การพัฒนาวิธีการตรวจวินิจฉัย คุณลักษณะทางโมเลกุล และประสิทธิภาพของวัคซีนหลอดลมอักเสบติดต่อในไก่ในประเทศไทย

นาย ธวัชชัย โพธิ์เฮือง

วิทยานิพนธ์นี้เป็นส่วนหนึ่งของการศึกษาตามหลักสูตรปริญญาวิทยาศาสตรดุษฎีบัณฑิต
สาขาวิชาอายุรศาสตร์สัตวแพทย์ ภาควิชาอายุรศาสตร์
คณะสัตวแพทยศาสตร์ จุฬาลงกรณ์มหาวิทยาลัย
ปีการศึกษา 2553

DEVELOPMENT OF DIAGNOSTIC METHOD, MOLECULAR CHARACTERIZATION AND EFFICACY OF INFECTIOUS BRONCHITIS VIRUS VACCINES IN CHICKENS IN THAILAND

Mr. Tawatchai Pohuang

A Dissertation Submitted in Partial Fulfillment of the Requirements
for the Degree of Doctor of Philosophy Program in Veterinary Medicine
Department of Veterinary Medicine
Faculty of Veterinary Science
Chulalongkorn University
Academic year 2010

Thesis Title	Development of diagnostic method, molecular characterization and efficacy of infectious bronchitis virus vaccines in chickens in Thailand
By	Mr. Tawatchai Pohuang
Field of Study	Veterinary Medicine
Thesis Advisor	Professor Jiroj Sasipreeyajan, D.V.M., Ph.D.
Thesis Co-Advisor	Associate Professor Niwat Chansiripornchai, D.V.M., Ph.D.

Accepted by the Faculty of Veterinary Science, Chulalongkorn University in Partial Fulfillment of the Requirements for the Doctoral Degree

..... Dean of the Faculty of Veterinary Science
(Professor Mongkol Techakumphu, D.V.M., Doctorat de 3e cycle)

THESIS COMMITTEE

..... Chairman
(Associate Professor Ratanaporn Brahmasa, D.V.M., M.P.H)

..... Thesis Advisor
(Professor Jiroj Sasipreeyajan, D.V.M., Ph.D.)

..... Thesis Co-Advisor
(Associate Professor Niwat Chansiripornchai, D.V.M., Ph.D.)

..... Examiner
(Associate Professor Achara Tawatsin, B.Sc., M.Sc.)

..... Examiner
(Associate Professor Somsak Pakpinyo, D.V.M., Ph.D.)

..... Examiner
(Associate Professor Kanisak Oraveerakul, D.V.M., Ph.D.)

..... External Examiner
(Associate Professor Thaweesak Songserm, D.V.M., Ph.D.)

ธวัชชัย โพธิ์เฮือง: การพัฒนาวิธีการตรวจวินิจฉัย คุณลักษณะทางโมเลกุล และประสิทธิภาพของวัคซีนหลอดลมอักเสบติดต่อในไก่ในประเทศไทย (DEVELOPMENT OF DIAGNOSTIC METHOD, MOLECULAR CHARACTERIZATION AND EFFICACY OF INFECTIOUS BRONCHITIS VIRUS VACCINES IN CHICKENS IN THAILAND) อ.ที่ปรึกษาวิทยานิพนธ์หลัก: ศ.น.สพ.ดร. จิโรจ ศศิปรียจันทร์, อ.ที่ปรึกษาวิทยานิพนธ์ร่วม: รศ.น.สพ.ดร. นิวัตร จันทร์ศิริพรชัย, 83 หน้า

วัตถุประสงค์ของการศึกษาในครั้งนี้คือ 1) พัฒนาวิธี nested RT-PCR ในการตรวจหาเชื้อไวรัสหลอดลมอักเสบติดต่อรวมทั้งสายพันธุ์ที่แยกได้ในประเทศไทย 2) วิเคราะห์ลักษณะและศึกษาความหลากหลายภายในยีนเอสวันของเชื้อไวรัสหลอดลมอักเสบติดต่อ ที่แยกได้จากไก่ป่วยในประเทศไทยในปัจจุบัน 3) ศึกษาประสิทธิภาพของโปรแกรมวัคซีนที่เกิดจากการให้วัคซีน 2 สายพันธุ์ต่อการป้องกันการติดเชื้อไวรัสหลอดลมอักเสบติดต่อสายพันธุ์ที่แยกได้ในประเทศไทย สำหรับการพัฒนาวิธี nested RT-PCR นั้น ได้ทำการออกแบบ primer ขึ้นมาใหม่ แล้วทดสอบความสามารถในการตรวจหาเชื้อไวรัสหลอดลมอักเสบติดต่อ พบว่ามีความไวมากกว่าการเพาะแยกเชื้อด้วยไข่ไก่ฟัก 10 เท่า และมีความไวมากกว่าวิธี RT-PCR 10-100 เท่า นอกจากนี้ยังพบว่าเป็นวิธีที่มีความจำเพาะสูงต่อเชื้อไวรัสหลอดลมอักเสบติดต่อ จากนั้นตรวจหาเชื้อที่มีการระบาดของโรคในฟาร์มไก่ช่วงปี พ.ศ. 2551-2552 ซึ่งสามารถแยกเชื้อได้จำนวน 32 isolate แล้วทำการถอดรหัสพันธุกรรมภายในยีนเอสวัน และศึกษาความสัมพันธ์เชิงวิวัฒนาการกับสายพันธุ์ที่เคยมีรายงานในประเทศอื่นๆ พบว่าเชื้อที่แยกได้ในประเทศไทยแบ่งเป็น 3 กลุ่มคือ กลุ่มที่ 1 สายพันธุ์ที่พบเฉพาะในประเทศไทย กลุ่มที่ 2 สายพันธุ์ที่เหมือนกับ QXIBV และกลุ่มที่ 3 สายพันธุ์ที่เหมือนกับ Massachusetts type ที่น่าสนใจคือพบการกลายพันธุ์แบบ recombination ในเชื้อกลุ่มที่ 1 และ 2 จากนั้นทำการศึกษาการป้องกันโรคด้วยการให้วัคซีนเชื้อเป็น 2 สายพันธุ์ คือ H120 และ 4/91 โดยไก่ได้รับวัคซีนด้วยโปรแกรมที่แตกต่างกันเมื่ออายุ 1 และ 14 วัน แล้วทำการให้เชื้อพิษทับ (isolate THA80151) เมื่ออายุ 28 วัน พบว่าไก่ทุกกลุ่มมีการติดเชื้อไม่แตกต่างกัน แต่ไก่ที่ได้รับวัคซีนทุกกลุ่มมีน้ำหนักตัวดีกว่าไก่ที่ไม่ได้รับวัคซีนแต่ได้รับเชื้อพิษ ($p<0.05$) รวมทั้งอาการป่วยและรอยโรคทางจุลพยาธิวิทยาที่ท่อลมน้อยกว่าไก่ที่ไม่ได้รับวัคซีนแต่ได้รับเชื้อพิษ ($p<0.05$) แสดงว่าโปรแกรมวัคซีนที่ใช้ในการศึกษาครั้งนี้ช่วยลดการสูญเสียน้ำหนัก อาการและรอยโรค แต่ไม่สามารถป้องกันการติดเชื้อได้

ภาควิชา.....อายุรศาสตร์..... ลายมือชื่อนิสิต.....

สาขาวิชา.....อายุรศาสตร์สัตวแพทย์... ลายมือชื่ออาจารย์ที่ปรึกษา.....

ปีการศึกษา....2553..... ลายมือชื่ออาจารย์ที่ปรึกษาร่วม.....

5075961931: MAJOR VERINARY MEDICINE

KEYWORDS: INFECTIOUS BRONCHITIS VIRUS/ NESTED RT-PCR/ S1 GENE/ VACCINE

TAWATCHAI POHUANG: DEVELOPMENT OF DIAGNOSTIC METHOD, MOLECULAR CHARACTERIZATION AND EVALUATION OF INFECTIOUS BRONCHITIS VIRUS VACCINES IN CHICKENS IN THAILAND. THESIS ADVISOR: PROF. JIROJ SASIPREEYAJAN, Ph.D., THESIS CO-ADVISOR ASSOC. PROF. NIWAT CHANSIRIPORNCHAI, Ph.D., 83 pp.

The aims of this study were firstly to establish a nested RT-PCR assay for detection of infectious bronchitis virus (IBV) including IBV isolated in Thailand, secondly to determine the molecular characterization of the recent Thai IBV by analysis of the S1 genes, and lastly to evaluate the levels of protection generated by 2 live attenuated vaccine strains against an IBV isolated in Thailand. In the development of nested RT-PCR, we designed the new primer sets for this assay and the results showed that the sensitivity of the nested PCR was increased 10 fold from virus isolation and 10-100 folds from non-nested RT-PCR. There were no cross-reactions with other avian viruses. These results suggest that the nested RT-PCR can be a sensitive and specific method for the diagnosis of IBV infection. In the molecular characterization, we collected thirty-two Thai IBV isolates from the outbreaks of disease in commercial chicken farms during 2008-2009. After sequencing of the S1 gene, phylogenetic analysis was performed and this found that the Thai IBV isolates were divided into three distinct groups, unique to Thailand (group I), QX-like IBV (group II) and Massachusetts type (group III). Moreover, the recombination events were found in groups I and II, but not in group III Thai IBV. Based on these facts, the field IBV in Thailand has undergone genetic recombination and evolution. In the protection study by using live attenuated vaccine strains H120 and 4/91, the chickens were vaccinated at 1 and 14-day-old with different vaccination programs and challenged with Thai IBV isolate THA80151 at 28-day-old. The results showed that the body weight gains of the vaccinated chickens were higher than the infected but non vaccinated chickens ($p<0.05$). Furthermore, the morbidity rates and tracheal histopathologic lesion scores of vaccinated chickens were lower than the infected chickens that were not vaccinated ($p<0.05$) although the infection rates of the tracheas were similar. These suggested that the live attenuated vaccines used in this study could induce clinical protection when administered at an interval of 2 weeks but could not protect against the infection of a challenge strain.

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

Field of study.....Veterinary Medicine..... Advisor's Signature.....

Academic year....2010..... Co-advisor's Signature.....

ACKNOWLEDGMENTS

I would like to express my sincere gratitude to my advisor, Prof. Dr. Jiroj Sasipreeyajan, for giving me an opportunity to perform the advance research in avian medicine projects, lots of suggestions, kindly support, helpfulness and constant encouragement through the whole course of this work. I would like to thank my co-advisor, Assoc. Prof. Dr. Niwat Chansiripornchai for his valuable suggestions on my thesis work. I wish to thank Assoc. Prof. Achara Tawatsin and Assoc. Prof. Dr. Somsak Pakpinyo for their kind suggestions. My sincere thank is also expressed to Assoc. Prof. Dr. Kanisak Oraveerakul and Dr. Pravina Kitikoon for their valuable suggestions and allowing me to conduct my molecular works in laboratory of Virology unit. I am grateful to Miss Suwarak Wanaratana and Miss Kridda Chukiatsiri for helpful cooperation in many ways that support me in performing this work. I wish to thank the staffs of Virology unit for their help through my laboratory work. I am grateful to my colleague, Assoc. Prof. Kanlaya Chuchan, for her encouragement and valuable suggestions. I am very thankful to the Graduate School of the Faculty of Veterinary Science of Chulalongkorn University, Intervet Schering-Plough Animal Health Ltd., Bangkok, Thailand, and the 90th Anniversary of Chulalongkorn University fund (Ratchadaphiseksomphot Endowment Fund), Chulalongkorn University for granting me the research fund.

Finally, I wish to express my most sincere thank to my family especially, father, mother and sister for their endless love, understanding and encouragement given during my whole life.

CONTENTS

LIST OF TABLES

LIST OF FIGURES

CHAPTER I

INTRODUCTION

Infectious bronchitis (IB) is an acute viral respiratory disease of chickens and results in a significant economic loss to commercial chicken industries in many countries of the world. The disease is characterized by respiratory signs including gasping, coughing, sneezing, tracheal rales and nasal discharge (Parsons et al., 1992). All ages of chickens are susceptible to IBV infection, but the clinical signs are more severe in young chickens (Animas et al., 1994). In hens, respiratory distress and a decrease in egg production have been reported (Gough et al., 1992). Some strains of IBV can cause acute nephritis and urolithiasis associated with a high mortality of infected chickens (Ziegler et al., 2002; Liu and Kong, 2004). In addition, IBV has also been reported to cause proventriculitis (Yu et al., 2001). Furthermore, the disease is a risk factor for secondary bacterial infection resulting in an even higher morbidity and mortality (Ziegler et al., 2002).

Infectious bronchitis virus (IBV), the causative agent of IB, is a coronavirus. The genome of IBV consists of positive sense single stranded RNA, approximately 27.6 kilobases in length (Boursnell et al., 1987), that is encoded for four structural proteins: nucleocapsid (N) protein, envelope (E) protein, membrane (M) glycoprotein, and spike (S) glycoprotein (Cavanagh and Naqi, 2003). The S glycoprotein is post-translationally cleaved into the S1 and S2 subunits (Cavanagh et al., 1986). The S1 subunit, located on the outside of virion, is responsible for the fusion between the virus envelope and the cell membrane of the host (Boursnell et al., 1987). It contains virus neutralization and serotype-specific epitopes that are formed by amino acid within the defined hypervariable region (HVR); therefore, the molecular characterization of IBV is based on an analysis of the S1 gene (Kingham et al., 2000).

The continuous emergence of new serotypes or variant strains of IBV has been reported world-wide (Gelb et al., 1991; Gough et al., 1992; Jia et al., 1995; Liu and Kong, 2004; Pohuang et al., 2009). The events are thought to be generated by mutation processes including deletion and insertion of the nucleotides within IBV genome;

moreover, the evolution by genetic recombination has also been reported (Jia et al., 1995). The new serotypes or variant strains of IBV can cause disease in vaccinated chickens (Gelb et al., 1991; Gough et al., 1992; Liu and Kong, 2004). Therefore, these emergences are of great concern to poultry producers.

Current diagnosis of IBV infection includes virus isolation, serological test and molecular assay. The virus isolation and serological methods have several problems in that they are expensive, time-consuming, labor intensive and lack sensitivity and specificity (De Wit, 2000). Moreover, it is difficult to provide the standard methods that can be used to detect all IBV strains because different serotypes of IBV have been reported world wide and new variant serotypes continue to be recognized (Gelb et al., 1991; Liu and Kong, 2004). Several times, the previous published diagnostic methods can not detect those new variant strains (Lee et al., 2001; Mondal et al., 2001; Worthington et al., 2008). However, it is necessary and important to be able to diagnose these new variant strains and determine the type of them because it is an important factor for the selection of an appropriate vaccine against IBV infection in the next flocks.

Vaccination for the prevention of IB is widely practiced. However, IBV has been isolated in vaccinated chickens (Gelb et al., 1991; Gough et al., 1992; Liu and Kong, 2004). The vaccine failure may be caused by the differences in the genotypes or serotypes of vaccine strains and field strains (Pensaert and Lambrechts, 1994). In some cases, improvements in protection might be achieved by the use of a different IB vaccination (Fabio et al., 2000). At present, many strains of vaccine are commercially available. It is important to periodically evaluate the cross-protective capabilities of vaccines versus field isolates, because the outcomes of these studies will provide valuable information on the practical use of existing vaccines and the potential need for new ones.

In Thailand, the outbreak of IB was initially reported during 1953-1954 (Chindavanig, 1962). Since then, IB has continued to be an economically important disease in the Thai poultry industry and can be found all over the country (Upatoom et al., 1983; Antarasena et al., 1990). Upatoom et al. (1983) reported the incidence of disease outbreaks in the north-east of Thailand in broilers aged 14-28 days with 1-15% mortality rate. Among the reports of 35 incidences in the south of Thailand were 29 in

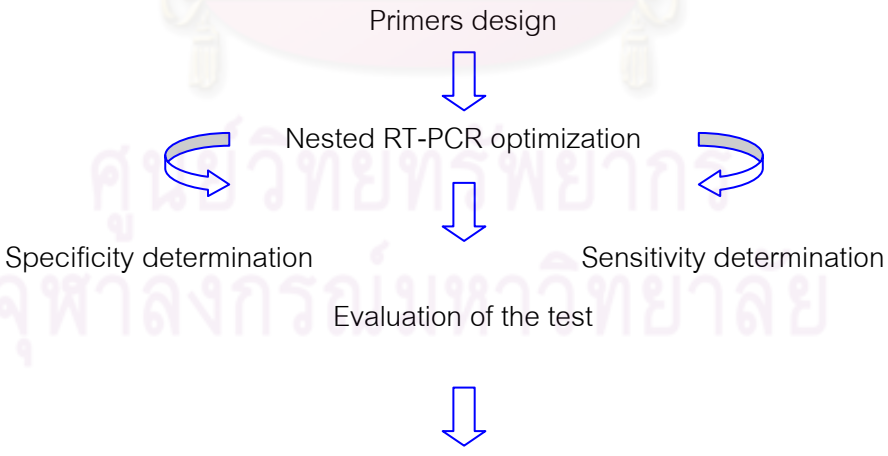
broilers aged 12-44 days, 5 in layers aged 8-40 weeks and 1 in 3 months old native chickens. Respiratory symptoms along with enlarged kidneys were observed in 21 incidences and enlarged kidneys only were observed in 4 incidences (Antarasena et al., 1990). Recently, we characterized IBV isolated in Thailand 1998 by analysis of the HVR of S1 genes and found that the Thai IBV isolates were difference from IBV in other countries and it was unique to Thai strain (Pohuang et al, 2009).

Objectives

1. To establish a nested RT-PCR assay for detection of IBV including IBV isolated in Thailand
2. To determine the molecular characterization of Thai IBV isolates in the recent disease outbreaks by analysis of the S1 genes and comparing them with previously published strains
3. To evaluate the levels of protection generated by 2 live attenuated vaccine strains against a recent IBV isolated in Thailand

Conceptual framework

Part 1: To establish a nested RT-PCR assay for detection of IBV



Part 2: To determine the molecular characterization of IBV isolated in Thailand

Detection of clinical cases associated with IBV infection by nested RT-PCR

Virus isolation and propagation

Amplification of S1 genes

Sequencing and sequence analysis

Part 3: To evaluate the protection generated by 2 live attenuated vaccine strains

IBV field isolate

Virus titration

Protection study in experimental chickens

Evaluation strategies

Virus detection, Body weight, Histopathology, Antibody response

Expected values

1) The results will provide a useful diagnostic method that can detect IBV including the IBV isolated in the recent disease outbreaks in Thailand.
2) The results will provide the information of the molecular characteristic and genetic evolution of IBV isolated in the recent outbreaks in Thailand.
3) The results will provide an appropriate vaccination protocol for the prevention of the chickens from infection with the recent Thai IBV isolate.

CHAPTER II

LITERATURE REVIEW

Infectious bronchitis virus

Infectious bronchitis virus (IBV), the causative agent of IB, is a group 3 coronavirus (Table 1). The four existing coronavirus groups were initially divided on the basis of a lack of antigenic relationships between the species of different groups (Cavanagh, 2003). IBV is enveloped, pleomorphic, with a mean diameter of approximately 120 nm. The virion has a large club-shaped on its surface (Figure 1). The genome of IBV consists of positive sense single stranded RNA, approximately 27.6 kilobases (kb) in length (Boursnell et al., 1987), which encoded for four structural proteins: nucleocapsid (N) protein, envelope protein, membrane (M) glycoprotein and spike (S) glycoprotein (Cavanagh and Naqi, 2003). The S glycoprotein is post-translationally cleaved into the S1 and S2 subunits (Cavanagh et al 1986).

Table 1 Coronavirus species (Cavanagh, 2003)

Group	Virus
Group1	Porcine transmissible gastroenteritis coronavirus
	Canine enteric coronavirus
	Feline coronavirus
	Porcine epidemic diarrhoea coronavirus
	Human coronavirus 229E
Group2	Murine hepatitis coronavirus
	Human coronavirus OC43
	Bovine coronavirus
	Canine respiratory coronavirus
	Porcine haemagglutinating encephalomyelitis coronavirus
Group3	Infectious bronchitis coronavirus
	Turkey coronavirus
	Pheasant coronavirus
Group4	SARS coronavirus

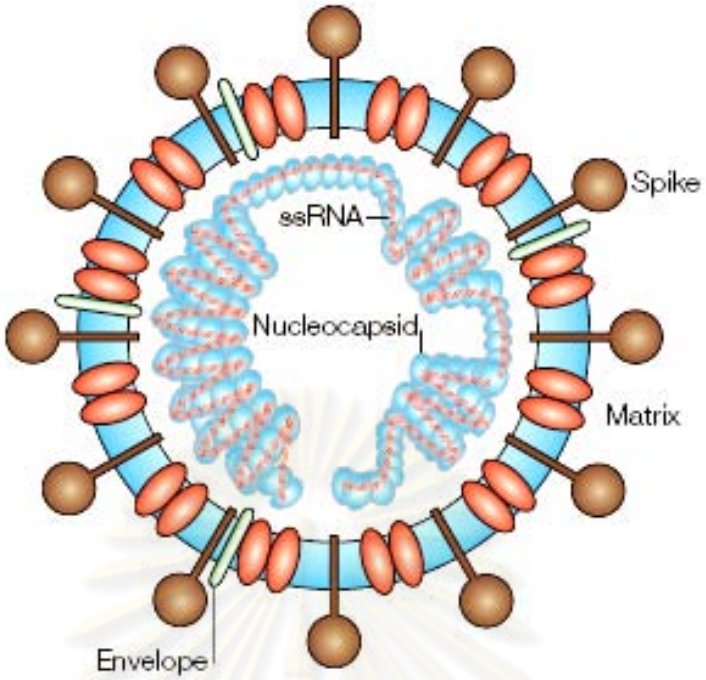


Figure 1 Coronavirus genome and virion (Perlman and Dandekar, 2005)

Antigenic determinants of Spike glycoprotein

It has been shown for IBV that the S glycoprotein is involved in the induction of neutralizing, serotype specific and protective immunity. The studies of epitopes on S glycoprotein indicate that the neutralizing epitopes are found on both S1 and S2 subunit (Cavanagh et al., 1988; Koch et al., 1990). Koch et al. (1990) found that the S glycoproteins of D207, D3896, D274, D3128, H120 and B801 strain were composed of eight epitopes. Six epitopes located in S1 subunit and two epitopes located in S2 subunit.

Parr and Collisson (1993) characterized the antigenic determinants of the S glycoprotein by using eleven monoclonal antibodies produced from the nephropathogenic Gray strain. They found that four monoclonal antibodies, the conformation-independent monoclonal antibodies, were considered group specific because they reacted with Arkansas DPI, Massachusetts 41 (M41) and Gray strains. Seven monoclonal antibodies, the conformation-dependent monoclonal antibodies, were

considered type specific because they reacted with Gray strain only. These indicated that eleven epitopes were present on S glycoprotein. Moreover, two monoclonal antibodies provided protection of chickens from kidney damage after challenge suggested that the epitopes on S glycoprotein reacted with these monoclonal antibodies associated with IBV-cell interaction that provide link to virus tropism and pathgenesis. The other reports have shown that conformation-independent neutralizing epitopes are located to the S2, whereas the conformation-dependent neutralizing epitopes are assigned to the S1 (Cavanagh et al., 1988; Lenstra et al., 1990).

