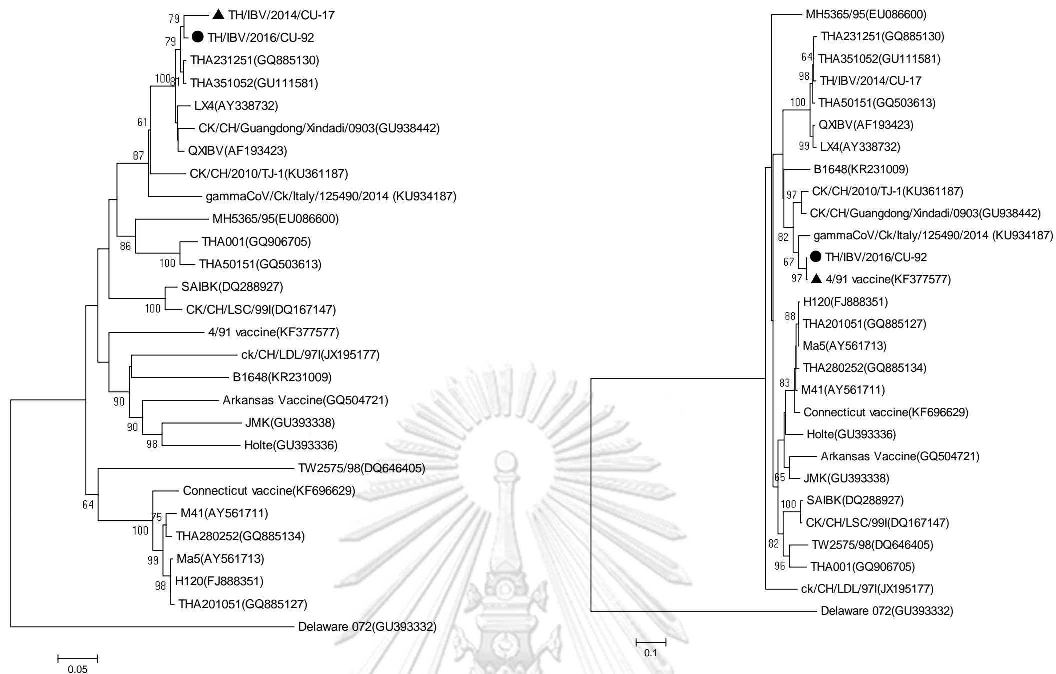



Figure 10 Recombination analysis of the novel recombinant Thai IBVs identified in this study (TH/IBV/2014/CU-179 (A, B, C and D) and TH/IBV/2016/CU-92 (E, F, G and H)).

The results from RDP4 and SimPlot software showing the possible recombination events in TH/IBV/2014/CU-179 (A and B) and TH/IBV/2016/CU-92 (E and F), respectively. The positions of recombination break point are shown as black arrows. Phylogenetic trees of each recombination region of the S1 gene of the novel recombinant Thai IBVs (TH/IBV/2014/CU-179 (C, D) and TH/IBV/2016/CU-92 (G, H)). The phylogenetic trees were constructed in MEGA v.6.0 using the neighbor-joining algorithm with the Kimura-2 parameter model applied to 1,000 replications of bootstrap (Tamura et al., 2013). Circles indicate the novel recombinant Thai IBVs (TH/IBV/2014/CU-179 and TH/IBV/2016/CU-92). Triangles indicate the potential parent strains involved in the recombination events of TH/IBV/2014/CU-179 and TH/IBV/2016/CU-92.

# CHAPTER 6

# Discussion

Infectious bronchitis (IB), caused by infectious bronchitis virus (IBV), is a highly contagious viral disease in chickens. This disease primarily causes an upper respiratory disease; however, some strains of IBV can cause clinical diseases in the urogenital, the reproductive and the gastrointestinal tracts, resulting in nephritis, reduced egg production and quality in layers, enteritis and significant mortality. Currently, IB causes significant economic losses to the poultry industry worldwide due to the frequent emergence of novel IBV variants, which can potentially lead to vaccination breaks (Reddy et al., 2015). This finding highlights the importance of continuous monitoring of IBV genotypes in chickens worldwide for effective control and prevention of IB. In this study, we investigated the genetic characteristic of IBV strains circulating in Thai chicken flocks during 2014-2016. Our results demonstrated that IBV infection is widely distributed throughout Thailand, indicating that IBV infection is endemic in Thai chicken flocks. In contrast to previous reports in 2008-2013 (Pohuang et al., 2011; Promkuntod et al., 2015), Thai IBVs circulating in Thailand during 2014-2016 were classified into 5 distinct genotypes, including QX-like IBV, Massachusetts, 4/91, Connecticut and a novel IBV genotype. Among Thai IBVs characterized, QX-like IBV was predominantly circulated, while a unique Thai IBV (THA50151) and THA001 reported previously disappeared from Thai chicken flocks. In addition, a novel IBV genotype derived from Thai QX-like IBV and 4/91 strain was identified in Thai chicken flocks in 2014 and 2016. To the best of our knowledge, this is the first report on the emergence of a novel recombinant IBV genotype, which is originated from Thai QX-like IBV and 4/91 strain.