S1 subunit

The S1 subunit of IBV forms a distal globular part of S glycoprotein, extending outwardly, and anchors to the S2 subunit which located in the virus envelope (Cavanagh, 1983). It has been shown for the S1 subunit that this part is responsible for the biological function of IBV including induction of neutralizing antibodies (Cavanagh et al., 1986; Ignjatovic and Galli, 1994), hemagglutinin activity and virus attachment to host cells (Cavanagh et al., 1986; Cavanagh and Davis, 1986).

The different serotypes, subtypes or IBV variant strains are thought to be occurred by amino acid changes in S1 subunit resulting from nucleotide deletion, insertion point mutation and recombination events (Cavanagh et al., 1986; Lee and Jackwood, 2000). Different IBV serotypes usually differ 20%-25% of amino acid in S1 subunit (Gelb et al., 1997). However, the S1 subunit of the same serotype may differ by 8%, whereas a variation of only 4.2% may also change the serotype because of the occurrence of amino acid change in S1 subunit (Moore et al., 1998).

Cavanagh et al. (1992) analyzed nucleotide and amino acid identity in S1 gene among three IBV serotypes including serotype1 (UK/6/82, NL/D207/79 and UK/142/86 strain), serotype2 (UK/167/84 strain) and serotype3 (UK/123/82 and NL/D3896/78 strain). They found that the difference in 19-32 nucleotides and 11 nucleotides were found between the strains within serotype1 and within serotype3, respectively. Nucleotide comparisons between serotypes showed that the difference in 19-34 nucleotides, 26-45 nucleotides and 19-34 nucleotides were found between serotype1 and 2, between serotype1 and 3 and between serotype2 and 3, respectively. The difference 6-14 amino

acids and 8 amino acids were found between the strains within serotype1 and within serotype3, respectively. Amino acid comparisons between serotypes showed that the difference in 10-17 amino acids, 16-23 amino acids and 20-23 amino acids were found between serotype1 and 2, between serotype1 and 3 and between serotype2 and 3, respectively.

The variable regions observed in S1 gene have been identified and designated as HVR which can be separated into two regions including HVR1 (residues 54-68) and HVR2 (residues 116-141) (Figure 2) (Kusters et al., 1989). After that other variable regions have been reported. Cavanagh et al. (1992) found that the most variable regions were observed within amino acid residues 19-122 and 251-374. Kant et al. (1992) found that the variable regions between D207 strain and its variant was observed within amino acid residues 26-61, 132-149 and 291-398 of S1 gene. Similar to the sequence variations among 19 IBV isolates from the United States, the sequence variations were observed between amino acids 55-96, 115-149, 255-309 and 378-395 (Moore et al., 1998).

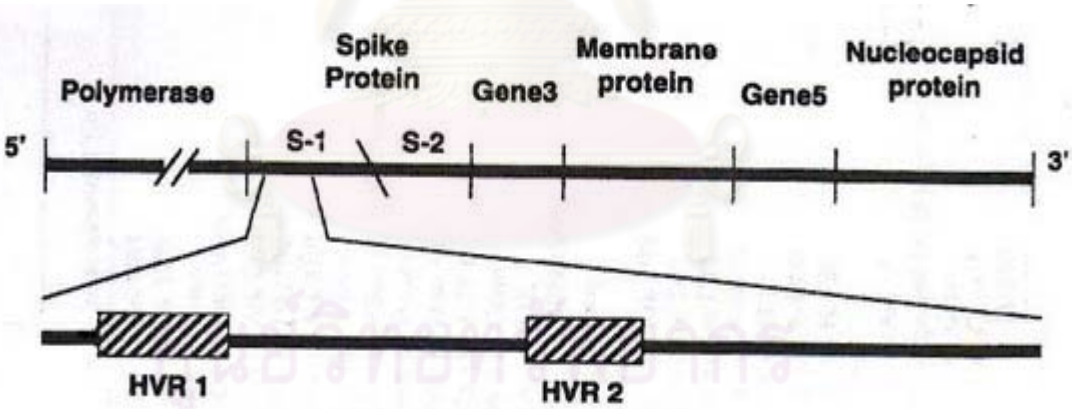


Figure 2 IBV genomic organization and hypervariable region in S1 gene. Schematic representation of the single stranded RNA genome of IBV. The cross-hatched boxes indicate the location of HVR1 and HVR2 (Kingham et al., 2000).

Clinical signs

IBV can cause an acute respiratory disease of chickens. The infected chickens show respiratory signs including gasping, coughing, sneezing, tracheal rales and nasal

discharge within 2 days post-infection, the mortality occur within 5-9 days post-infection (Chen et al., 1996). Occasionally, conjunctivitis, lacrimation, edema and cellulitis of the periorbital tissues and swollen sinuses may be seen (Parsons et al., 1992, Terregino et al., 2008). In addition the chickens appear lethargic, reluctant to move and in some case present with dyspnea (Terregino et al., 2008). The chickens can recover within 2 weeks post-infection (Chen et al., 1996).

IBV infection in hens is known to cause decline in egg production (Crinion et al., 1971; Crinion, 1972; Lucio and Fabricant, 1990; Parsons et al., 1992; Wang et al., 1996; Wang and Khan, 2000; Mondal et al., 2001) and deterioration of egg quality (Crinion et al., 1971; Crinion, 1972; Cook and Huggins, 1986; Wang et al, 1996; Wang and Khan, 2000). The appearance of paler shells, change in egg shape (Cook and Huggins, 1986; Chousalkar and Roberts, 2007a), soft-shell eggs (Wang and Khan, 2000) and laying eggs with watery albumin are found in infected hens (Crinion, 1972; Chousalkar and Roberts, 2007b). The severity of oviduct infection varies with the age of chickens at the time of infection and strains of IBV. The infection of hens at an early age can cause permanent damage to the developing reproductive tract, resulting in false layers at the laying period (Jones and Jordan, 1970; Crinion et al, 1971).

Some strains of IBV can cause diarrhea in the infected chickens. Some of the Australian T strain infected chicks are developed diarrhea and they have feces and urine matted to the cloaca (Chong and Apostolov, 1982). Villarreal et al. (2007) had reported the outbreak of moderate to severe enteric disease in 17 broilers and one laying farm. The infected chickens showed watery diarrhea, poor general condition and increased feed conversion ratio but without respiratory or reproductive signs. IBV could be isolated from the enteric samples of infected chickens and the strain appeared to be closely related to the D274 serotype. In the report of Yu et al. (2001), diarrhea is also found in the chickens infected with IBV strains that cause proventriculitis.

IBV-host cell interaction

The interaction of IBV with its cellular receptor is an importance step that will enable entry of virus into the cells. It has been shown that IBV uses an α2,3-linked sialic acid as a receptor determinant for attachment to red blood cells (Schultze et al., 1992).

Recently, the confirmation of IBV-receptor interaction on chicken cells has been reported. Winter et al. (2006) had shown that the primary chicken kidney cells incubated with neuraminidase enzyme became resistant to infection by IBV strain Beaudette and M41. It is known that neuraminidase enzyme has the ability to remove α2,3-linked sialic acid from the cell surfaces (Schultze et al., 1992; Ruano et al., 2000). Therefore, IBV recognize α2,3-linked sialic acid for its initial infection mechanism (Winter et al., 2006). Abd El Rahman et al. (2009) had demonstrated that there were many cells expressing α2,3-linked sialic acid on the tracheal epithelium. Moreover, IBV Beaudette, 4/91, Italy-02 and QX strain had the ability to use α2,3-linked sialic acid on the tracheal epithelium as a receptor determinant but the strains differed in the strength to use the receptor.

IBV can enter a host cell through endocytosis process (Chen and Itakura, 1996). Virus particles are found in endocytotic vesicles in the infected cells (Chong and Apostolov, 1982; Chen et al., 1996). Invaginations of cell membrane, resembling coated pits, in association with the encirclement of virus particles are observed (Chen and Itakura, 1996). The infected cells are swollen accompany with increasing in number of free ribosomes and polyribosomes. Mitochondria are swollen with increasing number of cisternae. The Golgi complex, rough endoplasmic reticulum (RER) and smooth endoplasmic reticulum (SER) are also dilated (Chen and Itakura, 1996; Chousalkar and Roberts, 2007b). The replication progressed changes are manifest by large numbers of virus particles present mostly in the dilated cisternae of RER and in the cytoplasmic vesicles (Chen and Itakura, 1996; Chousalkar and Roberts, 2007a). In the late change, the infected cells are markedly swollen, degenerated or detached. The mitochondria are swollen with disrupted cristae. The Golgi complex, RER and SER are dilated with varying sizes of cytoplasmic vesicles containing virus particles. At the free surface of cells, the cytoplasmic vesicles are fused with the cell membrane and the releasing of virus particles by exocytosis is observed (Chen and Itakura, 1996).

Pathogenesis and tissues tropism

Respiratory tract

The upper respiratory tract is the primary site of IBV infection. The replication of IBV is restricted to the ciliated epithelial cells and the mucus producing cells (Nakamura

et al., 1991). However, the strains of IBV differed in their efficiency to infect the tracheal epithelial cells (Abd El Rahman et al., 2009). The infection causes inhibition of ciliary activity (Benyeda et al., 2009) and deciliation of epithelial cells (Chousalkar, et al., 2007). The highest virus titers in the trachea are observed within 5-10 days post-infection (Otsuki et al., 1990).

The tracheal gross lesions are congested and it has excess mucus in the lumen (Parsons et al., 1992). In the air sacs, fine to marked thickening of the wall is recorded (Benyeda et al., 2009). It is assumed that IBV is detected in the air sac lining even though the membranes are visibly normal (Hofstad and Yoder, 1966). The respiratory tissues appear normal after 7 days post-infection (Dhinakar Raj and Jones, 1996b). The main histopathological lesions observed including loss of cilia, degenerative and hyperplastic changes in the epithelial cells, depletion of goblet cells and alveolar mucus glands and infiltration of inflammatory cells including heterophils, plasma cells and lymphocytes into the mucosa of the trachea (Nakamura et al., 1991).

In the tracheas, the lesions based on histopathological changes can be divided into three stages, acute (degenerative), hyperplastic and recovery stage. The most prominent finding in acute stage is degeneration and desquamation of ciliated epithelial cells and mucus producing cells (Nakamura et al., 1991; Benyeda et al., 2009). The degeneration and desquamation also occur in Goblet cells and mucus-secreting cells. The edema, mild infiltration of heterophils, lymphocytes and vascular congestions in the lamina propria and underlying connective tissue (between cartilaginous rings) are observed (Nakamura et al., 1991). In the hyperplastic stage, mononuclear cells infiltration, lymphocytic infiltration and newly formed of epithelial cells are observed (Nakamura et al., 1991; Benyeda et al., 2009). Sometimes hyperplasia of fibroblast-shaped epithelial cells (perhaps of basal cell origin) is found. In this stage, there are neither goblet cells nor alveolar mucus glands (Nakamura et al., 1991). The reparative processes and complete recovery of epithelial cells are the characteristic change found in recovery stage (Benyeda et al., 2009). There is slight infiltration of lymphocytes and plasma cells and a few lymphoid follicles in the lamina propria of the tracheas (Nakamura et al., 1991).

Kidney

Besides the respiratory tract, some strains of IBV can replicate and cause kidney damage. These strains are known as nephropathogenic IBV (NIBV). Up to date, NIBV has been reported world wide (Animas et al., 1994; Ziegler et al., 2002; Liu and Kong, 2004; Pohuang et al., 2009). It has been reported that NIBV can infect the epithelial cells of many parts of the kidney including proximal convoluted tubules, collecting tubules (Owen et al., 1991; Chen and Itakura, 1996), collecting ducts, distal convoluted tubules and Henle's loop (Chen and Itakura, 1996). The viral particles are observed in the kidney from 4-13 days post-infection. The maximum levels of the number of infected cells are observed at 6-8 days post-infection (Chen and Itakura, 1996).

Histopathological changes in the kidney of IBV-infected chickens are characterized by varying degrees of ducto-tubular interstitial nephritis (Chen et al., 1996). The progression of the kidney lesions can be divided into three phases: acute, subacute and chronic phase. In acute phase of infection, the dilatation of tubules and ducts are found (Chandra 1987; Tsukamoto et al., 1997). Some degenerated epithelial cells have swollen nuclei and vacuolated cytoplasm (Tsukamoto et al., 1997). A few heterophils and lymphocytes infiltrate into the interstitium of tubules and ducts (Chandra 1987; Tsukamoto et al., 1997). In subacute phase, the degenerated and necrotic epithelial cells appear in all segments of the nephrons (Tsukamoto et al., 1997). There are numerous of heterophils and lymphocytes infiltrate into the interstitium, while a few macrophages are found (Chandra 1987; Tsukamoto et al., 1997). Mitotic figures are occasionally present in hyperplastic epithelial cells (Tsukamoto et al., 1997). In chronic phase, numerous of the epithelial cells display regenerative processes. Diffused and scattered lymphocytic infiltration and fibrosis are commonly observed (Chandra 1987; Tsukamoto et al., 1997).

Reproductive tract

Lesions in the ovary of infected chickens develop rarely (Benyeda et al., 2009) or appear to be functioning normally (Chousalkar and Roberts, 2007b). IBV may be present at detectable levels in ovary until 28 days post-infection, but in low virus titers.

The most prominent alterations are calcium deposition in the primer oocytes accompanied with mononuclear cell infiltrations (Benyeda et al., 2009).

Crinion and Hofstad (1972) reported that there were the differences in virulence of IBV strains for immature chicken oviduct. Massachusetts strain produced the greatest number of the oviduct changes, followed by T strain, while Connecticut and Iowa 609 were not. Gross lesions of cystic or dilatation of the oviduct filled with a serous fluid may be seen in infected chickens (Crinion and Hofstad, 1972; Benyeda et al., 2009). Benyeda et al. (2009) reported that the characteristic dilatation of the oviduct developed in the chickens infected with the QX-like strains, while no change was observed in the chickens infected with M41 or 793/B strain. Some of infected hens might have an atrophied oviduct.

IBV has the potential to infect all part of the oviduct. In the upper part of reproductive tract (infundibulum and magnum), the patchy losses of cilia are found in the infundibulum. There are the losses of cilia from ampulla, middle and chalaziferous regions. The presence of plasma cells and lymphoid nodules in the lamina propia and muscularis region are reported. The losses of cilia from the surface epithelial cells also occur in the magnum. The lymphoid nodules are observed in the interglandular space and muscularis area of the magnum (Chousalkar and Roberts, 2007b). In the eggshell-forming regions (isthmus, tubular shell gland and shell gland pouch), there are the losses of cilia from the surface epithelium and glandular epithelium. The lymphocyte infiltrations are found in the interglandular space and muscularis area of the isthmus. The glands in the tubular shell gland and shell gland pouch are dilated (Chousalkar and Roberts, 2007a).

Digestive tract

It has been reported that digestive tract are the replication site of IBV. Several strains of IBV have been isolated from digestive tract including esophagus (Lucio and Fabricant, 1990), proventriculus (Lucio and Fabricant, 1990; Yu et al., 2001), duodenum, jejunum (Lucio and Fabricant, 1990), ileum (Benyeda et al., 2009) and rectum (Ganapathy et al., 2005). IBV has also been isolated from enteric contents and cloacal swabs (Chong and Apostolov, 1982; Lucio and Fabricant, 1990; Villarreal et al., 2007).

The study of tissues distribution of three IBV isolates (ECV-1, ECV-2 and ECV-3) recovered from cloacal swabs of chickens found that all of the isolates had a broad tissues distribution included respiratory, digestive and urinary tract. Although the tissue tropism and the persistence of infection found to be differed among the isolates, all of them could be isolated from esophagus, proventriculus, duodenum and cecal tonsil. Moreover, the isolations of these IBV were highest from cecal tonsil, followed by kidney, trachea, duodenum, esophagus and proventriculus (Lucio and Fabricant, 1990). Yu et al. (2001) demonstrated that three IBV isolates (Q1, J2 and T3) were isolated from proventricular tissues of 25 to 70-day-old chickens in China between 1996 and 1998. At necropsy, the proventriculus was enlarged and its mucosa was thickened. Light hemorrhagic lesions were predominantly observed. At the later stage of the disease, ulcer of proventricular papillary, hemorrhagic lesions of papillary groove and cecal tonsil, and thining of duodenum were also found.

Other tissues

It has been reported that an outbreak of a new IBV strain in broiler breeder flocks in Great Britain in 1992 associated with myopathy. Common finding had been a bilateral myopathy of both deep and superficial pectoral muscles. The marked pallor and swelling of the deep pectoral muscle together with the presence of gelatinous oedema over its surface had been observed. The histological examination had confirmed the presence of acute myodegenerative lesions. At the time, IBV could be isolated from the chickens and the strain characterization demonstrated the significant antigenic differences from M41 and Dutch variant strains (Gough et al., 1992). The experimental infection of the 793/B-like IBV in chickens showed that the mild pallor of the pectoral muscle was noticed on day 3 post-inoculation. No virus was isolated from the muscle and the histopathological examination of the muscle revealed not significant lesions, except a patchy oedematous separation of muscle fibres. Moreover, there was no significant increase in serum creatine kinase concentration. These revealed that the virus was not involved directly to pathological change of muscle (Dhinakar Raj and Jones, 1996b).

Hofstad and Yoder (1966) showed that the different types of IBV (isolate 33 and 766 (Massachusetts), isolate A5968 (Connecticut), isolate 1199, isolate 609 and isolate 97) were detected in the non-respiratory tissues, pancreas, spleen, liver and bursa of Frabicius but the concentration of virus was lesser than that of respiratory tissues. The persistence of IBV in non-respiratory tissues was also observed by Lee et al. (2002). They found that the eight strains of IBV (Arkansas DPI, Connecticut, CV56b, CWL0470, Gray, JMK, M41 and Wolgemuth) were detected in the trachea, lung, intestine and bursa of Frabicius but no virus was detected in the spleen. Kapczynski et al. (2002) demonstrated that IBV antigens were detected at the epithelium of bursa of Frabicius but the antigens were not detected within the follicles of bursa of Frabicius. Although IBV has been isolated from other non-respiratory tissues it has not been documented to be involved with any functional damage.

Several strains of IBV have been isolated from cecal tonsil (Lucio and Fabricant, 1990; Wang and Khan, 2000; Yu et al., 2001). In cecal tonsil, IBV is detected primarily in the cells which morphologically resembled histiocytes but can also be seen occasionally in lymphocytic cells (Owen et al., 1991). Benyeda et al. (2009) reported that IBV strains vary in the virus titers and the persistence of infection in the cecum. They showed that QX-like strains were considerably higher in the virus titers and longer in duration of infection than M41 and 793/B strain.

Factors associated with virulence

It is clearly that strains of IBV have wide and variable in tissue tropisms and the clinical manifestations of the diseases can be diverse. Albassam et al. (1986) inoculated 2-day-old, white leghorn chickens with one of 4 different strains of IBV (Gray, Holte, Australian T and Italian 731). When comparison of the virulence, the severe kidney lesions were observed in chickens infected with Australian T strains, whereas moderate to mild lesions were observed in chickens infected with Italian 731, Gray and Holte strains, respectively. Chandra (1987) inoculated 2-week-old, white leghorn chickens with one of 4 different strains of IBV (Gray, Holte, Australian T and M41). Inoculated chickens showed clinical signs associated with IBV infection within 2-7 day post-inoculation. The clinical sign was 30, 45, 55 and 100% in the chickens infected with Australian T, Holte,

Gray and M41, respectively. The kidney lesion was 5, 20, 35 and 85% in the chickens infected with M41, Gray, Holte and Australian T, respectively.

Although chickens of all ages are susceptible to infection with IBV, the clinical signs of infected chickens are more severe in young chickens. Animas et al. (1994) inoculated chickens at different ages (2, 4 and 6-week-old) with IBV Kagoshima-34 strain. At 3-6 days post-inoculation, chickens inoculated at 2-week-old had clinical signs more than the others. Ignjatovic et al. (2003) demonstrated that chickens inoculated with N1/62 strain at 2-week-old had mortality rate more than chickens inoculated at 4-week-old. Pohuang et al. (2009) showed that mortality rate and the average gross and histopathological lesion scores in the tracheas and the kidneys of chickens inoculated with Thai IBV isolate (THA001) at 2-day-old were higher than chickens inoculated at 14-day-old.

The genetic lineage of chicken is also reported as a factor influencing IBV pathogenesis. Ignjatovic et al. (2003) showed that three lines of white leghorn chickens which were S (inbred), HWL (non-inbred) and W (inbred) differed greatly in their susceptibility to IBV infection. The S and HWL chicken lines had morbidity rate more than W breed after infection with N1/62 strain. Virus titer is one of a factor influencing the clinical showing of IBV infected chickens. The 2-week-old, W breed chickens inoculated with $2x10^2$ CD_{50} of N1/62 strain do not showed any of clinical signs, but the clinical signs was 70% observed in the chickens inoculated with $2x10^4$ CD_{50}. Similar to the results observed in 4-week-old, S breed chickens, the 45 and 75% mortality rate were observed when inoculated with $2x10^2$ CD_{50} and $2x10^4$ CD_{50}, respectively (Ignjatovic et al., 2003).

Detection of IBV antigens

Detection by virus isolation

There are a number of biological systems used for isolation of IBV including embryonated chicken eggs, chicken organ cultures and cell cultures. Virus isolation in 9 to 11-day-old embryonated chicken eggs is the most common used method but it can be laborious and time consuming (Owen et al., 1991; Abdel-Moneim et al., 2009). In general, the initial inoculation of field virus isolates using embryonated chickens eggs has no effect on the embryo unless the virus is already egg-adapted. Therefore, the

several sequential passages are recommended (OIE, 2004). Typically, the visible changes in the embryos induced by IBV are stunting, curling, clubbing down feathers and the presence of urate in the kidney (Ziegler et al., 2002; OIE, 2004). The IBV titers in inoculated eggs increase rapidly at 24 hour post-inoculation. The allantoic fluids show high virus titers at 24-87 hour post-inoculation (Abdel-Moneim et al., 2009).

The chicken organ cultures including tracheal organ culture (TOC) and oviduct organ culture (OOC) can be used for the isolation of IBV. TOC prepared from 20-day-old embryos have proved very successful for the isolation of IBV directly from field materials. The isolation of IBV using TOC has the advantages that no adaptation of the virus is required (Cook et al., 1976). A visible change in TOC is ciliostasis of ciliated epithelial cells occurred within 1-2 days after inoculation but it can also be induced by many other agents (OIE, 2004). The OOC has been used for studying the pathogenicity (Pradhan et al., 1984) and the virulence (Dhinaka Raj and Jones, 1996a) of IBV. Assessment of ciliary activity of OOC is used as the criteria for the interaction of IBV to the oviduct ciliated epithelium (Dhinaka Raj and Jones, 1996a; Dhinaka Raj and Jones, 1997).

Compared with some other coronaviruses, IBV is less readily grown in cell cultures (Chen et al., 2007). The primary isolation of IBV directly from infected materials using cell cultures are not success (Cook et al., 1976). Before isolation in cell cultures, it is necessary to adapt IBV isolates by growing in chicken embryos in order to induction of its cytopathic effect (CPE) (OIE, 2004). The most susceptible cells are chicken kidney (CK) cells or chicken embryo kidney (CEK) cells because several strains can be isolated by using these cell cultures.

Otsuki et al. (1979) demonstrated that 10 IBV, Beaudette-42, M41, Connecticut A-5968, Connaught, Holte, Iowa-609, KH, Nerima, Ishida and Shiga strains replicated well in CK cells. Virus yield increased logarithmically within 24 hours. The maximum yield was detected at 36 hours for all strains. Only a few strains have been shown to replicate in mammalian cell lines. Beaudette-42 and Holte strains can replicate in BHK-21 (Otsuki et al., 1979). The M41 has ability to grow in chicken embryo rough cell line but CPE is observed after the fifth passage (Ferreira et al., 2003). The IBV strains M41, H52, H120 and Gray can replicate in HeLa cell line but the most efficient replication is observed in the fresh dispersed cells (Chen et al., 2007).

Detection by immunoassay

There are several techniques used for IBV antigen detection and all of the techniques use IBV-specific antibodies. These antibodies are polyclonal and monoclonal antibody. The polyclonal antibody produced from the animals, especially chickens, is relatively simple (Clarke et al., 1972; Chong and Apostolov, 1982; Bhattacharjee et al., 1994; Pensaert and Lambrechts, 1994) and sensitive for IBV detection (Yagyu and Ohta, 1990). The disadvantages of using polyclonal antibody are the occurrence of non-specific reaction and the different results occurred by using different lots of polyclonal antibody in other laboratories (Yagyu and Ohta, 1990). The monoclonal antibody is a useful material for detection of IBV antigen. Since a monoclonal antibody only reacts with one epitope, it provides a well-defined and specific product (De Wit et al., 1995). Another advantage of monoclonal antibody is that the hybridoma cell line produces a large constant volume of monoclonal antibody, therefore the detection of IBV among infected flocks are performed with constant monoclonal antibody in all laboratory assays (Yagyu and Ohta, 1990). The problem of using monoclonal antibody is that when the missing or antigenic changes occurred in an epitope, it can prevent binding of the monoclonal antibody to this epitope (Ignjatovic and Ashton, 1996).

Immunofluorescent assay (IFA) is a useful technique for detection of IBV antigens. It can be used for detection of IBV antigens in inoculated embryos (Clarke et al., 1972), infected tissues (Chong and Apostolov, 1982; Yagyu and Ohta, 1990; Owen et al., 1991; Pensaert and Lambrechts, 1994; De Wit et al., 1995), TOC (Bhattacharjee et al., 1994) and cell culture (Otsuki et al., 1979; Ferreira et al., 2003; Winter et al., 2006). IFA is an easy and rapid techinique, but requires specialized equipment and histological characterization of lesion is difficult (Owen et al., 1991). The cells recovered from the IBV inoculated eggs can be used for detection of IBV by IFA. The technique is found to be faster and more sensitive than virus isolation in embryonated eggs. The time required for detection is within 48 hour post-inoculation (Clarke et al., 1972). It has been reported that a simple method for rapid identification of IBV by IFA is the staining in TOC. In vitro, IBV can be detected as soon as 6 hours after inoculation with the high virus titers (3.5-

4.5 $\log_{10}$ CD_{50}). For the inoculation with the low virus titers (0.5-2.5 $\log_{10}$ CD_{50}), specific staining is detected before ciliostasis appeared (Bhattacharjee et al., 1994).

Immunohistochemistry (IH), like IFA, is a staining technique. It can be used for detection of IBV antigens in infected tissues (Nakamura et al., 1991; Owen et al., 1991; Chen et al., 1996; Kapczynski et al., 2002; Ziegler et al., 2002). IH does not require specialized equipment, such as fluorescent microscope, for visualize the antigens. Additionally, the slides obtained from IH are permanent and can be counterstained in order to improve morphological diagnosis of antigen location. The problems occurred when using IH include high background staining due to the using of polyclonal antibody, non-specific background staining by the enzymes naturally present in the sample and the section loss from the glass slides due to the many steps required for IH procedure (Owen et al., 1991).

Detection of IBV genome

The detection of IBV genome using molecular assays are commonly used because they provide highly specific and sensitive results in a timely manner (Callison et al., 2006). The reverse transcriptase-polymerase chain reaction (RT-PCR) can be used to detect IBV genome directly from clinical samples (Kwon et al., 1993; Ganapathy et al., 2005; Worthington et al., 2008) or from virus isolated in a laboratory host system (Keeler et al., 1998; Kingham et al., 2000; Lee et al., 2000; Yu et al., 2001). The problem occurred in the test is the false negative due to the high genomic variability among IBV strains. It is difficult to design the primer sets that can be used to detect all IBV strains. Mondal et al. (2001) reported that the attempts to amplify the S1 gene of DE072 isolates are unsuccessful by using a set of primers that amplify all standard IBV serotypes. As a report of Worthington et al. (2008), the genotype D1466 is not detected by using the universal primers. Therefore, the selection of appropriate primers is an important procedure to increase the specificity and sensitivity of the assay (Lee et al., 2000).

Several RT-PCR assays have been established for detection of IBV. The two-step RT-PCR is used by many researchers. It consists of two-step, RT reaction and PCR amplification. The RT reaction is a step to synthesize the cDNA by using reverse transcriptase enzyme. After that, the PCR amplification is performed by the step of

denaturation, annealing and polymerization (Kwon et al., 1993; Wang et al., 1996; Yu et al., 2001). The one-step RT-PCR, a more rapid and easy assay than two-step RT-PCR, is used for detection of IBV. The RT reaction and PCR amplification of the assay are performed in the same tube. This can reduce the contamination resulting to false positive at the time of performing the test (Lee et al., 2000; Lee et al., 2001; Lee et al., 2003).

The nested RT-PCR has been developed for detection of IBV (Falcone et al., 1997). In general, the nested RT-PCR is a more sensitive than conventional RT-PCR (Kho et al., 2000). It appears that nested RT-PCR has the ability to detect IBV from infected samples at similar rate as embryonated chicken eggs, and is better than TOC (Ganapathy et al., 2005). This allows a marked reduction in the handling of the specimens and reduces the time required for the diagnosis by eliminating the complex adaptation of field isolates to the growth in laboratory host systems (Falcone et al., 1997). Due to their extreme sensitivity, the risk of laboratory contamination may be occurred; therefore optimal the test conditions are even more critical for performing the nested RT-PCR than conventional RT-PCR (De wit, 2000). A nested RT-PCR is performed by using the 2 pairs of the highly conserved primers. The procedure is carried out in the double-PCR amplifications (Falcone et al., 1997; Kho et al., 2000). The nested RT-PCR is regularly used for detection of IBV directly from infected samples. Ganapathy et al. (2005) demonstrated that direct swabs from homogenized tissues can be used for IBV detection by nested RT-PCR. Villarreal et al. (2007) showed that the nested RT-PCR can be used for detection of IBV directly from pooled enteric contents and pooled respiratory tissues and kidneys.

Recently, Callison et al. (2006) developed a real-time RT-PCR assay for early and rapid detection of IBV. The test is extremely sensitive and specific, and can be used for quantitative of the genomic RNA of IBV in the samples. The comparison of the ability to detection of IBV in tracheal swabs showed that 79.04% of the swabs were positive by real-time RT-PCR, whereas only 27.51% were positive by virus isolation. Although a real-time RT-PCR assay is an extremely sensitive and specific technique, it requires specialized equipment to perform the test.

Strain classification

Protectotype

The major goal of identification of IBV strains is to establish the relationship between IBV and specific vaccine strains because vaccination is the controlling methods of IBV infection (Cook et al., 1999). Therefore the protectotype concept has been described. This classification system is a functional test which regards the biological function of the virus (De Wit, 2000). It is performed by measuring the degrees of cross-protection in chickens against a heterologous strain. Ladman et al. (2006) described the protective relatedness values (PRV) for the evaluation of cross-challenge. They found that the results of cross-challenge with homologous strains show the protection ranging from 82-100%. Although protectotype is considered the most accurate test, it is impractical to test every field isolates in cross-challenge test.

Serotype

Serotype is a classical functional typing system which is based on the reaction between an IBV strain and IBV serotype-specific antibody raised in chickens (De Wit, 2000). Virus neutralization (VN) test is a traditional method used for IBV serotyping (Lee et al., 2003). A test is divided in to two different method, α- and β-method. The α-method is performed with constant serum and variable virus (Yachida et al., 1978; Wang and Khan, 2000) whereas the β-method is performed with constant virus and diluted serum (Cook et al., 1996; Case et al., 1997). The highest reaction that completely neutralizes the virus or prevents changing of laboratory host systems is used as an end point of VN titer (Mondal et al., 2001).

Sometimes an attempt to characterize the IBV isolates is not successful by the α-method but they can be characterized by β-method, therefore the beta method is preferred over the α-method because of the more sensitivity and precision obtained (De Wit, 2000). VN test can be performed in several laboratory host systems such as embryonated chicken eggs (Cubillos et al., 1991; Case et al., 1997; Wang and Huang, 2000; Mondal et al., 2001; Lee et al., 2003), TOC (Ignjatovic and Ashton, 1996; Fabio et al., 2000) and CKC (Lucio and Fabricant, 1990).

Genotype

Genotype is a grouping method based on genetic characterization of the genome. Several methods can be used for genotyping of IBV including genotypic-specific RT-PCR (Keeler et al., 1998; Wang and Khan, 2000; Kingham et al., 2000), restriction fragment length polymorphism (RFLP) (Kwon et al., 1993; Wang et al., 1996; Lee et al., 2000; Mondal et al., 2001; Jang et al., 2007), and genomic sequencing (Cavanagh et al., 1992; Kant et al., 1992). The methods provide a good information about the evolution of IBV and essential information for epidemiological studies.

The serotype-specific RT-PCR had been described by Keeler et al. (1998). The method could differentiate the IBV serotype Massachusetts, Connecticut, Arkansas, JMK, Delaware (DE/072/92) and California (CA/633/85). Multiplex RT-PCR is also used for detection and differentiation between Massachusetts and Arkansas serotypes. If the samples contain both Massachusetts and Arkansas, two serotype-specific PCR products will be amplifiled, 1026 bp for Massachusetts and 869 bp for Arkansas (Wang and Khan, 2000). Although, an identification of IBV serotypes by serotype-specific RT-PCR offers a rapid diagnostic method the specific primers are needed to add in the test in order to identify the new serotypes occurred in the present time (Kingham et al., 2000).

Kwon et al. (1993) had demonstrated the identification of IBV by using RFLP. The method could identify IBV including variant strains and the results agreed with the VN test. The sequences of complete S1 gene were amplified, purified and digested with endonuclease enzymes, *Hae*III, *Xcm*I and *Bsty*I. IBV isolates in the same group or came from the same original strains should have the same RFLP patterns. At the time, the IBV Holte, Arkansas DPI, SE17, Md27 and Iowa 97 strain could be differentiated from each other by using *Hae*III enzyme. The Beaudette, Massachusetts 41, Connecticut and Florida 80 strain could be differentiated from each other by using *Xcm*I and *Bsty*I enzyme. Later, these methods had been used for identification of IBV strains world wide (Wang et al., 1996; Lee et al., 2000; Mondal et al., 2001; Jang et al., 2007). In spite of the advantage, RFLP have a limitation. A mutation at an endonuclease recognition site may prevent an enzyme activity and the mutation within the restriction regions may not be shown by the method (Kingham et al., 2000). Furthermore, the RFLP restriction

patterns of some IBV serotypes may be the same and difficult to distinguish each other (Keeler et al., 1998)

Due to the most antigenic of the virus neutralization antibody-inducing epitopes located in the S1 part of S gene (Cavanagh et al., 1992; Kant et al., 1992; Jang et al., 2007), this part is commonly used for sequencing. The using of S1 gene identity values as a typing of IBV is a more readily achievable alternative to perform than other laboratory typing (Ladman et al., 2006). The S1 gene identity is performed by comparison of the obtained sequences with the known sequences deposited in GenBank Database. Both the complete and partial part of S1 genes are used to sequence analysis. Genotyping based on the HVR of S1 gene is reported to correlate with the using of complete S1 gene (Wang and Huang, 2000), VN test (Lee et al., 2003) and cross-challenge study (Ladman et al., 2006). However, the genotyping based on HVR may not always correlate with VN test because there may be other regions that play a role in determining the test. Furthermore, there is always a chance that new virus can not be the primers used for genotyping by HVR (Lee et al., 2003).

Prevention and control

To prevent economic losses associated with IBV infection, IB vaccines are routinely administered. It has been reported that complete protection is provided when vaccine and infectious virus were homologous strain or serotype. A low level or no protection is observed when heterologous vaccines are administered. Al-Tarcha and Sadoon (1991) immunized 3-day-old chickens by intra-tracheal or oculo-nasal route with vaccine H120 strain, after that they were challenged with one of 8 heterologous IBV at 4 weeks post-vaccination. The evaluation performed by observation of the ciliary activity of individual chickens showed that protection was found in the chickens challenged with homologous serotype (M41). Pensaert and Lambrechts (1994) reported that vaccination with homologous vaccine induced protection of mortality and virus infection superior to that induced by heterologous vaccines. Liu et al. (2009) immunized 15-day-old chickens by inoculated intra-nasally with different IBV strains. At 20 day post-vaccination, the chickens were challenged with CK/CH/LDL/97I strain. A complete protection (assessed

by morbidity, mortality and virus recovery from tracheas and kidneys of individual chickens) was only observed following vaccination with homologous strain.

Although vaccination with one heterologous strain does not protect chickens from IBV infection, sometimes, broad spectrum protection can be achieved using a combined vaccination program incorporating with different live attenuated vaccine strains. Cook et al. (2001) reported that vaccination using a combination of Ma5 and 4/91 vaccine strains could provide a good protection of the kidney against heterologous NIBV. Martin et al. (2007) demonstrated that chickens vaccinated Holland combined with Arkansas strains had a protection against challenged with isolate CA99 which was typed to be difference from vaccine serotypes.

CHAPTER III

MATERIALS AND METHODS

Part1: Development of nested RT-PCR for the detection of infectious bronchitis virus

Viruses

Six strains of IBV were used to calibrate the test. Three IBV vaccine strains, Ma5 (Intervet international, Holland), H120 (Intervet international, Holland) and 4/91 (Intervet international, Holland) were obtained from Intervet, Thailand. Two IBV strains Arkansas and Connecticut were obtained from Saha Farm, Thailand. A Field isolate (THA001) was preserved at -70 °C in Department of Veterinary Medicine, Faculty of Veterinary Science, Chulalongkorn University.

RNA extraction

Viral RNA was extracted from 200 µl of the samples containing different IBV stains described above by using a Viral Nucleic Acid Extraction Kit (Real Biotech, Taiwan) following the manufacturer's instructions. RNA was dissolved in 50 µl of RNase free water and processed to nested RT-PCR reaction.

Design of primers

The primers were designed by identifying conserved regions of the S1 gene sequences available in GenBank database. All of the selected S1 gene sequences were aligned by using Clustal W multiple alignment method deposited in BioEdit program version 7.0.5.2 (Hall, 1999). After that, the primer sets related to the highly conserved region of the S1 genes of IBV were selected. Primer sequences were analyzed for secondary structure formation, G + C content, primer dimer formation, hairpin formation, and their compatibility in the nested RT-PCR using the OLIGO primer analysis software (Institute of Biotechnology, Unversity of Helsinki, Finland). The selected primers showed no major unspecific homologies in a BLAST search via the National Center of Biotechnology Information (USA).

Amplification reaction

The first amplification reaction was carried out with one-step RT-PCR (AccessQuick™ RT-PCR System, Promega, USA) in a final volume of 25 µl. The reaction mixture was composed of 12.5 µl of 2x AccessQuick™ Master Mix, 0.5 µl of AMV reverse transcriptase, 8 µl of RNase free water, 0.5 µl (25 µM) of each outer primer and 3 µl of RNA template. The one-step RT-PCR was conducted by 45 min of RT reaction at 48 °C, heating at 94 °C for 5 min and 35 cycles of denaturation at 94 °C for 30 sec, annealing at 54 °C for 30 sec and polymerization at 72 °C for 45 sec with a final elongation step of 10 min at 72 °C.

The second amplification reaction (nested-PCR) was carried out in a final volume of 25 µl. The reaction mixture was composed of 12.5 µl of 2x AccessQuick™ Master Mix, 10.5 µl of RNase free water, 0.5 µl (25 µM) of each inner primer and 1 µl of a 1:10-diluted products of the first amplification. The PCR was conducted by heating at 94 °C for 5 min and 35 cycles of denaturation at 94 °C for 30 sec, annealing at 54 °C for 30 sec, and polymerization at 72 °C for 30 sec with a final elongation step of 10 min at 72 °C. The amplified PCR products were analyzed in 1.2% agarose gel.

Sensitivity determination

A serial 10-fold dilution of the stock IBV isolate THA001 was performed. Then, 100 µl was subjected to RNA extraction and the remainder was inoculated into five embryonated chicken eggs (100 µl per egg). The extracted RNA was tested by the nested PCR and non-nested RT-PCR (using both outer and inner primers). The protocol for non-nested RT-PCR was performed by using one-step RT-PCR (AccessQuick™ RT-PCR System, Promega, USA) as described by the manufacturer. The thermal profile was similar to the first amplification reaction of the one-step RT-PCR. The inoculated eggs were opened and observed for stunted embryos on day 5 post-inoculation. The positive results were recorded when one or more than one egg had embryonic change.

Specificity determination

The specificity was evaluated with three other avian infectious viruses. Avian influenza virus (A/chicken/Nakorn-Pathom/Thailand/CU-K2/2004 (H5N1)) was obtained

from Virology unit, Department of pathology, Faculty of Veterinary Science Chulalongkorn University. Newcastle disease virus (NDV-CU-1) and Infectious bursal disease virus (IBDV-CU-1) were provided by Prof. Dr. Jiroj Sasipreeyajan.

Part 2: Detection and molecular characterization of infectious bronchitis virus isolated from recent outbreaks in broiler flocks in Thailand

This part was divided into 2 phases including:

1) The first phase: the characterization by using partial S1 gene sequencing
2) The second phase: the characterization by using complete S1 gene sequencing

First phase: The molecular characterization by using partial S1 gene sequencing

Viruses

In the initial working, between January and June, 2008, thirteen poultry farms had an outbreak of a mild-to-moderate respiratory disease. All flocks had been vaccinated against IB with commercial live attenuated H120. Chickens showed respiratory symptoms including gasping, coughing, sneezing, and tracheal rales. Sick chickens were selected and sent to the Department of Veterinary Medicine, Faculty of Veterinary Science, Chulalongkorn University. Necropsy was performed and gross lesions were evaluated. Gross lesions showed mild- to moderated tracheitis and non-purulent airsacculitis. No gross lesions were found in the kidneys. The trachea and lung samples were taken as pools of chickens from the same farm. The samples were prepared as 10% w/v suspensions in phosphate-buffered saline (pH 7.4) and centrifuged at 1,800 × g for 10 min. The supernatants were then collected for analysis.

RNA extraction

Viral RNA was extracted by using Viral Nucleic Acid Extraction Kit (Real Biotech, Taiwan) following the manufacturer's instructions directly from 200 µl of the supernatant of 10% w/v sample suspension and from 200 µl of the allantoic fluid of embryonated chicken eggs used for virus isolation.

Virus screening with nested RT-PCR

Viral RNA, extracted directly from the supernatants of 10% w/v sample suspensions, was screened for the presence of IBV by using a nested RT-PCR described above.

Virus isolation and propagation

For virus isolation, the supernatants of IBV-positive samples determined by RT-PCR were inoculated into 10-day-old embryonated chicken eggs. For each sample to be examined, five embryonated chicken eggs were used. The eggs were inoculated with 0.2 ml of the sample into the allantoic cavity. The inoculated eggs were incubated at 37°C and candled daily. Allantoic fluids were harvested at 96 h postinoculation. A further blind serial passage was performed in a similar way. All of the allantoic fluids were harvested and stored at –70°C.

RT-PCR amplification for sequencing

The allantoic fluid from the second passage of each sample positive for virus screening was submitted to another RT-PCR for amplification of a segment of 878-bp of the S1 gene coding region by using an outer primer set of nested RT-PCR described above. The amplification reaction was carried out with one-step RT-PCR (AccessQuick™ RT-PCR System, Promega, USA) in a final volume of 50 µl. The reaction mixture was composed of 25 µl of 2x AccessQuick™ Master Mix, 1 µl of AMV reverse transcriptase, 17 µl of RNase free water, 1 µl (25 µM) of each outer primer and 5 µl of RNA template. The one-step RT-PCR was conducted by 45 min of RT reaction at 48 °C, heating at 94 °C for 5 min and 35 cycles of denaturation at 94 °C for 30 sec, annealing at 54 °C for 30 sec and polymerization at 72 °C for 45 sec with a final elongation step of 10 min at 72 °C. The amplified RT-PCR product was analyzed in 1.2% agarose gel. Then it was stained with ethidium bromide (0.5 µg/ml) and visualized by using an ultraviolet transilluminator.

RT-PCR product purification and sequencing

The RT-PCR products were cut from the gel and purified using the Wizard SV Gel and PCR Clean-Up system (Promega, USA) according to the manufacturer's

protocol. Purified RT-PCR products were sequenced in a forward direction using a forward primer of an outer primer set of nested RT-PCR and in a reverse direction using a reverse primer of an outer primer set of nested RT-PCR described above. The purified RT-PCR products were sequenced by commercial service (First Base, Selangor, Malaysia).

Sequences and phylogenetic analysis

To identify the Thai IBV isolates, sequences of the S1 gene of the Thai IBV isolates were compared with published IBV sequences deposited in the GenBank database using a BLAST search via the National Center of Biotechnology Information (USA). Sequence identities by BLAST analysis were included in 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 using MEGA version 4 (Tamura et al., 2007). The S1 gene sequences of the thirteen IBV isolates were submitted to the GenBank database. The other S1 gene sequences from the GenBank database were used for comparison or phylogenetic analysis in this study including M41 (AY561711), Ma5 (AY561713), H120 (M21970), IBN (AAW83034), W93 (AY427818), Connecticut 46 (L18990), Florida 18288 (AF027512), JMK (L14070), Spain/99/319 (DQ064810), Spain/00/337 (DQ064813), J2 (AF286303), BJQ (DQ070839), QXIBV (AF193423), LC2 (DQ480154), A2 (AY043312), SH (DQ480156), K069-01 (AY257061), 4/91 (AF093794), UK2/91 (Z83976), Ark DPI (AF006624), Australian T (AY775779), N1/62 (AIU29522), Armidale (DQ490205), GA/7994/99 (AF338717), GA/8077/99 (AF338718), DLD (EU589323), THA001 (DQ449628).

Second phase: The molecular characterization by using complete S1 gene sequencing

Viruses

Thirty-two Thai IBV isolates were used in this study. All of them were isolated from commercial poultry farms in Thailand which had been experiencing of respiratory disease between January 2008 and October 2009.

Virus isolation and RNA extraction

Before the virus isolation, all of the samples were screened and had positive results with nested-PCR for the presence of IBV described above. Briefly, the trachea and lung samples were taken from pools of chickens from the same farm. The samples were prepared as 10% w/v suspensions in phosphate-buffered saline (pH 7.4) and centrifuged at 1,800 × g for 10 min. The supernatants were then collected for RNA extraction using Viral Nucleic Acid Extraction Kit (Real Biotech, Taiwan) following the manufacturer's instructions. The extracted RNA was subjected to nested-PCR using the primer sets and reaction conditions described above. The supernatants of IBV-positive samples were inoculated into 9-11-day-old embryonated chicken eggs. Each egg received 200 µl of the supernatant. The inoculated eggs were incubated at 37°C and candled daily. After 96 h post-inoculation, allantoic fluids were harvested. A further blind serial passage was performed in a similar way. All of the allantoic fluids were harvested and stored at –70°C. Then, the allantoic fluids were used for RNA extraction as described above.