Our data showed that IBVs were detected predominantly in the eastern, the central and the northeastern regions of Thailand, especially Saraburi, Chonburi and

Nakhon Rachasima provinces. This may result from the geographic distribution of chicken farms in Thailand, which are mostly located in those particular regions (Bureau of Livestock Standard and Certification, 2016) In addition, our result demonstrated that most of the IBV positive samples were obtained from broilers, which possibly correlates with the higher numbers of broiler chickens in Thailand when compared to other types of chickens (Bureau of Livestock Standard and Certification, 2016).

Genetic analysis revealed that five IBV genotypes, including QX-like IBV, Massachusetts, 4/91, Connecticut and a novel IBV genotype, had been circulating in Thailand during 2014-2016. It is noted that IBV genotypes circulating in Thai chicken flocks among 2014, 2015 and 2016 were slightly different. Overall, our result showed that the genetic characteristic of IBVs in Thailand has changed when compared to the previous reports (Pohuang et al., 2011; Promkuntod et al., 2015). This finding suggested that IBVs circulating in Thailand have been continuously evolving, possibly leading to evade the immunization of currently used IBV vaccines. In addition, our result showed that amino acid mutations of Thai IBVs characterized in this study were observed across the S1 protein. Since S1 protein is involved in the induction of virus-neutralizing antibodies (Montassier, 2010), the amino acid mutations identified in the 2014-2016 Thai IBVs may affect the cross protection reactivity between the 2014-2016 Thai IBVs and other IBVs strains, particularly vaccine strains. Therefore, the cross-protective efficiency between field Thai IBVs and vaccine strains required further investigation.

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Our results also showed that QX-like IBV has become the predominant genotype currently circulating in chickens in Thailand, replacing unique Thai IBV (THA50151) and THA001 genotypes (Pohuang et al, 2011). This finding corresponded to several previous studies, which demonstrated that QX-like IBV is one of the most predominant IBV genotypes extensively circulating in chicken flocks worldwide, causing the major problem in the IBV-vaccinated chicken flocks in many countries (De Wit et al., 2011a). In addition to field IBV genotypes (QX-like IBV and a novel genotype), the remaining IBV genotypes circulating in Thailand were closely related to currently used

live attenuated IBV vaccine strains, including Massachusetts, 4/91 and Connecticut. Based on the complete S1 gene analysis, most strains were vaccine-derived strains (98.3%-98.7% amino acid identity with vaccine strains), while some strains were mutated vaccine strains, showing only 94.6%-97.8% amino acid identity with vaccine strains. This finding indicated that the use of IBV vaccines in chickens might lead to the emergence of mutated vaccine strains with potentially causing disease in the vaccinated chickens (Ball et al., 2016; Abozeid et al., 2017). Several previous studies showed that some mutated IBV vaccine strains could cause disease in vaccinated chickens, including ck/CH/LHLJ/140906, which emerged from recombination between 4/91 and H120 vaccine strains (Zhang et al., 2015) and ZJ971, which mutated from H120 vaccine strain (Zhang et al., 2010). However, whether the mutated IBV vaccine strains identified in this study can cause disease in chickens remain to be investigated.