Primers and RT-PCR amplification

The primer sets used in this study were newly designed to amplify the full length of the S1 gene. The procedure of primer design was performed as described above. At the time, two primer sets were selected: a primer set for amplifying the region from 5' terminus to the middle of S1 gene (GCCAGTTGTTAATTTGAAAAC and TAATAACCACTCTGAGCTGT) and another for amplifying the region from the middle to 3' terminus S1 gene (ACTGGCAATTTTTCAGATGG and AACTGTTAGGTATGAGCACA). These primer sets had an overlapping region between each other about 200 bp. The amplification reaction was carried out with one-step RT-PCR (AccessQuick™ RT-PCR System, Promega, USA) in a final volume of 50 µl. The reaction mixture was composed of 25 µl of 2x AccessQuick™ Master Mix, 1 µl of AMV reverse transcriptase, 17 µl of RNase free water, 1 µl (25 µM) of each outer primer and 5 µl of RNA template. RT was performed at 48°C for 45 min and heating at 94°C for 5 min. PCR was then performed by 35 cycles of denaturation at 94°C for 60 sec, annealing at 54°C for 30 sec, extension at

72°C for 60 sec and final extension at 72°C for 10 min. The PCR product was analyzed by electrophoresis on 1.2% agarose gel, followed by staining with ethidium bromide (0.5 µg/ml) and then was visualized by using an ultraviolet transilluminator.

Product purification and sequencing

The RT-PCR products were cut from the gel and purified using the Wizard SV Gel and PCR Clean-Up system (Promega, Madison, WI, USA) as the protocols suggested by the manufacturer. The purified RT-PCR products were sequenced in both of the forward and the reverse direction by commercial service (First Base, Selangor, Malaysia)

Sequence and phylogenetic analysis

The nucleotide sequences of the S1 gene from the ATG start site to the cleavage recognition site of thirty-two Thai IBV isolates were assembled, aligned and compared with published IBV sequences deposited in the GenBank database. The first time they were compared with published IBV 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 S1 gene sequences of the thirty-two IBV isolates were submitted to the GenBank database (Table 1). The other S1 gene sequences from the GenBank database which were used for comparison or phylogenetic analysis in this study including M41 (AY561711), Ma5 (AY561713), H120 (M21970), IBN (AAW83034), Connecticut 46 (L18990), Florida 18288 (AF027512), JMK (L14070), BJQ (DQ070839), QXIBV (AF193423), LC2 (DQ480154), A2 (AY043312), SH (DQ480156), K069-01 (AY257061), 4/91 (AF093794), UK2/91 (Z83976), Ark DPI (AF006624), Australian T (AY775779), N1/62 (AIU29522), Armidale (DQ490205), DLD (EU589323), THA001 (GQ906705).

Recombination detection

Putative recombinant sequence and its parental strains were identified with Simplot version 3.5.1 (Lole et al., 1999). The nucleotide identity was performed by using Kimura (2-parameter) method with a transition-transversion ratio of 2. The window width and the step size were 200 bp and 20 bp, respectively. Bootscan analysis was also carried out employing subprogram embedded in SimPlot, using the signals of 70% or more of the observed permuted trees for indication of the potential recombination events (Salminen et al., 1995). Recombination breakpoints were analyzed by maximization of χ^2 using the program Findsites included in the SimPlot (Robertson et al., 1995).

Part 3: Evaluation of the effectiveness of vaccination with two live attenuated IBV vaccine strains against an IBV isolated from infected chickens in Thailand

Challenge virus

An IBV isolate THA80151 was isolated from the infected chickens by using the methods described above and preserved in allantoic fluid at -70°C. The preserved agent was thawed and then propagated by inoculation in embryonated chicken eggs as described above. The determination of the virus concentration in the stock solution was done by the 10 fold serial dilution method. After that, each dilution was inoculated into embryonated chicken eggs as described above. The embryo infectious dose 50% (EID_{50}/100 µl) was calculated according to Reed and Muench (1938).

Experimental design

Eighty-four, 1-day-old female Cobb 500 were randomly allocated into 6 groups with 14 chickens each and housed in separate experimental rooms. Feed and water were supplied ad lib. An infectious bronchitis vaccine was administered to the chickens via eye drops at 1-and 14-day-old. Two strains of vaccines including 4/91 (Intervet international, Holland) and H120 (Intervet international, Holland) provided by Intervet, Thailand were used for vaccination. The groups were treated with the following regime shown in table 2. This experiment has been approved by the Institutional Animal Care and Use Committee in accordance with university regulations and policies governing the

care and use of laboratory animals, approval number 0931008, issued by the Faculty of Veterinary Science, Chulalongkorn University.

Table 2 Experimental design used to study the efficacy of live attenuated vaccines

Group	Vaccination		Challenge
	1 day-old	14 day-old	28 day-old
1	H120	H120	Yes
2	4/91	H120	Yes
3	4/91	4/91	Yes
4	H120	4/91	Yes
5 (positive control)	No	No	Yes
6 (negative control)	No	No	No

Four weeks after the first vaccination, the chickens were weighed before challenge inoculation. After that, the chickens in groups 1-5 were inoculated with 100 µl of isolate THA80151 via eye drops. The virus concentration was approximately $10^{4.17}$ EID_{50}. At 7 dpi, all of the chickens were humanely killed, necropsy was performed and the tracheas were collected for evaluation of the protection.

Clinical signs and body weight gains

Clinical signs and mortalities were daily observed for 7 dpi. The chickens were weighed before the challenge inoculation and at 7 dpi.

Virus detection

The tracheas were individually collected from each chicken for virus detection. In each group, the proximal portion of the trachea was placed in a sterile plastic bag. The samples were prepared as 10% w/v suspensions in phosphate-buffered saline (pH 7.4) and centrifuged at 1,800 × g for 10 min. The supernatants were then collected for

RNA extraction using Viral Nucleic Acid Extraction Kit (Real Biotech, Taiwan) following the manufacturer's instructions. The extracted RNA was subjected to RT-PCR using a new primer set designed for detection of isolate THA80151 but not detection of 4/91 and H120 strain. The primers designed base on the S1 gene of THA80151, 4/91 and H120 strain were FOR216 (AAGGACGTCTATAATCAAAG) and RE769 (AGTACCATTAACAAAATAAGC). The RT-PCR was performed by using one-step RT-PCR (AccessQuick™ RT-PCR System, Promega, USA). The RT-PCR conditions were the same as the first amplification reaction of nested RT-PCR described above.

Histopathological examinations

The distal portion of the tracheas were placed in 10% buffered formalin, sectioned, stained with haematoxylin and eosin, and evaluated for histopathological lesion scores by the method of Ratanasethakul et al. (1999). Briefly, the histopathological lesions were evaluated as follows:

0 = no lesions

1 = epithelial deciliation and desquamation with minimal lymphoid infiltration in lamina propia and submucosa

2 = generalized epithelial deciliation and hyperplasia with moderate lymphoid infiltration in lamina propia and submucosa

3 = generalized epithelial deciliation and hyperplasia with heavy lymphoid infiltration in lamina propia and submucosa

Detection of IBV antibody titers

At 1-day-old, blood samples were randomly collected from 20 chickens. At 7-day-old, blood samples were randomly collected from 10 chickens of group 1, 3 and 6. At 14 and 21-day-old, blood samples were randomly collected from 10 chickens in each group, excepted group 6. Then, blood samples were randomly collected from 10 chickens in each group every week of age. The serum samples were kept at -20ºC. IBV antibody titer was determined using commercial Enzyme Linked Immunosorbent Assay (ELISA) test kit (BioChek, Holland). These ELISA plates were coated with inactivated IBV

antigen. Serum samples were diluted at 1:500 and the test was performed according to the manufacturer's instructions.

Statistical analysis

A comparison of body weight and antibody titer among the experimental groups was performed using one-way analysis of variance (ANOVA) and followed by the least significant difference (LSD). The morbidity rate and IBV detection rate among the groups were compared by Chi-square test. The histopathological lesion scores were analyzed by using Kruskal Wallis test.

CHAPTER IV

RESULTS

Part 1: Development of nested RT-PCR for the detection of infectious bronchitis virus

Primers

The primer sets designed for nested RT-PCR were FOR1 (5'-CTT TTG TTT GCA CTA TGT AG-3') and RE3 (5'-TAA TAA CCA CTC TGA GCT GT-3') for the outer primers and FOR2 (5'-CAG TGT TTG TCA CAC ATT GT -3') and RE2 (5'-CCA TCT GAA AAA TTG CCA GT-3') for the inner primers. The outer and inner primers amplified 878-bp and 400-bp fragments of the S1 gene, respectively.

Sensitivity determination

The results of sensitivity determination showed that non-nested RT-PCR using outer primers, non-nested RT-PCR using inner primers, and nested RT-PCR had detection limit of 10^{-3}, 10^{-4} and 10^{-5} dilution of stock virus, respectively (Figure 3 and Table 3). At the time, virus isolation in embryonated chicken eggs had a detection limit of 10^{-4} dilution of stock virus (Table 3).

Table 3 Comparison of the sensitivity of different IBV detection assays

Dilution	Detection assay			
	RT-PCR (outer primer)	RT-PCR (inner primer)	Virus isolation	Nested RT-PCR
10-2	+	+	+	+
10-3	+	+	+	+
10-4	-	+	+	+
10-5	-	-	-	+
10-6	-	-	-	-

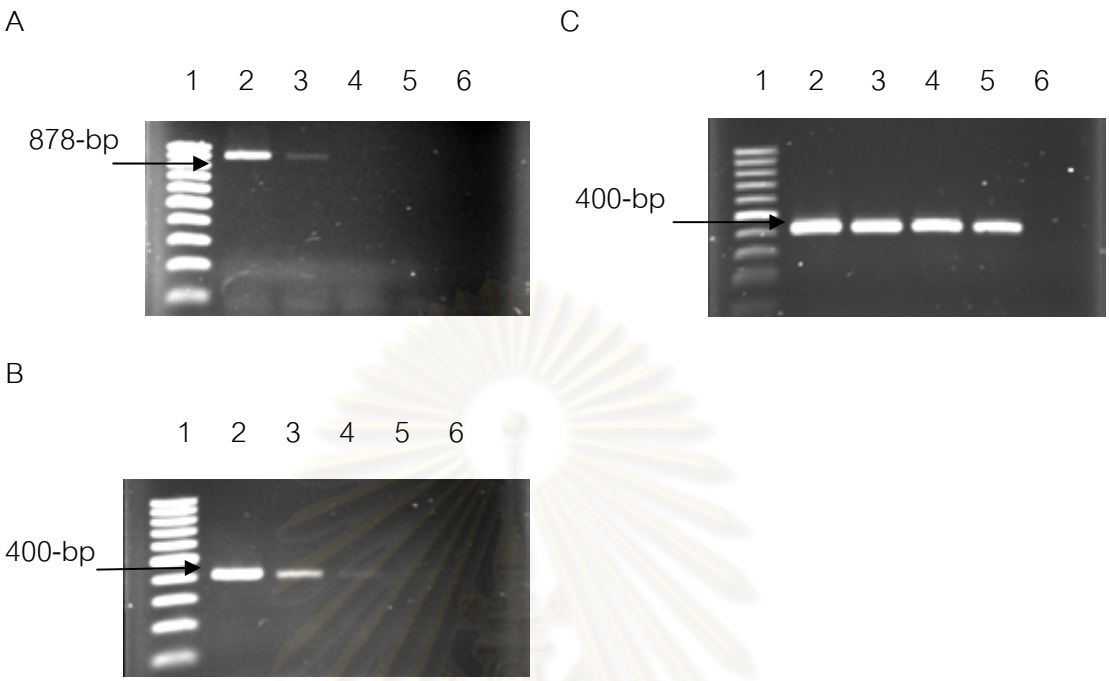


Figure 3 Sensitivity determination of different RT-PCR assays for detection of IBV; non-nested RT-PCR using outer primers (A), non-nested RT-PCR using inner primers (B), and nested PCR (C). Lane1: 100-bp marker, Lane2-6: 10^{-2}, 10^{-3}, 10^{-4}, 10^{-5} and 10^{-6} dilution of stock virus, respectively.

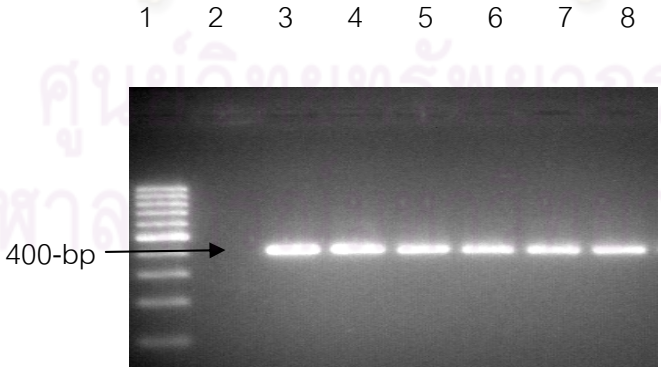


Figure 4 Specificity determination of nested RT-PCR for detection of different IBV strains. Lane1: 100-bp marker, Lane2: negative control, Lanes3-8: Field isolate (THA001), Ma5, H120, 4/91, Arkansas and Connecticut, respectively.

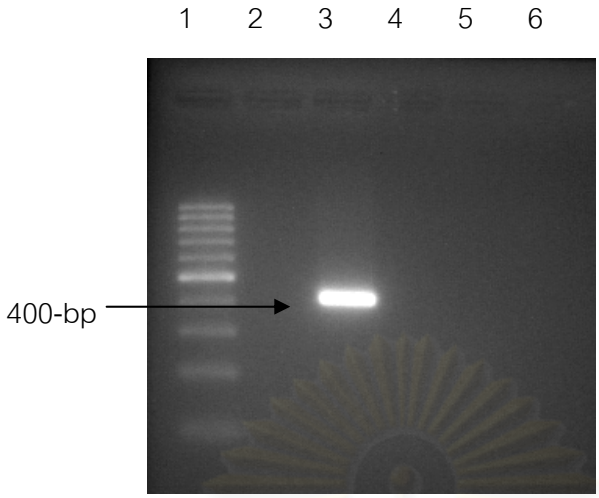


Figure 5 Specificity determination of nested RT-PCR for detection of other chicken infectious viruses. Lane1: 100-bp marker, Lane2: negative control, Lane3: Field isolate (THA001, Lane4: NDV, Lane5: AIV, Lane6: IBDV

Specificity determination

The primer sets for the nested RT-PCR were designed using the highly conserved region of S1 gene sequences deposited in GenBank database. In the specificity determination, RNA extracted from six strains, amplified by nested RT-PCR, showed target bands of 400-bp (Figure 4). None of other avian viruses showed any amplified product (Figure 5).

Part 2: Detection and molecular characterization of infectious bronchitis virus isolated from recent outbreaks in broiler flocks in Thailand

First phase: The molecular characterization by using partial S1 gene sequencing

Virus screening and isolation

For virus screening, pooled trachea and lung samples from each flock suspected of IBV infection were determined to be positive for IBV by screening with nested RT-PCR. A 400-bp fragment of the S1 gene was amplified in all 13 samples tested. The allantoic fluid from the second passage of each sample screened positive for the virus

was also determined to be positive with RT-PCR amplification and a segment of 878-bp of the S1 gene was obtained (Figure 6).

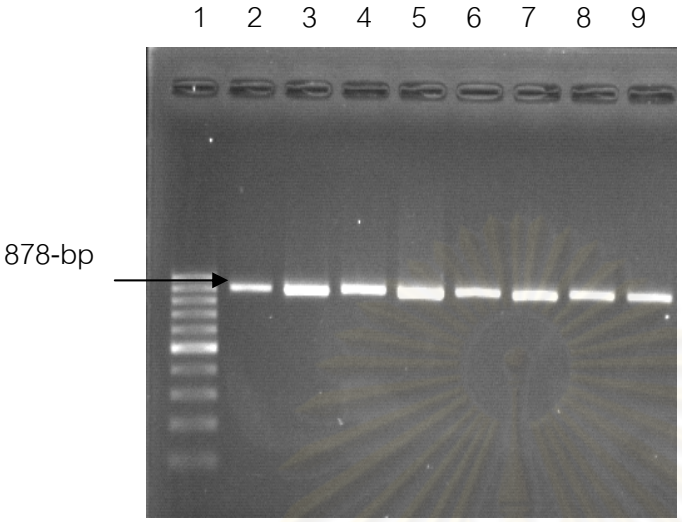
Phylogenetic analysis

To assess the genetic relationship among the IBV isolates, a phylogenetic tree was constructed from the nucleotide sequences of S1 genes (Figure 7). The thirteen IBV isolates were separated into two distinct groups. Group I consisted of five isolates including THA20151, THA40151, THA50151, THA60151, and THA90151. The isolates in group I showed evolutionary distances from each other. Group II consisted of eight isolates including THA30151, THA70151, THA80151, THA100151, THA110351, THA120351, THA130551, and THA140551, which had a close relationship with Chinese IBV isolates (strain A2, SH and QXIBV).

Nucleotide and amino acid sequence comparison

The S1 gene of the thirteen IBV isolates was sequenced to characterize the isolates. The nucleotide and deduced amino acid sequences were determined and compared among each other and with other IBV strains published in the GenBank database. Group I Thai IBV isolates had nucleotide and amino acid sequence identities between 99-100% with each other. They had nucleotide sequence identities less than 85% and amino acid sequence identities less than 84% with other IBVs published in the GenBank database. Group II Thai IBV isolates had nucleotide and amino acid sequence identities between 99-100% with each other. They had nucleotide sequence identities of 97-98% and amino acid sequence identities of 96-98% with Chinese IBVs (strain A2, SH and QXIBV).

A



B

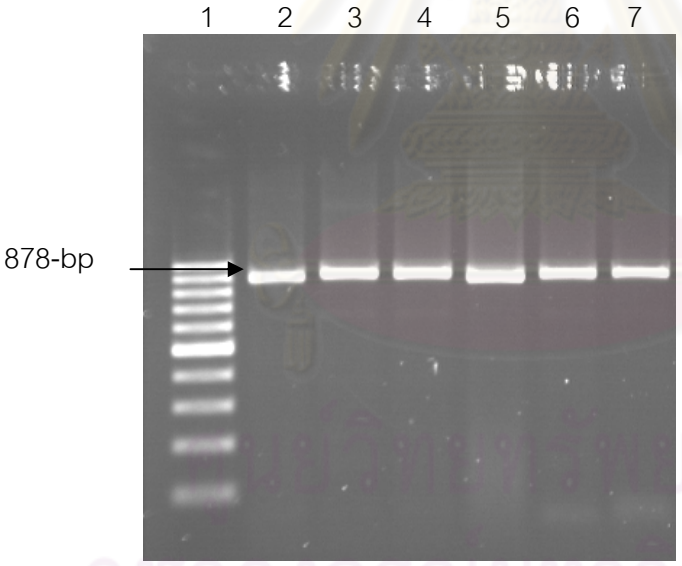


Figure 6 IBV detection from the second passage of allantoic fluid of each sample. A; Lane1: 100-bp marker, Lane2-9: isolate THA20151, THA30151, THA40151, THA50151, THA60151, THA70151, THA80151 and THA90151, respectively. B; Lane1: 100-bp marker, Lane2-7: positive control, isolate THA100151, THA110351, THA120351, THA130551, and THA140551, respectively.

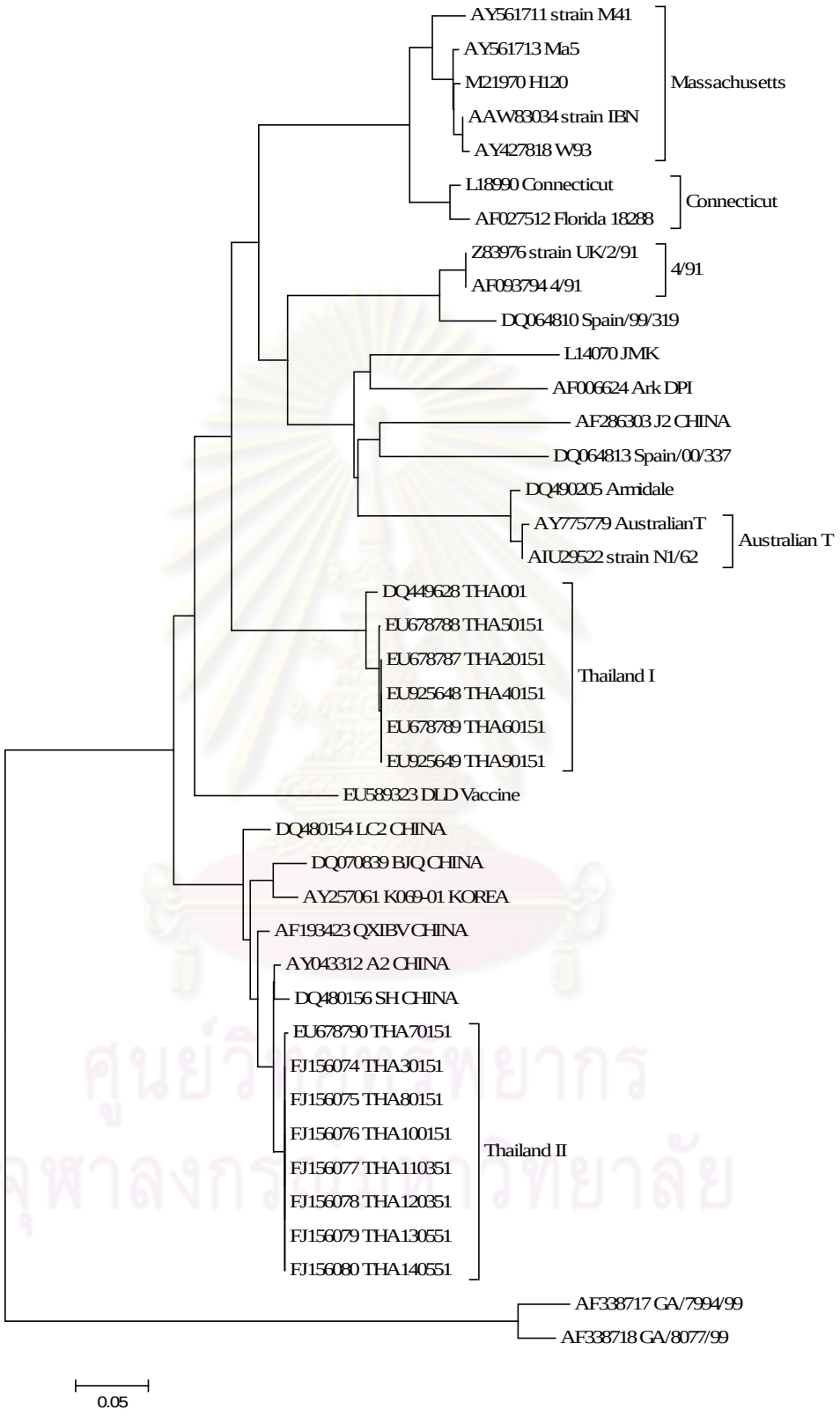


Figure 7 Phylogenetic tree based on the nucleotide sequences between aligned partial S1 genes of Thai IBV isolates and published sequences.

Second phase: The molecular characterization by using complete S1 gene sequencing

Primers

The S1 gene amplified by a new primer designed for amplifying the region from 5' terminus to the middle of S1 gene showed the positive RT-PCR band of 988-bp (Figure 8). Amplification by using a new primer designed for amplifying the region from the middle to 3' terminus showed the positive RT-PCR band of 1046-bp (Figure 9).

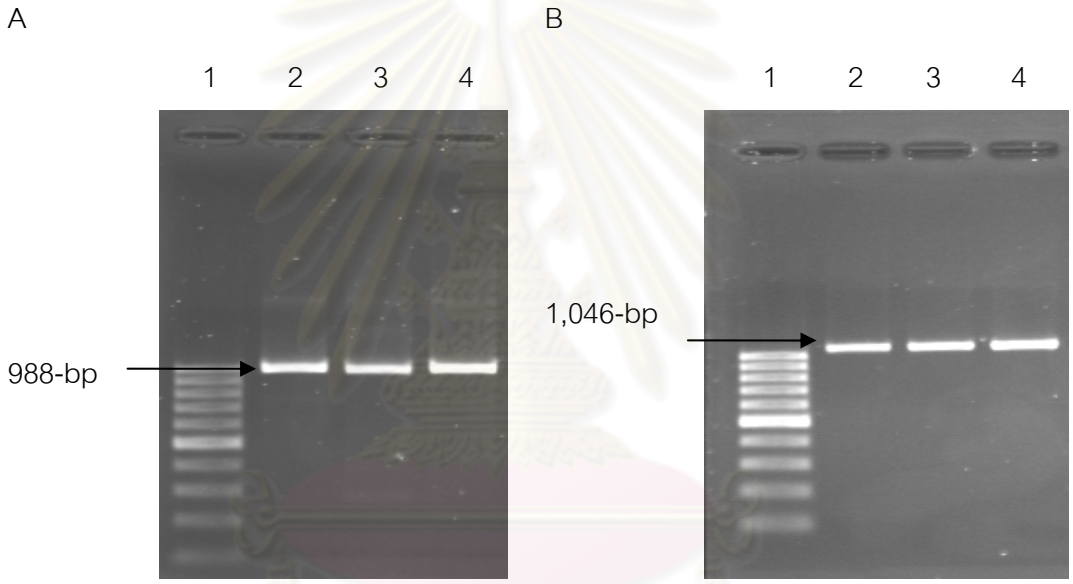


Figure 8 Detection of complete S1 gene by using a newly designed primer sets. Amplifying the region from 5' terminus to the middle (A); Lane1: 100-bp marker, Lane2-4: isolate THA20151, THA90151 and THA201051, respectively. Amplifying the region from the middle to 3' terminus (B); Lane1: 100-bp marker, Lane2-4: isolate THA20151, THA90151 and THA201051, respectively.

Table 4 Spike glycoprotein cleavage recognition sites of Thai IBV isolates

IBV isolates	Group of isolate	Years of isolation	Cleavage sites[a]	Accession number
THA10151	II	2008	Arg-Arg-His-Arg-Arg	GQ503609
THA20151	I	2008	Arg-Arg-His-Arg-Arg	GQ503610
THA30151	II	2008	Arg-Arg-His-Arg-Arg	GQ503611
THA40151	I	2008	Arg-Arg-His-Arg-Arg	GQ503612
THA50151	I	2008	Arg-Arg-His-Arg-Arg	GQ503613
THA60151	I	2008	Arg-Arg-His-Arg-Arg	GQ503614
THA70151	II	2008	Arg-Arg-His-Arg-Arg	GQ503615
THA80151	II	2008	Arg-Arg-His-Arg-Arg	GQ503616
THA90151	I	2008	Arg-Arg-His-Arg-Arg	GQ503617
THA100151	II	2008	Arg-Arg-His-Arg-Arg	GQ503618
THA110351	II	2008	Arg-Arg-His-Arg-Arg	GQ503619
THA120351	II	2008	Arg-Arg-His-Arg-Arg	GQ503620
THA130551	II	2008	Arg-Arg-His-Arg-Arg	GQ503621
THA140551	II	2008	Arg-Arg-His-Arg-Arg	GQ503622
THA150351	II	2008	Arg-Arg-His-Arg-Arg	GQ503623
THA171051	II	2008	Arg-Arg-His-Arg-Arg	GQ885126
THA201051	III	2008	Arg-Arg-Phe-Arg-Arg	GQ885127
THA211051	II	2008	Arg-Arg-His-Arg-Arg	GQ885128
THA221051	III	2008	Arg-Arg-Phe-Arg-Arg	GQ885129
THA231251	II	2008	Arg-Arg-His-Arg-Arg	GQ885130
THA241251	III	2008	Arg-Arg-Phe-Arg-Arg	GQ885131
THA250152	II	2009	Arg-Arg-His-Arg-Arg	GQ885132
THA260152	II	2009	Arg-Arg-His-Arg-Arg	GQ885133
THA280252	III	2009	Arg-Arg-Phe-Arg-Arg	GQ885134
THA290252	III	2009	Arg-Arg-Phe-Arg-Arg	GQ885135
THA300252	II	2009	Arg-Arg-His-Arg-Arg	GQ885136
THA310252	II	2009	Arg-Arg-His-Arg-Arg	GQ885137
THA320352	III	2009	Arg-Arg-Phe-Arg-Arg	GQ885138
THA330352	II	2009	Arg-Arg-His-Arg-Arg	GQ885139
THA340552	III	2009	Arg-Arg-Phe-Arg-Arg	GQ885140
THA351052	II	2009	Arg-Arg-His-Arg-Arg	GU111581
THA361052	II	2009	Arg-Arg-His-Arg-Arg	GU111582

[a] *Arg* arginine, *Phe* phenylalanine, *His* histidine

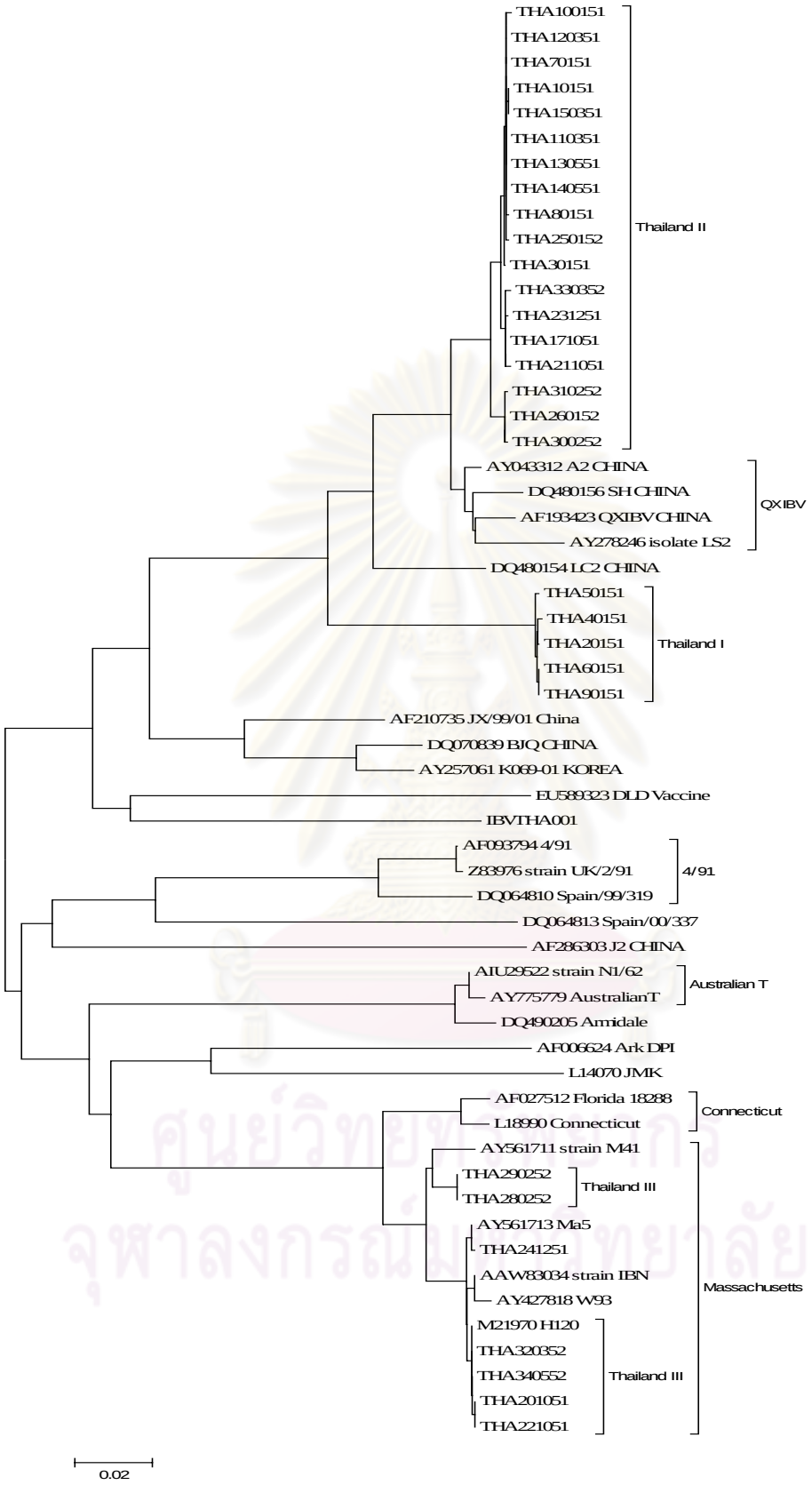


Figure 9 Phylogenetic tree based on the nucleotide sequences between aligned complete S1 genes of Thai IBV isolates and published sequences.

Phylogenetic analysis

A phylogenetic tree was constructed using the nucleotide sequences of the S1 genes (position from ATG start site to the cleavage recognition site) of the field Thai IBV isolates and the GenBank deposited sequences. Thirty-two Thai IBV isolates were separated into three distinct groups (Figure 9). Group I consisted of five isolates including THA20151, THA40151, THA50151, THA60151 and THA90151. The isolates in group I showed evolutionary distances from each other and this group was unique to Thailand. Group II consisted of twenty isolates including THA10151, THA30151, THA 70151, THA 80151, THA100151, THA110351, THA120351, THA130551, THA140551, THA150351, THA171051, THA211051, THA231251, THA250152, THA260152, THA300252, THA310252, THA330352, THA351052 and THA361052 and had a close relationship with Chinese QXIBV. Group III consisted of seven isolates including THA201051, THA221051, THA241251, THA280252, THA290252, THA320352 and THA340552 and had a close relationship with the Massachusetts type.

Analysis of the spike glycoprotein cleavage recognition site

There were two different cleavage recognition site sequences observed among the Thai IBV isolates (Table 4). The most common cleavage recognition site sequence was Arg-Arg-His-Arg-Arg observed in both the group I and the group II Thai IBV. Another cleavage recognition site sequence was Arg-Arg-Phe-Arg-Arg observed in the group III Thai IBV.

Comparison of complete S1 gene

When the BLAST search was performed, we found that groups I, II and III had a nucleotide identity of about 89%, 97% and 95-100% with other IBV strains deposited in GenBank database, respectively. The complete nucleotide and deduced amino acid sequences of the S1 gene of the fifteen Thai IBV isolates were determined and compared with each other. Group I Thai IBV isolates had nucleotide identities of 99.8-100% and amino acid identities of 99.3-100% with each other. Group II Thai IBV isolates had nucleotide identities of 98.8-100% and amino acid identities of 96.3-100% with each other. Group III Thai IBV isolates had nucleotide identities of 97.9-100% and amino acid

identities of 95.2-100% with each other. When comparison of the nucleotide sequences among the groups was made, less than 90.1 %, 74.5% and 73.5% identity was found between group I and II, between group I and III, and between group II and III, respectively. For the amino acid identity among the groups, less than 80.4%, 52.2% and 53.1% identity was found between groups I and II, between group I and III, and between group II and III, respectively.

Table 5 Nucleotide and amino acid identities of S1 gene among QXIBV

QXIBV in other countries	Group II Thai IBV	
	Nucleotide identiy (%)	Amino acid identity (%)
QXIBV China	95.5-96.0	90.7-92.8
LX4 China	91.6-91.5	89.6-90.4
LH2 China	91.0-91.5	88.6-90.7
LS2 China	90.3-90.7	87.5-88.3
LD3 China	91.0-91.5	87.5-89.6
LH10 China	90.9-91.1	88.3-89.3
NL/L-1449K/04 Netherlands	90.5-90.6	90.7-91.8
L-1148 Netherlands	90.6-90.8	90.6-91.4
Ck/SP/170/09 Spain	89.6-89.8	89.3-89.7
Ck/SP/79/08 Spain (2008)	89.6-89.8	90.0-90.7
K1583/04 South Korea	90.0-90.3	88.3-90.0
K1277/03 South Korea	90.5-90.7	89.7-90.4
K630/02 South Korea	90.2-90.4	89.0-89.6
UK/AV2150/07 United Kingdom	89.3-89.4	87.9-88.9
FR/L-1450T/05 France	90.5-90.6	90.4-91.5
IS/1201 Israel	90.0-90.4	89.0-90.4

The comparison of S1 genes between group II Thai IBV and QXIBV reported in other countries was shown in Table 5. Group II Thai IBV had nucleotide identity 89.3-96.0 % and amino acid identity 87.5-92.8% with QXIBV reported in other countries. When comparison by alignment of S1 genes, the results showed that group II Thai IBV had nucleotide sequences at 3' terminus differed from other QXIBV (Figure 10).

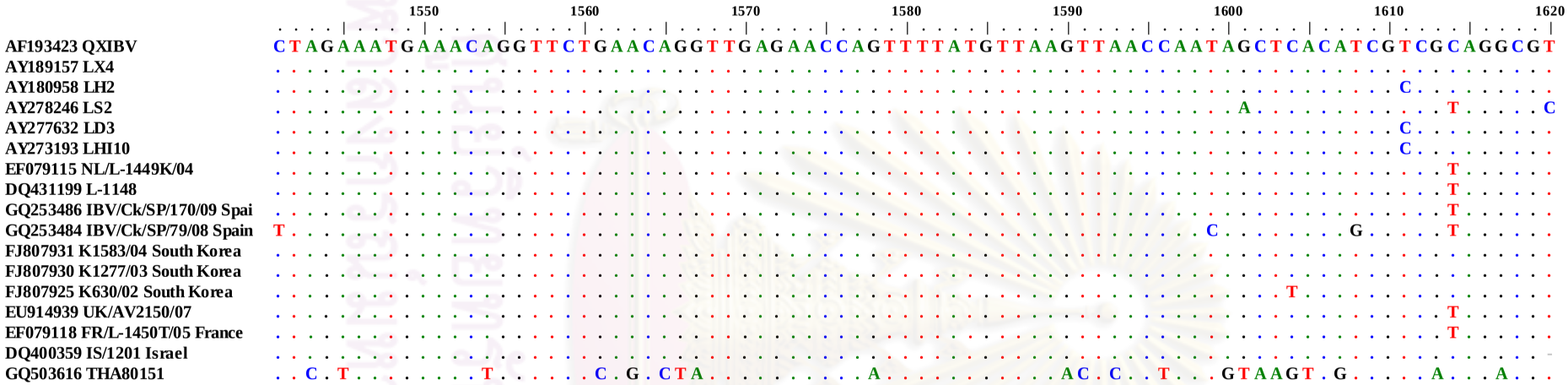


Figure 10 Nucleotide sequences at 3'-terminus of S1 gene of group II Thai IBV (represented by isolate THA80151) compared with QXIBV reported in other countries

Comparison of individual nucleotide of S1 gene

The individual nucleotides of the S1 gene were aligned and compared. We found an exchange of genetic information in groups I and II. The event was not found in group III. Overall, groups I and II were clustered into a different group based on phylogenetic analysis of the S1 gene (Figure 9). However, when individual nucleotides were aligned, it showed that the 3'-terminus of the S1 gene was found to be similar with each other (Figure 11). The 5'-terminus of the S1 gene of the group I was similar to isolate THA001 which was unique to Thailand, isolated in 1998 (Figure 12). Although the 5'-terminus region of the S1 gene of group II was found to be similar to Chinese QXIBV, the remaining region at 3 the 3'-terminus was similar with the Chinese strain JX/99/01 (Figure 13). The nucleotide sequences around the exchange regions seem to conservation. We found that the 45 nucleotides downstream from the exchange region between isolate THA001 and group II Thai IBV had 93% of nucleotide identity. Furthermore, the upstream 45 nucleotides from that site of JX/99/01 and QXIBV were also analyzed and found 93% of nucleotide identity.

Recombination in the S1 gene

The recombination events in the S1 gene sequences of Thai IBV were analyzed by using the Simplot analysis. In the similarity plot, the strains were considered as recombinants if any crossover event took place between two putative parental strains. By this analysis, the recombination events were found in groups I and II, but not in group III Thai IBV. When the similarity plot of group I (represented by isolate THA90151) was performed, the 3'-terminus of the S1 gene was found to be similar with each other (Figure 14). Interestingly, The 5'-terminus of the group I was similar to isolate THA001 which was unique to Thailand, isolated in 1998 (Figure 14). The positions of recombination breakpoints were estimated at nucleotide 679-699 (the maximization of χ^2 = 146.4). The similarity plot of group II (represented by isolate THA80151) showed that the 5'-terminus of the S1 gene was found to be similar to Chinese QXIBV but a short region at the 3'-terminus was similar to the Chinese strain JX/99/01 (Figure 15). The positions of recombination breakpoints were estimated at nucleotide 1531-1537 (the maximization of χ^2 = 71.0).

Figure 11 Nucleotide sequences at the 3'-terminus of S1 genes of group I Thai IBV compared with their potential parent strains. Dots indicate nucleotide identity at that position. Group I Thai IBV was represented by THA20151 and THA90151. The putative parent strains were THA001 and group II Thai IBV (represented by THA80151, THA100151 and THA310252).

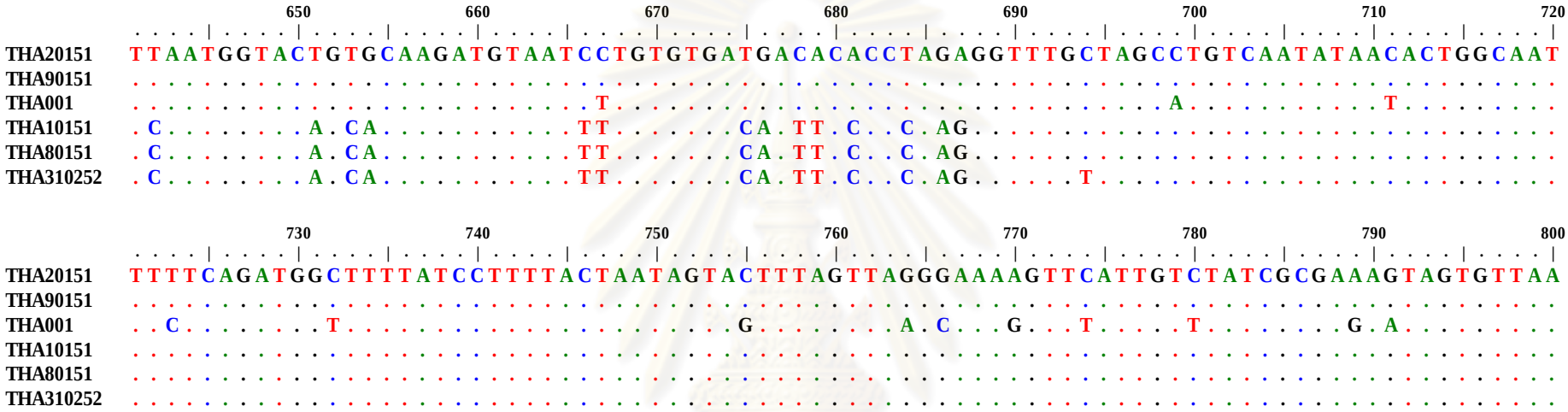


Figure 12 Nucleotide sequences at the 5'-terminus of S1 genes of group I Thai IBV compared with their potential parent strains. Dots indicate nucleotide identity at that position. Group I Thai IBV was represented by THA20151 and THA90151. The putative parent strains were THA001 and group II Thai IBV (represented by THA80151, THA100151 and THA310252).

Figure 13 Nucleotide sequences at the 3'-terminus of S1 genes of group II Thai IBV compared with their potential parent strains. Dots indicate nucleotide identity at that position. Group II Thai IBV was represented by isolate THA80151, THA100151 and THA310252. The putative parent strains were QXIBV, LS2, JX/99/01 and CK/CH/LAH/99I.

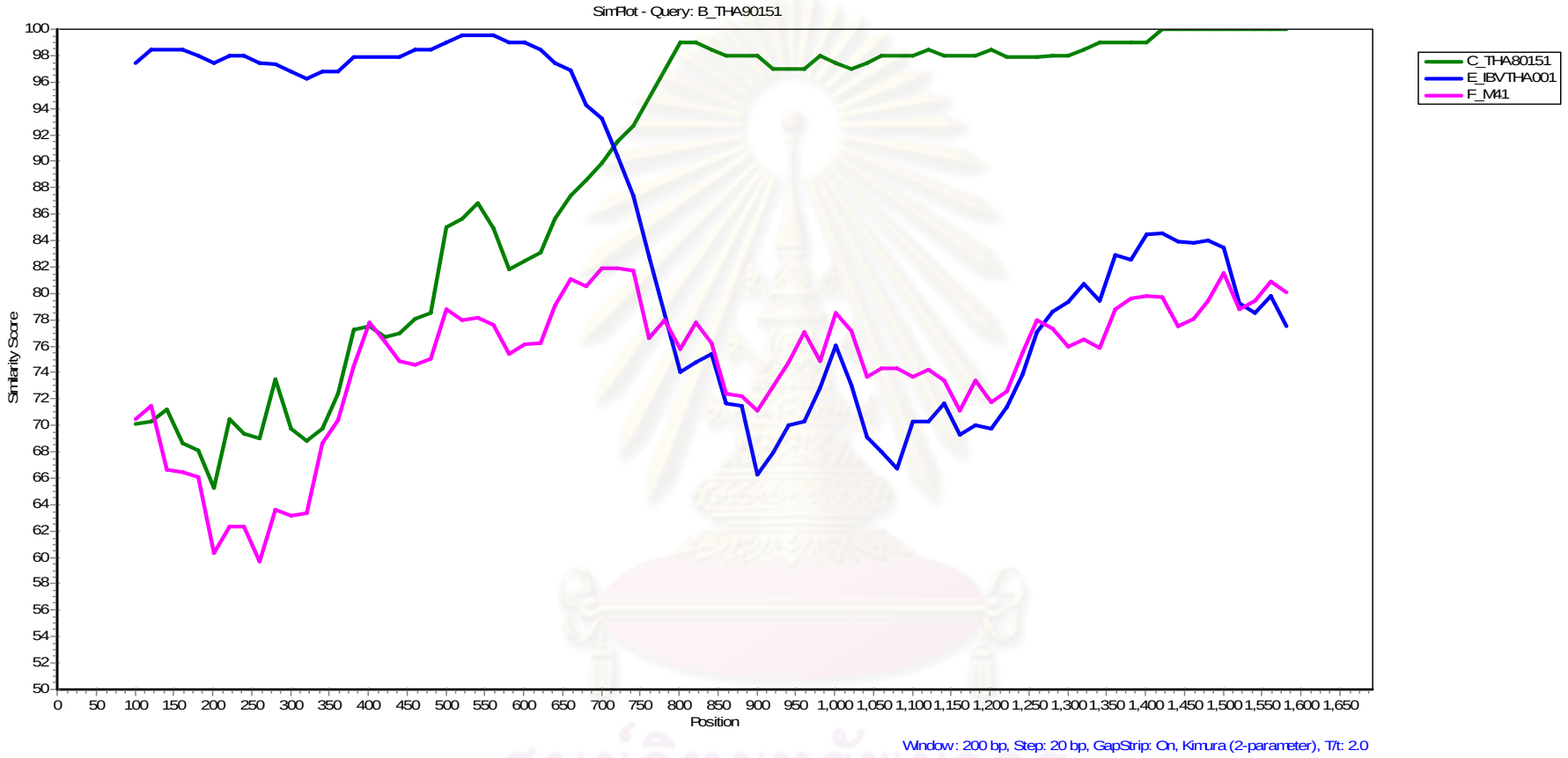


Figure 14 Similarity plot of the S1 gene of the group I Thai IBV (represented by THA90151). Isolate THA80151 (green) and isolate THA001 (blue) were used as putative parental strains when isolate THA90151 was queried. M41 (pink) was used as an outlier sequence.