The recombination analysis demonstrated that two novel recombinant variants first identified in this study were originated from Thai QX-like IBV and 4/91 vaccine strain, supporting the fact that the recombination of IBV can occur in not only between field strains, but also between field and vaccine strains (Moreno et al., 2017). The genetic recombination between vaccine and field strains has also frequently been detected in several countries (Bande et al., 2015). Our study showed that field QX-like IBV could be continuously isolated from vaccinated chickens in Thailand. This finding indicated that vaccination with ineffective live attenuated IBV vaccines possibly generates an environment where co-infection between circulating field and vaccine strains can increase the chance of recombination. It is noted that these two novel recombinant variants were isolated from chickens vaccinated with Massachusetts (H120 and Ma5) and 4/91 vaccine strains. However, no evidence of recombination between field and Massachusetts vaccine strains was found in this study, even though this vaccine have been extensively used to control IBV in Thailand. This may relate to the shorter persistence and shedding of Massachusetts vaccine in chickens, which might decrease the possibility of recombination between this vaccine and field strains (Bijlenga et al., 2004). Our finding showed that the recombination site of both novel

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recombinant variants was located at the HVR 3 of the S1 gene, indicating that the recombination event of these viruses frequently occurred in the HVR of the S1 gene corresponding to the previous reports (Zanaty et al., 2016). However, it is known that genetic recombination of IBV can occur in several genes during a natural infection (Thor et al., 2011b), further studies on the recombination analysis based on other genes of these novel recombinant variants should be conducted in order to provide more information on the genetic feature of these novel IBV variants. Although, the generation of many recombinant IBV variants can occur frequently in nature, these recombinant variants showed different levels of replicative fitness in a host (Montassier, 2010). Our result showed that a novel recombinant IBV genotype was detected repeatedly in different regions of Thailand in 2014 and 2016, suggesting the possibility of this novel virus to spread widely and become an epidemic strain in chickens in Thailand. Based on field observations, chickens infected with this novel recombinant IBV genotype had higher mortality rate (5.56%-16.8%) than those infected with commonly circulated QX-like IBV (1.48%-4.5%), indicating that this novel recombinant IBV genotype possibly enhanced virulence in infected chickens. However, additional studies will be required to investigate the replication and pathogenicity abilities of this novel recombinant genotype in chickens. Moreover, the cross-protective efficiency of commercial IBV vaccines against this novel genotype should be also evaluated for effective control and prevention of IB.

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In summary, our data collectively demonstrate the IBV genotypes currently circulating in Thailand and the emergence of a novel recombinant IBV genotype originating from Thai QX-like IBV and 4/91 vaccine strain. This study also indicates an increase in genetic diversity of IBVs circulating in Thai chicken flocks, possibly affecting the effectiveness of currently used IBV vaccines. Therefore, this study highlights the necessity of continued IBV surveillance in order to monitor the genetic evolution of IBVs for effective control and prevention of IB.

# CHAPTER 7

## Conclusion

The overall data collectively demonstrated the IBV genotypes circulating in Thailand during 2014-2016 and the emergence of a novel recombinant IBV genotype originating from Thai QX-like IBV and 4/91 vaccine strain. Our study indicated that the genetic characteristic of Thai IBVs has changed when compared to the characteristic described in previous reports, suggesting the continuing evolution of IBV in Thailand and an increase in genetic diversity of IBVs circulating in Thai chicken flocks. This possibly affects the effectiveness of currently used IBV vaccines. Therefore, this study highlights the necessity of continued IBV surveillance in order to monitor the genetic evolution of IBVs for effective control and prevention of IB. Additional studies on the cross-protective efficiency of commercial IBV vaccines against the 2014-2016 Thai IBVs and the pathogenesis study of these newly identified IBVs in chickens should be conducted in order to provide the valuable data for the development of an effective control and prevention plan of IB in Thailand.

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APPENDIX

# VITA

Sirorat Munyahongse was born on August 13, 1983 in Bangkok, Thailand. She received her Doctor of Veterinary Medicine (DVM) degree with the second-class honours from Faculty of Veterinary Science, Kasetsart University in 2007. Her research focuses on the infectious bronchitis virus in chickens. She has published a number of international scientific articles as follow;

The publication from her thesis:

Poster presentation

1. Munyahongse et al., 2016. Genetic characterization of infectious bronchitis viruses isolated from chickens in Thailand, 2014. Proceeding of The 15th Chulalongkorn University Veterinary Conference CUVC2016, Bangkok, Thailand 20-22 April 2016: 281-283.

2. Munyahongse et al., 2017. Genetic characterization of infectious bronchitis viruses isolated from chickens in Thailand, 2015. Proceeding of The 16th Chulalongkorn University Veterinary Conference CUVC2017, Bangkok, Thailand 22-24 March 2017: 225-226.

3. Munyahongse et al., 2017. Genetic characterization of infectious bronchitis viruses isolated from chickens in Thailand, 2016. Proceeding of The XXTH World Veterinary Poultry Association Congress 2017, Edinburgh, Scotland 4-8 September 2017: 364.

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