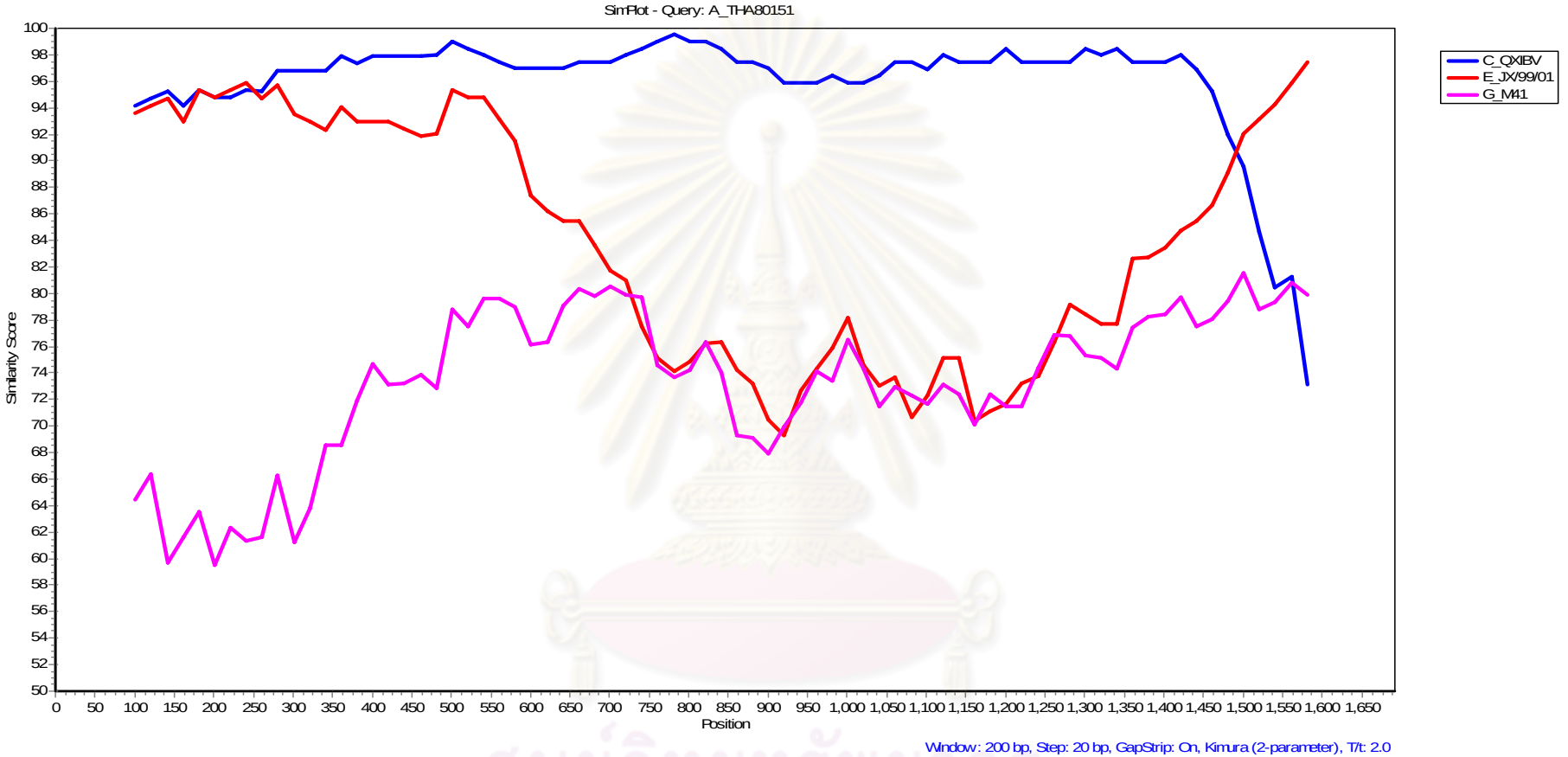


Figure 15 Similarity plot of the S1 gene of the group II Thai IBV (represented by THA80151). QXIBV (blue) and JX/99/01 (red) were used as putative parental strains when isolate THA80151 was queried. M41 (pink) was used as an outlier sequence.

Part 3: Evaluation of the effectiveness of vaccination with two live attenuated IBV vaccine strains against an IBV isolated from infected chickens in Thailand

Clinical signs and body weight gains

In the infected groups, some chickens showed tracheal rales, coughing and gasping at 2 dpi. Decreased feed consumption and ruffled feathers were observed. In vaccinated groups, the numbers of chickens which showed clinical signs were significantly lower ($p<0.05$) than those of the positive control group (Table 6). The dead chickens were not found in the entire group until the end of the experiment. There were neither clinical signs nor deaths in the negative control groups. At 7 dpi, the body weights of chickens in all infected group were significantly lower ($p<0.05$) than those of the negative control group. However, the body weights of chickens in vaccinated groups were significantly higher ($p<0.05$) than the positive control group (Table 7).

Table 6 The morbidity rate of chickens individually observed during 7 dpi

Group	Vaccination	Morbidity (%)
1	H120+H120	$3/14^{ac}$ (21.4)
2	4/91+H120	$2/14^{ac}$ (14.3)
3	4/91+4/91	$3/14^{ac}$ (21.4)
4	H120+4/91	$2/14^{ac}$ (14.3)
5	positive control	$14/14^{b}$ (100)
6	negative control	$0/14^{c}$ (0)

a, b, c- significantly different ($p<0.05$) from the same column

Table 7 The body weights of chickens at 28 and 35-day-old

Group	Vaccination	Body weight (g)[A]	
		28-day-old (0 dpi)	35-day-old (7 dpi)
1	H120+H120	1347.1±51.5	1842.9±126[a]
2	4/91+H120	1334.3±65.4	1854.3±103[a]
3	4/91+4/91	1366.4±57.5	1862.1±91.1[a]
4	H120+4/91	1352.9±61.3	1835.7±83.9[a]
5	Positive control	1332.9±47	1742.9±183.2[b]
6	Negative control	1330±65.5	1947.1±101.5[c]

[A] mean ± sd

a, b, c- significantly different ($p<0.05$) from the same column

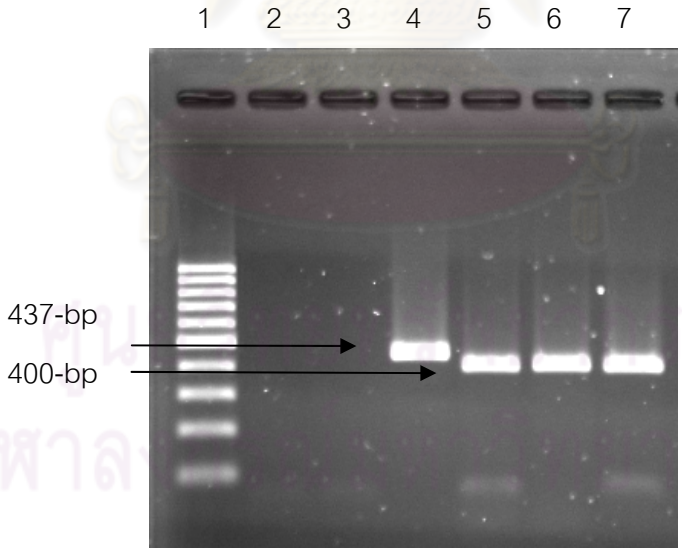


Figure 16 IBV detection using a primer set specific to THA80151. Lane1: 100-bp marker, detection with THA80151 specific primers; lane2-4, H120 4/91 and THA80151, respectively. Detection with IBV specific primers; Lane5-7, H120 4/91 and THA80151, respectively.

Virus detection

Detection of IBV was performed by RT-PCR. A new primer set could detect an isolate THA80151 but it could not detect vaccine strains H120 and 4/91 (Figure 16). IBV was 100% (Table 8) of detection in the tracheas of the groups 1, 3 and 5 (Figure 17, 19 and 21) and 76.8% (Table 8) of detection in the tracheas of the groups 2 and 4 (Figure 18 and 20). IBV was not detected in the tracheas of the negative control group (Figure 22).

Table 8 IBV detection (%) and average histopathologic lesion scores in the tracheas of the experimental chickens

Group	Vaccination	IBV detection (%)	Tracheal histopathologic lesion scores (average)
1	H120+H120	14/14[a] (100)	2.00[a]
2	4/91+H120	11/14[a] (78.6)	1.50[a]
3	4/91+4/91	14/14[a] (100)	1.93[a]
4	H120+4/91	11/14[a] (78.6)	1.57[a]
5	positive control	14/14[a] (100)	2.86[b]
6	negative control	0/14[b] (0)	0.00[c]

a, b, c- significantly different ($p<0.05$) from the same column

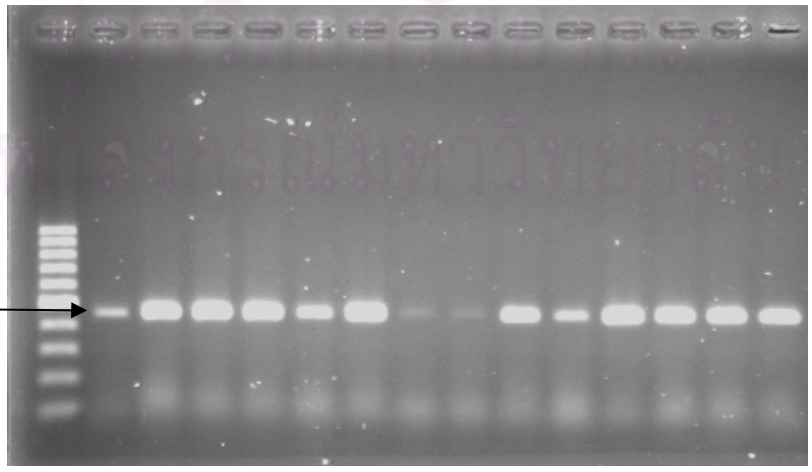


Figure 17 IBV detection in the tracheas of the vaccinated chickens in group 1

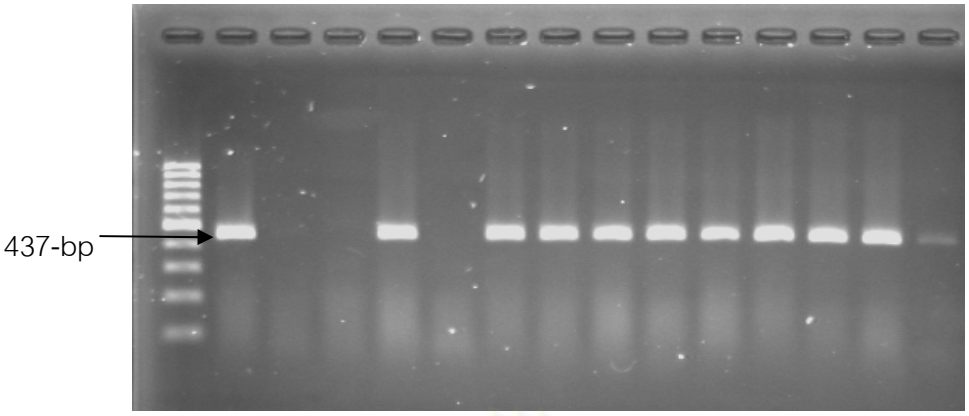


Figure 18 IBV detection in the tracheas of the vaccinated chickens in group 2

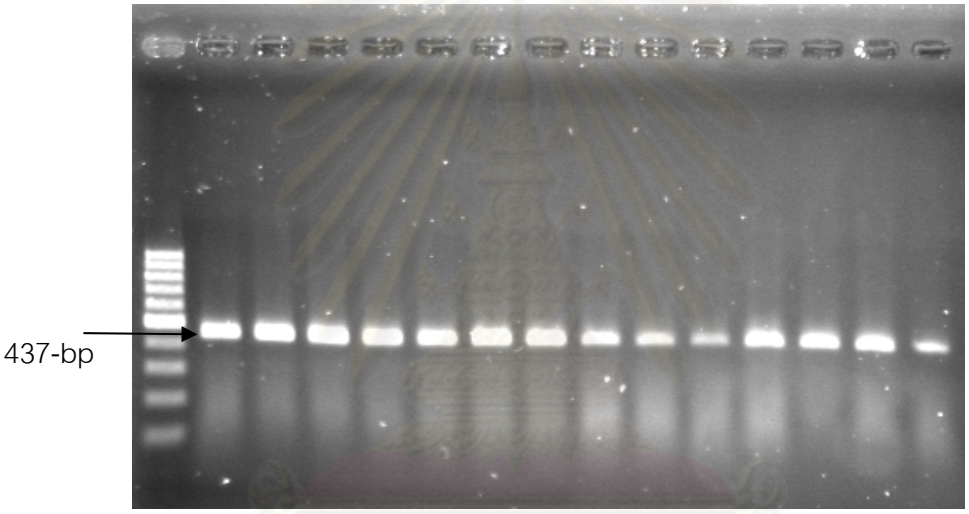


Figure 19 IBV detection in the tracheas of the vaccinated chickens in group 3

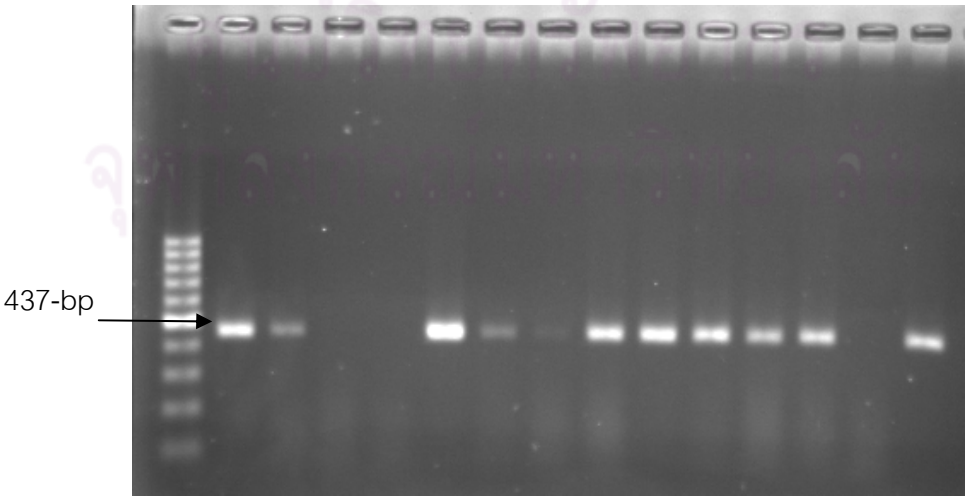


Figure 20 IBV detection in the tracheas of the vaccinated chickens in group 4

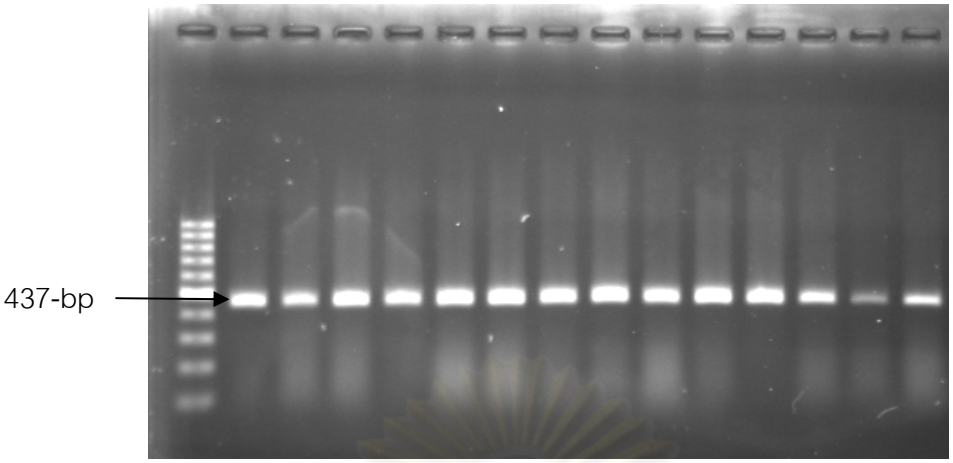


Figure 21 IBV detection in the tracheas of the positive control group

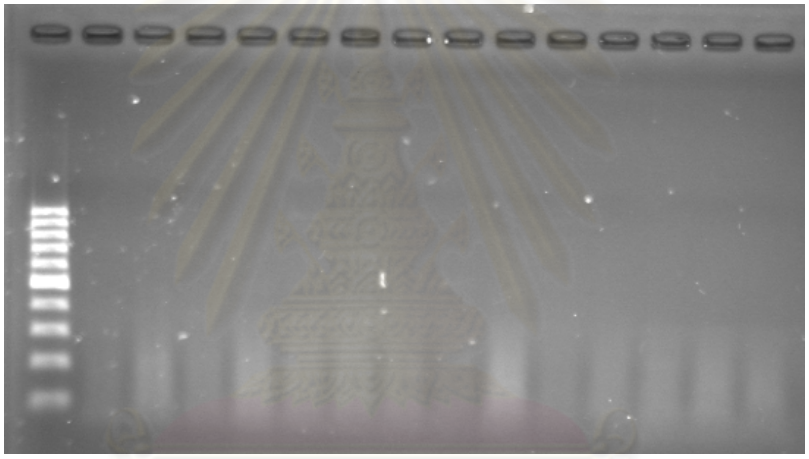


Figure 22 IBV detection in the tracheas of the negative control group

Histopathological examinations

At 7 dpi, the tracheas of vaccinated chickens and the positive control chickens had histopathologic lesions including loss of cilia from epithelial cells, desquamation of epithelial cells and thickening of the mucosa due to lymphoid infiltrations in lamina propia and submucosa. No prominent histopathologic lesions were observed in negative control chickens. The average histopathologic lesion scores in the tracheas of the vaccinated chickens were significantly lower ($p<0.05$) than non vaccinated chickens (Table 8).

IBV antibody response

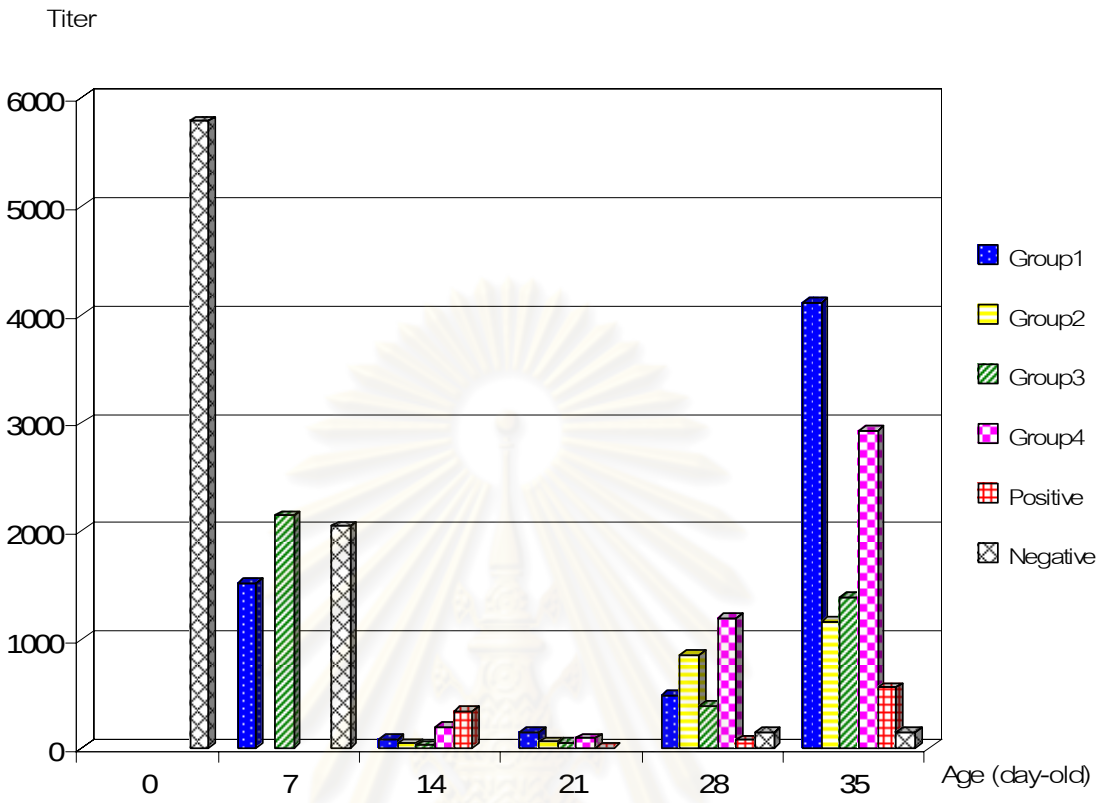


Figure 23 The average antibody titer levels comparing among the experimental groups

The average antibody titer levels comparing among the experimental groups were shown in Figure 23. At 1-day-old, the average IBV antibody titer of experimental chickens was 5783±2743. At 7-day-old, the average IBV antibody titer was not significant difference ($p>0.05$) among the groups. At 14-day-old, the average IBV antibody titres were 86.3±181, 41.5±85.5, 25.9±66.8, 196±541 and 344±388 in group 1, 2, 3, 4 and 5. The significant difference ($p<0.05$) was only found between group 3 and 5. At 21-day-old, the average IBV antibody titer was not significant difference ($p>0.05$) among the groups. At 28-day-old, the average IBV antibody titres were 482±437, 862±762, 395±314, 1202±704, 73.9±80.7 and 146.4±99.6 in group 1, 2, 3, 4, 5 and 6. The average titer of group 4 was significant difference ($p<0.05$) from group 1, 3, 5 and 6. The average titer of group 2 was not significant difference ($p>0.05$) from group 4 and 1 but significant difference ($p<0.05$) from the others. The average titer of group 1 was not significant difference ($p>0.05$) from group 2, 3, 5 and 6. At 35-day-old,

the average IBV antibody titres were 4113±919.8, 1167±649, 1396±681, 2932±1484, 140±180 and 560±584 in group 1, 2, 3, 4, 5 and 6. The average titer of group 1 and 4 was significant difference ($p<0.05$) from the others. The average titer of group 3 was not significant difference ($p>0.05$) from group 2 but significant difference from the others. The average titer of group 5 was not significant ($p>0.05$) difference from group 2 and 6 but significant difference ($p<0.05$) from the others.

CHAPTER V

DISCUSSION AND CONCLUSIONS

Discussion

One of the major problems of IBV is the frequent emergence of new variant serotypes. Different serotypes have been reported world-wide and new variant serotypes continue to be recognized (Gelb et al., 1991; Gough et al., 1992; Jia et al., 1995; Liu and Kong, 2004; Pohuang et al., 2009). Thus, it is necessary and important to be able to diagnose these new serotypes. Furthermore, determining the types as well as field isolates is important to select an appropriate vaccine against IBV infection in the next flock. Therefore, several tests have been employed to identify the isolates into serotypes or genotypes. Typing with RT-PCR and sequencing of the S1 gene is easier and faster than the more traditional virus neutralization methods, but it is difficult to design PCR primers that can be used for detect all of IBV isolates (Lee et al., 2001; Mondal et al., 2001; Worthington et al., 2008). Previously, we could not amplify a Thai IBV isolate THA001 (Pohuang et al., 2009) with the primer set and PCR method described by Kwon et al. (1993). To overcome this problem, the new primer sets were designed and the nested RT-PCR was developed in this study in order to increase the sensitivity and specificity of the test used to detect IBV isolated in Thailand.

IB is a highly infectious disease and a cause of economical loss in the poultry industry. Early diagnosis is essential for the effective control of an outbreak. Although an RT-PCR assay is commonly used for viral RNA detection (Kwon et al., 1993; Keeler et al., 1998), sometime there is a problem with sensitivity for the detection of low level viral RNA (Kim et al., 2001). Falcone et al. (1997) described a nested RT-PCR for IBV detection within 12 h but the step was carried out in separate tubes for RT and double-PCR amplification. Therefore, in this study, we established a nested PCR with initiating by one-step RT-PCR and followed with PCR reaction for the detection of IBV. The result showed that the whole procedure was easy to perform and allowed result to be obtained within 7 h.

The primer sets were selected from the highly conserved region of S1 gene because the S1 gene provides a good target for serotype identification by sequencing or restriction fragment length polymorphism (RFLP) (Keeler et al., 1998). Therefore, PCR-amplification products from our assay described here could be further analyzed by sequencing to confirm and identify the serotypes of IBV. The results of sensitivity determination showed that the nested RT-PCR was about 10 folds more sensitive than virus isolation in embryonated chicken eggs and 10-100 folds more sensitive than non-nested RT-PCR. A greater sensitivity of nested RT-PCR compared to the standard PCR has also been reported in the detection of other pathogens (Kho et al., 2000; Kim et al., 2001). Therefore, the nested RT-PCR enhances the sensitivity of IBV determination. This investigation is useful when detecting low levels of IBV particles. In the specificity determination, RNA extracted from six strains, amplified by the nested RT-PCR, showed target bands of 400-bp. This suggested that the regions selected for the primers were the conserved regions of the S1 gene of IBV. None of other avian viruses showed any amplified product. This indicated that the nested RT-PCR was not a cross-reaction with the other common avian viruses.

Genotyping of IBV on the basis of the S1 gene sequence, particularly the HVR region of the S1 gene, is the most common way to classify IBV isolates. It has been shown that the genetic typing based on HVR I (nucleotide position 114-325) of the S1 gene could represent the grouping method based on the whole S1 gene (Wang and Huang, 2000). Furthermore, in a recent study using an approximately 450-bp region covering HVR I and HVR II for IBV typing, it was found that typing by this region correlates with virus neutralization results (Lee et al., 2003). In our study, an approximately 878-bp region of S1 gene amplified by the outer primer of nested RT-PCR covering HVR I and HVR II (nucleotide position 31-908) were amplified and used for typing the field isolates in Thailand. Based on these results, a nested RT-PCR established in this study is a useful method for IBV detection; furthermore, PCR products amplified by using the outer primer sets can be used for genotyping of IBV.

In the genotyping by using partial S1 genes, we demonstrated that IBV isolates in Thailand between January and June, 2008 were divided into two distinct genotypic groups based on an analysis of the HVR of the S1 genes. After that, we continued to

collect the IBV samples until October 2009. Furthermore, the complete S1 gene sequences were determined in order to obtain more information about the evolution of Thai IBV. Herein, we found that fifteen Thai IBV isolated between 2008 and 2009 were divided into three distinct genotypic groups. When compared with the partial S1 genes, the group I Thai IBV was clustered into a different group but still unique to Thailand, the group II was clustered in the group of QX-like IBV and the group III reported only in the complete S1 gene study was clustered into the Massachusetts type. The result suggested that at least three groups of IBV are circulating at the present time in Thailand.

Although the basic amino acid residues of the cleavage recognition site of IBV did not correlate with cleavability, host cell range and virulence as orthomyxoviruses and paramyxoviruses, these sequences correlated with the geographic distribution of IBV (Jackwood et al., 2001). Two spike glycoprotein cleavage recognition sites were found in the Thai IBV isolates. The cleavage recognition site sequence, Arg-Arg-His-Arg-Arg, was found in the group I and the group II Thai IBV. This cleavage recognition site sequence had been previously reported in Chinese IBV isolates (Liu et al., 2006). Another cleavage recognition site sequence, Arg-Arg-Phe-Arg-Arg, was found in the group III Thai IBV. This cleavage recognition site sequence has been found in many countries (Jackwood et al., 2001; Liu et al., 2006). Based on these facts, the group I and the group II Thai IBV isolates have a close relationship with Chinese IBV isolates.

Overall, the group I Thai IBV appeared to be different from previous published strains by phylogenetic analysis. Interestingly, when the potential of recombination event was analyzed, the 5'-terminus of the S1 gene were similar to isolate THA001 which was isolated in Thailand in 1998 but the remaining sequences were similar to the group II Thai IBV. Surprisingly, when the S1 genes of the group II Thai IBV were analyzed, recombination event was also observed in the nucleotide sequences near the cleavage recognition site. Although the group II Thai IBV appeared to be similar to QXIBV, a region near the cleavage recognition site was found to be similar to JX/99/01 isolated in China in 1999 (Liu et al., 2006). Our primary concern was that the possible viral recombination resulting from the co-infection of heterologous field strains in the chicken flocks. The recombination may be subsequently occurring with co-infection with QXIBV

and JX/99/01 resulting for the occurrence of the group II Thai IBV. After that, the co-infection and exchange of genetic information between the group II Thai IBV and isolate THA001 resulted in the occurrence of the group I Thai IBV. As reported by Wang et al. (1993), the evidence of natural recombination could occur within the S1 gene of IBV field isolates. The natural recombination events observed here indicated that the S1 gene of IBV was the potential site for recombination and that the exchange of genetic information could occur in more than one region of the S1 gene.

The recombination event is thought to occur by the switching of the polymerase from one template to another during the genomic synthesis (Tolskaya et al., 1987). Specifically, an intergenic (IG) consensus sequences (CTGAACAA or CTTAACAA) serve as recombination “hot spots” (Lee and Jackwood, 2000). Sometime, the presence of homologous nucleotide sequence regions between the strains may serve as a potential recombination junction or cross-over site (Wang et al., 1993). Although the IG consensus sequences were not observed in this study we found the highly conserved nucleotide sequences present around the recombination breakpoint regions in the putative parental strains. The result suggested that the homologous nucleotide sequence regions between the strains may play a role as a cross-over site of our IBV isolates.

QXIBV was first described and identified in China, after that this IBV genotype spread and became one of the most prominent genotypes in many countries (Beato et al., 2005; Bochkov et al., 2006; Gough et al., 2008). Although the complete S1 gene of the group II Thai IBV was of 95.5-96.0% nucleotide identity to Chinese QXIBV, the nucleotide sequences at 3’-terminus near the cleavage recognition site were different from the Chinese QXIBV. We found that this region was closely related to the Chinese isolate JX/99/01. Interestingly, this change was not found when the comparisons of the S1 gene among QXIBV reported in others countries were analyzed. These findings suggested that the group II Thai IBV had gone through evident evolution change in Thailand.

The isolates in the group III were clustered into the Massachusetts type. The Massachusetts type was also isolated in many countries world-wide including Asian countries such as China, Japan and South Korea (Liu et al., 2006). Some of the

Massachusetts type isolated here may be a field challenge which comes from the point mutation of vaccine strains because they had 97.5-99.9% nucleotide and 93.2-99.7% amino acid identity to the vaccine strains (data not shown). It has been shown that the S1 gene of the 4/91 pathogenic virus differs only 0.6% from the vaccine strain whereas other genes were no different (Callison et al., 2001). However, the possibility that some of them were re-isolation of vaccine strains could not be excluded due to the 100% identity with the Massachusetts type, the vaccine strain used in Thailand. As indicated in the previous report, we could not conclusively distinguish between the vaccine strain and field challenge of the same genotype, especially when a sequence identity of between 99-100% was found (Worthington et al., 2008).

It is known that IB vaccines administered at 1-day-old are interfered by maternal derived antibodies (Pensaert and Lambrechts, 1994; Mondal and Naqi, 2001; Terregino et al., 2008). Therefore, it seems reasonable to vaccinate the chickens, particularly under field conditions, with a double vaccination. In the case of IB, although vaccination for the prevention is widely practiced, it still has been the incidence of disease outbreaks sporadically in vaccinated chicken flocks (Gelb et al., 1991; Gough et al., 1992; Liu and Kong, 2004). As observed in our study, the chicken flocks showed clinical signs associated with IBV infection including, gasping coughing, sneezing and tracheal rales although all of them had been vaccinated. It has been reported that complete protection is provided by homologous strains but a low level or no protection is observed when vaccination with one heterologous strain (Pensaert and Lambrechts, 1994; Al-Tarcha and Sadoon, 1991; Liu et al., 2009). However, the partial protection may be providing after vaccination with live attenuated vaccines (Darbyshire, 1985; Albassam et al., 1986; Wang et al., 1996; Liu et al., 2009). Furthermore, using a combined vaccination program incorporating with different live attenuated vaccine strains provide a good protection against heterologous strain (Cook et al., 2001; Martin et al., 2007).

In this study, the chickens were vaccinated with 2 live attenuated vaccine strains at 1 and 14-day-old and challenged with a heterologous strain, Thai IBV isolate THA80151, at 28-day-old. This isolate was representing for group II Thai IBV, the most frequently isolated type in Thailand during the time of study. Therefore, it was selected

as a challenged virus. At the time of evaluation, the tracheas were only used to evaluate of protection study because the tracheas were the primary site of IBV infection. Moreover, the results from the preliminary experiment showed that the virus did not cause prominent lesion in the kidney although it was clustered into QXIBV, a nephropathogenic strain. This observation was also observed by Benyeda et al. (2009). They reported that five QX-like IBV strains (Chinese, French, Slovakian, Greek and Hungarian) isolated from different countries had the difference in clinical signs and pathological lesions. The comparison of pathogenicity, under the experimental condition, showed that the respiratory lesions, nephritis and oviduct dilatation were observed in the infected chickens; however, variation in the pathogenicity was found among the strains.

The results of the protection study showed that the body weight gains of the vaccinated chickens were higher than the infected but non vaccinated chickens. Furthermore, the morbidity rate and tracheal histopathologic lesion scores of vaccinated chickens were lower than the infected chickens that were not vaccinated, although the number of infected chickens was not significantly difference. Interestingly, the clinical protection was also found in the chickens vaccinated 2 times with the same IBV vaccine strains; however, it appeared to have a slightly lower protection than vaccinated 2 times with the heterologous strains. These suggested that the live attenuated vaccines used in this study could induce clinical protection when administered at an interval of 2 weeks but it could not protect against the infection of a challenge strain. The results are consistent with other studies that show the level of protection of heterologous challenge test. Martin et al. (2007) reported that the vaccination protocol consisted of vaccination with Mass/Conn at hatch followed by Hol/Ark at 18-day-old could reduce the disease associated with IBV isolate CA99 which was typed into serotype CA99 distinct from Ark, Mass, and Conn serotypes. Terregino et al. (2008) described the vaccination program using Ma5 at 1-day-old and 4/91 at 14-day-old. It could protect the chickens from infection with the QX-like IBV variant strain isolated in Italy. They found that no clinical signs attributable to IBV infection were observed in vaccinated chickens and the number of IBV positive tracheal samples from the vaccinated SPF and vaccinated commercial broilers were lower than the infected non vaccinated chickens.

Several reports had shown that vaccination with live attenuated vaccines provide a protection against challenged with heterologous IBV strains. Darbyshire (1985) reported that chickens vaccinated with the H120 strain could reduce the amount of Australian T strain 30,000 times within 4 days post-infection. Albassam et al. (1986) showed that chickens vaccinated with the H120 strain at 10-day-old and challenged at 4 weeks later with one of 4 IBV strains; Gray, Holte, Australian T and Italian did not show tracheal lesions but 50-70% of the chickens showed renal lesions. Pensaert and Lambrechts (1994) demonstrated that vaccination at 1-day-old either with homologous strain, H120 or combined H120 and D274 vaccines reduced the duration of virus replication in the tracheas of the chickens inoculated with B1648 strain at 4-week-old. Wang et al. (1996) showed that in the H120 strain vaccinated chickens, only 1 of 10 that had been challenged with the 1171 strain and none of the chickens that had been challenged with the 1449 strain showed clinical signs associated with IBV infection. Liu et al. (2009) showed that the clinical protection against pathogenic CK/CH/LDL/97I was conferred following vaccination with the heterologous vaccine strains CK/CH/LDT3/03 P120 and J9 but the respiratory protection conferred by the heterologous vaccine strains was <50%, as determined by virus isolation from the tracheas. The reasons for the clinical protection against heterologous challenge may be that live attenuated vaccines induce the local immunity of the upper respiratory tract resulting to reduce the replication of challenge virus (Darbyshire, 1985; Terregino et al., 2008).

It is known that the trachea is a primary site for the IBV to propagate. Live vaccines can reduce mortality in infected animals due to mucosal immunity induced by the virus. The mucosal immunity prohibits invasion and propagation of the virus in the tracheal mucosa. Thompson et al. (1997) examined the mucosal immunity of infected chickens and found that 70% of the samples contained IgA, 52% and 56% of the samples contained IgG and IgM, respectively. Nakamura et al. (1991) found IgM, IgA, and IgG in the trachea more often in chickens that were resistant to the disease compared to susceptible chickens. Live vaccines can also induce cellular immunity, which can prohibit virus attack as well. Pei et al. (2003) found that transfer of CD8+ T cell isolated during 3-6 weeks after infection caused by IBV to 6-day-old chicks can protect the chicks from infection.

The antibody titres in chicken serums collected before challenge inoculation date gradually declined with time, which is consistent with the reported on the changes in maternal antibody titres (Al-Taracha et al., 1991). Hens transmit maternal antibodies to their offspring by depositing the antibodies in the eggs. The percentage of IgY transfer from hens plasma to their chicks is estimated to be 30%. The levels of anti-IBV antibody in the chicks serum detected by ELISA kit are highest on 3-day-old and decrease substantially on 7 and 14-day-old, respectively (Hamel et al., 2006). Although the immune responses of young chickens are interfered by maternal derived antibodies, the protection can be provided after vaccination. Al-Taracha and Sadoon (1991) showed that chickens having maternal derived antibodies can be protected against intra-tracheal challenge with IBV strain M 41 by vaccination via intranasal-eye drop at 1-day-old. Pensaert and Lambrechts (1994) showed that vaccination of 1-day-old chicks with maternal derived antibodies induced protection against a challenge strain but the protection was lower than the chicks without maternal derived antibodies.

At the day of challenge inoculation, the average antibody titres of vaccinated groups appeared to be higher than the positive control group but the infection rate at the time of evaluation was high. These antibodies might not be the primary protection mechanism against IBV infection. The humoral antibodies might play a role on reducing the tissues damage by challenge virus. It had been shown that humoral immunity to IBV played a role directly against viremia of the viruses; therefore, it was important in protection of non respiratory tissues (Terregino et al., 2008; Lui et al., 2009). However, the complete protection was not occurring in this study because the vaccine strains and a challenge virus were heterologous. The antibody titers induced by vaccines might be occurring from the group specific epitopes located on the enveloped of virus (Parr and Collisson, 1993) and they induced partial protection against a challenge virus.

Conclusions

The data obtained from this study indicated that firstly, the nested RT-PCR established in this study can be a sensitive, specific and practical method for the diagnosis of IBV infection. Secondly, at least three groups of IBV are circulating at the present time in Thailand and they undergo genetic recombination and evolution. The

natural recombination is contributing to the emergence of a new genotypes or IBV variants in the field. Lastly, for the protection study, vaccination with the live attenuated vaccine strains used in this study administered at an interval of 2 weeks could induce clinical protection, reduce body weight gain loss and reduce tracheal histopathologic lesions but could not protect against the infection of a challenge strain.

REFERENCES

Abdel-Moneim, A.S., Zlotowski, P, Veits, J., Keil, G.M. and Teifke, J.P. 2009. Immunohistochemistry for detection of avian infectious bronchitis virus strain M41 in the proventriculus and nervous system of experimentally infected chicken embryos. Virol. J. 6: 15.

Abd El Rahman, S., El-Kenawy, A.A., Neumann, U., Herrler, G. and Winter, C. 2009. Comparative analysis of the sialic acid binding activity and the tropism for the respiratory epithelium of four different strains of avian infectious bronchitis virus. Avian Pathol. 38: 41-45.

Albassam, M.A., Winterfield, R.W. and Thacker, H.L. 1986. Comparison of the nephropathogenicity of four strains of infectious bronchitis virus. Avian Dis. 30: 468-476.

Al-Tarcha, B. and Sadoon, S.A. 1991. Cross-protection studies with vaccine strain H120 of infectious bronchitis virus using ciliary activity. Acta Vet. Hung. 39: 95-101.

Animas, S.B., Otsuki, K., Hanayama, M., Sanekata, T. and Tsubokura, M. 1994. Experimental infection with avian infectious bronchitis virus (Kagoshima-34 strain) in chicks at diferrent ages. J. Vet. Med. Sci. 56: 443-447.

Antarasena, C., Sahapong, S., Aowcharoen, B., Choe-ngern, N., Kongkanunt, R. 1990. Avian infectious bronchitis in the Southern part of Thailand. Songklanakarin J. Sci. Technol. 12: 273-279.

Beato, M.S., De Battisti, C., Terregino, C., Drago, A., Capua, I. and Ortali, G. 2005. Evidence of circulation of Chinese strain of infectious bronchitis virus (QXIBV) in Italy. Vet. Rec. 156: 720.

Benyeda, Z., Mato, T., Suveges, T., Szabo, E., Kardi, V., Abonyi-Toth, Z., Rusvai, M. and Palya, V. 2009. Comparison of the pathogenecity of QX-like, M41 and 793/B infectious bronchitis strains from different pathological conditions. Avian Pathol. 38: 449-456.

Bhattacharjee, P.S., Naylor, C.J. and Jones, R.C. 1994. A simple method for immunofluorescence staining of tracheal organ cultures for the rapid identification of infectious bronchitis virus. Avian Pathol. 23: 471-480.

Bochkov, Y.A., Batchenko, G.V., Shcherbakova, L.O., Borisov, A.V. and Drygin, V.V. 2006. Molecular epizootiology of avian infectious bronchitis in Russia. Avian Pathol. 35: 379-393.

Boursnell, M.E.G., Brown, T.D.K., Foulds, I.J., Green, P.F., Tomley, F.M., Binns, M.M. 1987. Completion of the sequence of the coronavirus avian infectious bronchitis virus. J. Gen. Virol. 68: 57-77.

Callison, S.A., Hilt, D.A., Boynton, T.O., Sample, B.F., Robison, R., Swayne, D.E. and Jackwood, M.W. 2006. Development and evaluation of a real-time RT-PCR assay for the detection of infectious bronchitis virus from infected chickens. J. Virol. Methods. 138: 60-65.

Callison, S.A., Jackwood, M.W., Hilt, D.A. 2001. Molecular characterization of infectious bronchitis virus isolates foreign to the United States and comparison with United States isolates. Avian Dis. 45: 492-499.

Case, J.T., Sverlow, K.W. and Reynolds, B.J. 1997. A novel protein polymorphism differentiates the California serotype of infectious bronchitis from other serptypes common to California. J. Vet. Diag. Invest. 9: 149-155.

Cavanagh, D. 1983. Coronavirus IBV: Structural characterization of the spike protein. J. Gen. Virol. 64: 2577-2583.

Cavanagh, D. 2003. Severe acute respiratory syndrome vaccine development: experiences of vaccination against avian infectious bronchitis coronavirus. Avian Pathol. 32: 567-582.

Cavanagh, D. and Davis, P.J. 1986. Coronavirus IBV: removal of spike glycopolypeptide S1 by urea abolishes infectivity and hemagglutination but not attachment to cells. J. Gen. Virol. 67: 1443-1448.

Cavanagh, D. and Naqi, S.A. 2003. Infectious bronchitis. In: Diseases of poultry. 11th ed. A.M. Saif, Y.M. Fadly, L.R. McDougald and D.E. Swayne (ed.). Iowa State University Press, Ames, IA. 101-119.

Cavanagh, D., Davis, P.J., Cook, J.K.A., Li, D., Kant, A. and Koch, G. 1992. Location of the amino acid differences in the S1 spike glycoprotein subunit of closely related serotypes of infectious bronchitis virus. Avian Pathol. 21: 33-43.

Cavanagh, D., Davis, P.J. and Mockett, A.P.A. 1988. Amino acids within hypervariable region1 of avian coronavirus IBV (Massachusetts serotype) spike glycoprotein are associated with neutralization epitopes. Virus Res. 11:141-150.

Cavanagh, D., Davis, P.J., Darbyshire, H. and Peters, R.W. 1986. Coronavirus IBV: Virus retaining spike glycopeptide S2 but not S1 is unable to induce virus-neutralizing or haemagglutination-inhibiting antibody, or induce chicken tracheal protection. J. Gen. Virol. 67:1435-1442.

Chandra, M. 1987. Comparative nephropathogenicity of different strains of infectious bronchitis virus in chickens. Poult. Sci. 66: 954-959.

Chen, B.Y. and Itakura, C. 1996. Cytopathology of chick renal epithelial cells experimentally infected with avian infectious bronchitis virus. Avian Pathol. 25: 675-690.

Chen, B.Y., Guo, A.Z., Peng, B., Zhang, M.F., Guo, H.Y. and Chen, H.C. 2007. Infection of HeLa cells by avian infectious bronchitis virus is dependent on cell status. Avian Pathol. 36: 269-274.

Chen, B.Y., Hosi, S., Nunoya, T. and Itakura, C. 1996. Histopathology and immunohistochemistry of renal lesions due to infectious bronchitis virus in chicks. Avian Pathol. 25: 269-283.

Chindavanig, P. 1962. Studies on the attenuated on infectious bronchitis virus. J. Thai Vet. Med. Assoc. 12: 1-7.

Chong, K.T. and Apostolov, K. 1982. The pathogenesis of nephritis in chickens induced by infectious bronchitis virus. J. Comp. Pathol. 92: 199-211.

Chousalkar, K.K. and Roberts, J.R. 2007a. Ultrastructural observations on effects of infectious bronchitis virus in eggshell-forming regions of the oviduct of the commercial laying hen. Poult. Sci. 86: 1915-1919.

Chousalkar, K.K. and Roberts, J.R. 2007b. Ultrastructural study of infectious bronchitis virus infection in infundibulum and magnum of commercial laying hens. Vet. Microbiol. 122: 223-236.

Chousalkar, K.K., Roberts, J.R. and Reece, R. 2007. Histopathology of two serotypes of infectious bronchitis virus in laying hens vaccinated in rearing phase. Poult. Sci. 86: 59-62.

Clarke, J.K., McFerran, J.B. and Gay, F.W. 1972. Use of allantoic cells for the detection of avian infectious bronchitis virus. Arch. ges. Virusforsch. 36: 62-70.

Cook, J.K.A. and Huggins, M.B. 1986. Newly isolated serotypes of infectious bronchitis virus : their role in disease. Avian Pathol. 15: 129-138.

Cook, J.K.A., Darbyshire, J.H. and Peters, R.W. 1976. The use of chicken tracheal organ cultures for the isolation and assay of avian infectious bronchitis virus. Arch Virol. 50: 109-118.

Cook, J.K.A., Orbell, S.J., Woods, M.A. and Huggins, M.B. 1996. A survey of the presence of a new infectious bronchitis virus designated 4/91. Vet. Rec. 138: 178-180.

Cook, J.K.A., Orbell, S.J., Woods, M.A. and Huggins, M.B. 1999. Breadth of protection of the respiratory tract provided by different live-attenuated infectious bronchitis vaccines against challenge with infectious bronchitis virus of heterologous serotypes. Avian Pathol. 28: 477-485.

Cook, J.K.A, Chesher, J., Baxendale, W., Greenwood, N., Huggins, M.B. and Orbell, S.J. 2001. Protection of chickens against renal damage caused by a nephropathogenic infectious bronchitis virus. Avian Pathol. 30: 423-426.

Crinion, R.A.P. 1972. Egg quality and production following infectious bronchitis virus exposure at one day old. Poult. Sci. 51: 582-585.

Crinion, R.A.P., Ball, A.R. and Hofstad, M.S. 1971. Abnormalities in laying chickens following exposure to infectious brobchitis virus at one day old. Avian Dis. 15: 42-48.

Crinion, R.A.P. and Hofstad, M.S. 1972. Pathogenicity of four serotypes of avian infectious bronchitis virus for the oviduct of young chickens of various ages. Avian Dis. 16: 351-363.

Cubillos, A., Ulloa, J., Cubillos, V. and Cook, J.K.A. 1991. Characterisation of strains of infectious bronchitis virus isolated in Chile. Avian Pathol. 20: 85-99.

Darbyshire, J.H. 1985. A clearance test to assess protection in chickens vaccinated against avian infectious bronchitis virus. Avian Pathol. 14: 497-508.

De Wit, J.J. 2000. Detection of infectious bronchitis virus. Avian Pathol. 29: 71-93.

De Wit, J.J., Koch, G., Kant, A. and van Roozelaar, D.J. 1995. Detection by immunofluorescent assay of serotype-specific and group-specific antigens of infectious bronchitis virus in tracheas of broilers with respiratory problems. Avian Pathol. 24: 465-474.

Dhinakar Raj, G. and Jones, R.C. 1996a. An in vitro comparison of the virulence of seven strains of infectious bronchitis virus using tracheal and oviduct organ cultures. Avian Pathol. 25: 649-662.

Dhinakar Raj, G. and Jones, R.C. 1996b. Immunopathogenesis of infection in SPF chicks and commercial broiler chickens of a variant infectious bronchitis virus of economic importance. Avian Pathol. 25: 481-501.

Dhinakar Raj, G. and Jones, R.C. 1997. Growth of infectious bronchitis virus vaccines in oviducts derived from oestrogen-treated chicks and embryos. Vaccine. 15: 163-168.

Fabio, J.D., Rossini, L.I., Orbell, S.J., Pual, G., Huggins, M.B., Malo, A., Silva, B.G.M. and Cook, J.K.A. 2000. Characterization of infectious bronchitis viruses isolated from outbreaks of disease in commercial flocks in Brazil. Avian Dis. 44: 582-589.

Falcone, E., D' Amore, E., Trani, L.D., Sili, A., and Tollis, M. 1997. Rapid diagnosis of avian infectious bronchitis virus by the polymerase chain reaction. J. Virol. Methods. 64: 125-130.

Ferreira, H.L., Pliz, D., Mesquita, L.G. and Cardoso, T. 2003. Infectious bronchitis virus replication in the chicken embryo related cell line. Avian Pathol. 32: 413-417.

Ganapathy, K., Cargill, P.W. and Jones, R.C. 2005. Effects of cold storage on detection of avian infectious bronchitis virus in chicken carcasses and local antibodies in tracheal washes. J. Virol. Methods. 126: 87-90.

Gelb, J., Jr., Keeler, C.L., Jr, Nix, W.A., Rosenberger, J.K. and Cloud, S.S. 1997. Antigenic and S1 genomic characterization of the Delaware variant serotype of infectious bronchitis virus. Avian Dis. 41: 661-669.

Gelb, J., Jr, Wolff, J.B. and Moran, C.A. 1991. Varient serotypes of infectious bronchitis virus isolated from commercial layer and broiler chickens. Avian Dis. 35: 82-87.

Gough, R.E., Cox, W.J., Welchman, D., Worthington, K.J., Jones, R.C. 2008. Chinese QX strain of infectious bronchitis virus isolated in UK. Vet. Rec. 162: 99-100.

Gough, R.E., Randall, C.J., Dagless, M., Alexander, D.J., Cox, W.J. and Pearson, D. 1992. A 'new' strain of infectious bronchitis virus infecting domestic fowl in Great Britain. Vet. Rec. 130: 493-494.

Hall, T.A. 1999. BioEdit: a user friendly biological sequence alignment editor and analysis program for Windows 95/98/NT. Nulc. Acids. Symp. Ser. 41: 95-98.

Hamel, K.R., Burgess, S.C., Pevzner, I.Y. and Erf, G.F. 2006. Maternal antibody transfer from dams to their egg yolks, egg whites, and chicks in meat lines of chickens. Poult. Sci. 85: 1364-1372.

Hofstad, M.S. and Yoder, Jr., H.W. 1966. Avian infectious bronchitis –virus distribution in tissues of chicks. Avian Dis. 10: 230-139.

Ignjatovic, J. and Ashton, F. 1996. Detection and differentiation of avian infectious bronchitis virus using a monoclonal antibody-based ELISA. Avian Pathol. 25: 721-736.

Ignjatovic, J. and Galli, L. 1994. The S1 glycoprotein but not the N or M proteins of avian infectious bronchitis virus induces protection in vaccinated chickens. Arch. Virol. 138: 117-134.

Ignjatovic, J., Reece, R. and Ashton, F. 2003. Susceptibility of three genetic lines of chicks to infection with a nephropathogenic T strain of avian infectious bronchitis virus. J. Com. Pathol. 128: 92-98.

Jackwood, M.W., Hilt, D.A., Callison, S.A., Lee, C.W., Plaza, H. and Wade, E. 2001. Spike glycoprotein cleavage recognition site analysis of infectious bronchitis virus. Avian Dis. 45: 366-372.

Jang, J.H., Sung, H.W., Song, C.S. and Kwon, H.M. 2007. Sequence analysis of S1 glycoprotein gene of infectious bronchitis viruses: identification of a novel phylogenetic group in Korea. J. Vet. Sci. 8: 401-407.

Jia, W., Karaca, K., Parrish, C.R. and Naqi, S.A. 1995. A novel variant of avian infectious bronchitis virus resulting from recombination among three different strains. Arch. Virol. 140:259-271.

Jones, R.C. and Jordan, F.T.W. 1970. The exposure of day-old chicks to infectious bronchitis and subsequent development of the oviduct. Vet Rec. 87: 504-505.

Kant, A., Koch, G., Roozelaar, D.J., Kusters, J.G., Poelwijk, F.A.J. and van der Zeijst, B. A.M. 1992. Location of antigenic sites defined by neutralizing monoclonal antibodies on the S1 avian infectious bronchitis virus glycopeptide. J. Gen. Virol. 73: 591-596.

Kapczynski, D.R., Sellers, H.S., Rowland, G.N. and Jackwood, M.W. 2002. Detection of in ovo-inoculated infectious bronchitis virus by immunohistochemistry and in situ hybridization with a riboprobe in epithelial cells of the lung and cloacal bursa. Avian Dis. 46: 679-685.

Keeler, C.L., Jr., Reed, K.L., Nix, W.A. and Gelb, J., Jr. 1998. Serotype identification of avian infectious bronchitis virus by RT-PCR of the peplomer (S1) gene. Avian Dis. 42: 275-284.

Kho, C.L., Mohd-Azmi, M.L., Arshad, S.S. and Yusoff, K. 2000. Performance of an RT-nested PCR ELISA for detection of Newcastle disease virus. J. Virol Methods. 86: 71-83.

Kim, Y.H., Cho, K.W., Youn, H.Y., Yoo, H.S. and Han, H.R. 2001. Detection of canine distemper virus (CDV) through one step RT-PCR combined with nested PCR. J. Vet. Sci. 2: 59-63.

Kingham, B.F., Keeler, C.L., Jr, Nix, W.A., Ladman, B.S. and Gelb, J., Jr. 2000. Identification of avian infectious bronchitis virus by direct automated cycle sequencing of the S1 gene. Avian Dis. 44: 325-335.

Koch, G., Hartog, L., Kant, A. and van Roozelaar, D.J. 1990. Antigenic domains on the peplomer protein of avian infectious bronchitis virus: correlation with biological functions. J. Gen. Virol. 71: 1929-1935.

Kusters, G.J., Niesters, M.G.H., Lenstra, A.J., Horzinek, C.M. and van der Zeijst, M.A.B. 1989. Phylogeny of antigenic variants of avian coronavirus IBV. Virology. 169: 217-221.

Kwon, H.M., Jackwood, M.W., Brown, T.P. and Hilt, D.A. 1993. Polymerase chain reaction and a biotin-labeled DNA probe for detection of infectious bronchitis virus in chickens. Avian Dis. 37: 149-156.

Ladman, B.S., Loupos, A.B. and Gelb, J., Jr. 2006. Infectious bronchitis virus S1 gene sequence comparison is a better predictor of challenge of immunity in chickens than serotyping by virus neutralization. Avian Pathol. 35: 127-133.

Lee, C.W. and Jackwood, M.W. 2000. Evidence of genetic diversity generated by recombination among avian coronavirus IBV. Arch. Virol. 145:2135-2148.

Lee, C.W., Brown, C. and Jackwood, M.W. 2002. Tissue distribution of avian infectious bronchitis virus following in ovo inoculation of chicken embryos examinated by in situ hybridization with antisense digoxigenin-labeled universal riboprobe. J. Vet. Diagn. Invest. 14: 377-381.

Lee, C.W., Hilt, D.A. and Jackwood, M.W. 2000. Redesign of primer and application of the reverse transcriptase-polymerase chain reaction and restriction fragment length polymorphism test to the DE072 strain of infectious bronchitis virus. Avian Dis. 44: 650-654.

Lee, C.W., Hilt, D.A. and Jackwood, M.W. 2001. Identification and analysis of the Georgia 98 serotype, a new serotype of infectious bronchitis virus. Avian Dis. 45: 164-172.

Lee, C.W., Hilt, D.A. and Jackwood, M.W. 2003. Typing of field isolates of infectious bronchitis virus based on the sequence of the hypervariable region in the S1 gene. J. Vet. Diagn. Invest. 15: 344-348.

Lenstra, J.A., Kusters, J.G., Koch, G. and van der Zeijst, B.A.M. 1990. Mapping of viral epitopes with prokaryotic expression products. Arch. Virol. 100:1-24.

Liu, S.W. and Kong, X.G. 2004. A new genotype of nephropathogenic infectious bronchitis virus circulating in vaccinated and non-vaccinated flocks in China. Avian Pathol. 33: 321-327.

Liu, S.W., Zhang, Q.X., Chen, J.D., Han, Z.X., Liu, X., Feng, L., Shao, Y.H., Rong, J.G., Kong, X.G. and Tong, G.Z. 2006. Genetic diversity of avian infectious bronchitis coronavirus strains isolated in China between 1995 and 2004. Arch. Virol. 151: 1133-1148.

Liu, S.W., Zhang, X.N., Wang, Y., Li, C.G., Liu, Q., Han, Z.X., Zhang, Q.X., Kong, X.G. and Tong, G.Z. 2009. Evaluation of the protection conferred by commercial

vaccines an attenuated heterologous isolates in China against the CK/CH/LDL/97I strain of infectious bronchitis coronavirus. Vet. J. 179: 130-136.

Lole, K.S., Bollinger, R.C., Paranjape, R.S., Gadkari, D., Kulkarni, S.S., Novak, N.G., Ingersoll, R., Sheppard, H.W. and Ray, S.C. 1999. Full-length human immunodeficiency virus type1 genomes from subtype C-infected seroconverters in India, with evidence of intersubtype recombination. J. Virol. 73: 152–160.

Lucio, B. and Fabricant, J. 1990. Tissue tropism of three cloacal isolates and Massachusetts strain of infectious bronchitis virus. Avian Dis. 34: 865-870.

Martin, M.P., Wakenell, P.S., Woolcock, P. and O'Connor, B. 2007. Evaluation of the effectiveness of two infectious bronchitis virus vaccine programs for preventing disease caused by California IBV field isolate. Avian Dis. 51: 584-589.

Mondal, S.P. and Naqi, S.A. 2001. Maternal antibody to infectious bronchitis virus: its role in protection against infection and development of active immunity to vaccine. Vet. Immuno. Immunopathol. 79: 31-40.

Mondal, S.P., Lucio-Martinez, B. and Naqi, S.A. 2001. Isolation and characterization of a novel antigenic subtype of infectious bronchitis virus serotype DE072. Avian Dis. 45: 1054-1059.

Moore, K.M., Bennett, J.D., Seal, B.S. and Jackwood, M.W. 1998. Sequence comparison of avian infectious bronchitis virus S1 glycoproteins of the Florida serotype and five variant isolates from Georgia and California. Virus Genes. 17: 63-83.

Nakamura, K., Cook, J.K.A., Otsuki, K., Huggins, M.B. and Frazier, J.A. 1991. Comparative study of respiratory lesion in two chicken lines of different susceptibility infected with infectious bronchitis virus: histology, ultrastructure and immunohistochemistry. Avian Pathol. 20: 241-257.

Office International des Epizooties (OIE). 2004. Avian infectious bronchitis: Manual of diagnostic test of vaccine for terrestrial animals, 5th edition [Online]. Available: http://www.oie.int/eng/normes/ mmanual/A_summry.htm.

Otsuki, K., Huggins, M.B. and Cook, J.K.A. 1990. Comparison of the susceptibility to avian infectious bronchitis virus infection of two inbred lines of white leghorn chickens. Avian Pathol. 19: 467-475.

Otsuki, K., Noro, K., Yamamoto, H. and Tsubokura, M. 1979. Studies on avian infectious bronchitis virus (IBV): Propagation of IBV in several cultured cells. Arch. Virol. 60: 115-122.

Owen, R.L., Cowen, B.S., Hattel, A.L., Naqi, S.A., and Wilson, R.A. 1991. Detection of viral antigen following exposure of one-day old chickens to the Holland-52 strain of infectious bronchitis virus. Avian Pathol. 20: 663-673.

Parr, R.L. and Collisson, E.W. 1993. Epitopes on the spike protein of a nephropathogenic strain of infectious bronchitis virus. Arch. Virol. 133: 369-383.

Parsons, D., Ellis, M.M., Cavannagh, D. and Cook, J.K.A. 1992. Characterisation of an infectious bronchitis virus isolated from vaccinated broiler breeder flocks. Vet. Rec. 131: 408-411.

Pei, J., Briles, W.E. and Collisson, E.W. 2003. Memory T cells protect chicks from acute infectious bronchitis virus infection. Virology. 306: 376-384.

Pensaert, M. and Lambrechts, C. 1994. Vaccination of chickens against a Belgian nephropathogenic strain of infectious bronchitis virus B1648 using attenuated homologus strains. Avian Pathol. 23: 631-641.

Perlman, S. and Dandekar, A.A. 2005. Immunopathogenesis of coronavirus infection implications for SARS. Nature reviews. 5: 917-927.

Pohuang, T., Chansiripornchai, N., Tawatsin, A. and Sasipreeyajan, J. 2009. Pathogenesis of a new genotype infectious bronchitis virus isolated in chickens. Indian Vet. J. 86:1110-1112.

Pradhan, H.K., Mohanty, G.G. and Rajya, B.S. 1984. Comparative sensitivity of oviduct and tracheal organ cultures and chicken embryo kidney cultures to infectious bronchitis virus. Avian Dis. 27: 594-601.

Ratanasethakul, C., Chuachan, K., Sukulapong, V. and Sarachoo, K. 1999. Pathogenesis of nephritis in chickens induced by infectious bronchitis virus. Proceeding of quality control in animal production: nutrition, management, health and product. Chiang Mai, Thailand. 8-10 December: 243-252.

Reed, L.R. and Muench, H. 1938. A simple method of estimating fifty percent endpoints. Am. J. Hyg. 27: 493-497.

Robertson, D.L., Hahn, B.H. and Sharp, P.M. 1995. Recombination in AIDS viruses. J. Mol. Evol. 40: 249–259.

Ruano, M., El-Attrache, J. and Villegas, P. 2000. Rapid-plate hemagglutination assay for the detection of infectious bronchitis virus. Avian Dis. 44: 99-104.

Salminen, M.O., Carr, J.K., Burke, D.S. and McCutchan, F.E. 1995. Identification of breakpoints in intergenotypic recombinants of HIV type 1 by bootscanning. AIDS Res. Hum. Retroviruses. 11: 1423–1425.

Schultze, B., Cavanagh, D. and Herrler, G. 1992. Neuraminidase treatment of avian infectious bronchitis coronavirus reveals a haemaaglutinating activity that is dependent on sialic acid-containing receptor on erythrocytes. Virology. 189: 792-794.

Tamura, K., Dudley, J., Nei, M. and Kumar, S. 2007. MEGA4: molecular evolutionary genetics analysis (MEGA) software version 4.0. Mol. Biol.Evol. 24:1596-1599.

Terregino, C., Toffan, A., Beato, M.S., Nardi, R.D., Vascellari, M., Meini, A., Ortali, G., Mancin, M. and Capua I. 2008. Pathogenicity of a QX strain of infectious bronchitis virus in specific pathogen free and commercial broiler chickens, and evaluation of protection induced by vaccination programme based on the Ma5 and 4/91 serotypes. Avian Pathol. 37(5): 487-493.

Thompson, G., Mohammed, H., Buaman, B. and Naqi, S. 1997. Systemic and local antibody responses to infectious bronchitis virus in chickens inoculated with infectious bursal diseases virus and control chickens. Avian Dis. 41: 519-527.

Tolskaya, E.A., Romanova, L.A., Blinow, V.M., Virtorova, E.G., Sinyakov, A.N., Kolesnikova, M.S. and Agol, V.I. 1987. Studies on the recombination between RNA genomes of poliovirus: the primary structure and nonrandom distribution of crossover regions in the genomes of intertypic poliovirus recombinants. Virology. 161:54-61.

Tsukamoto, Y., Matsumoto, T., Kotani, T., Taira, E, Takaha, N., Miki, N., Yamate, J., and Sakuma, S. 1997. The expression of gicerin, a cell adhesion molecule, in regenerating process of collecting ducts and ureters of the chickens kidney following infection with a nephropathogenic strain of infectious bronchitis virus. Avian Pathol. 26: 245-255.

Upatoom, N., Jirathanawat, V., Srihakim, S., Leesirikul, N., Chirawatanapong, W., Bunyahotra, R., Siriwan, P. and Likitdecharoj, B. 1983. Infectious bronchitis (nephritis-nephrosis) in broilers. Thai. J. Vet. Med. 13: 36-43.

Villarreal, L.Y.B., Brandao, P.E., Chacon, J.L., Saidenberg, A.B., Assayag, M., Jones, R.C. and Ferreira, A.J.P. 2007. Molecular characterization of infectious bronchitis virus strains isolated from the enteric contents of Brazilian laying hens and broilers. Avian Dis. 51: 974-978.

Wang, C.H. and Huang, Y.C. 2000. Relationship between serotypes and genotypes based on the hypervariable region of the S1 gene of infectious bronchitis virus. Arch. Virol. 145: 291-300.

Wang, C.H., Hsieh, M.C. and Chang, P.C. 1996. Isolation, pathogenicity, and H120 protection efficacy of infectious bronchitis virus isolated in Taiwan. Avian Dis. 40: 620-625.

Wang, L., Junker, D., Collisson, E.W. 1993. Evidence of natural recombination within the S1 gene of infectious bronchitis virus. Virology. 192:710-716.

Wang, X. and Khan, M. 2000. Molecular characterization of an infectious bronchitis virus strain isolated from an outbreak in vaccinated layers. Avian Dis. 44: 1000-1006.

Winter, C., Schwegmann-Webels, C., Cavanagh, D., Neumann, U. and Herrler, G. 2006. Sialic acid is a receptor determinant for infection of cells by avian infectious bronchitis virus. J. Gen. Virol. 87: 1209-1216.

Worthington, K.J., Currie, R.J.W. and Jones, C.R. 2008. A reverse transcriptase-polymerase chain reaction survey of infectious bronchitis virus genotypes in Western Europe from 2002 to 2006. Avian Pathol. 37: 247-257.

Yachida, S., Aoyama, S., Takahashi, N., Iritani, Y. and Katagiri, K. 1978. Plastic multiwell plates to assay avian infectious bronchitis virus in organ cultures of chicken embryo trachea. J. Clin. Microbiol. 8: 380-387.

Yagyu, K. and Ohta, S. 1990. Detection of infectious bronchitis virus antigen from experimentally infected chickens by indirect immunofluorescent assay with monoclonal antibody. Avian Dis. 34: 246-252.

Yu, L., Jiang, Y., Low, S., Wang, Z., Nam, S.J., Liu, W. and Kwang, J. 2001. Characterization of three infectious bronchitis virus isolates from China associated with proventriculitis in vaccinated chickens. Avian Dis. 45: 416-424.

Ziegler, F.A., Ladman, S.B., Dunn, A.P., Schneider, A., Davison, S., Miller, G.P., Lu, H., Weinstock, D., Slem, M., Eckroade, J.R. and Gelb, J, Jr. 2002. Nephropathogenic infectious bronchitis in Pennsylvania chickens 1997-2000. Avian Dis. 46: 847-858.

BIOGRAPHY

Mr. Tawatchai Pohuang was born on January, 28, 1976 at Mahasarakham province, Thailand. He graduated Doctor of Veterinary Medicine from Faculty of Veterinary Science, Chulalongkorn University in 2001. After graduated, he was appointed as an instructor in Faculty of Veterinary Medicine, Chiangmai University during 2001-2002. At present, he has been appointed as an instructor in Department of Veterinary Medicine, Faculty of Veterinary Medicine, Khon Kaen University. He got a scholarship for proceed his Master degree at Faculty of Veterinary Science, Chulalongkorn University during 2004-2006. After that, he received a scholarship for proceed his PhD program in Veterinary Medicine at Faculty of Veterinary Science, Chulalongkorn University in 2007.