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## Molecular characterization of infectious bronchitis virus (IBV) isolated from commercial chicken farms

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### ABSTRACT

Infectious bronchitis virus (IBV) primarily causes highly infectious bronchitis (IB) in commercial chickens. Virus isolation and identification are necessary for diagnosis of the disease. In this study, a total of 5 IBV isolates from commercial poultry farms were isolated by inoculation in 9 day-old embryonated specific pathogen free (SPF) eggs. After few passages, the embryos showed typical lesions of IB like stunted and curled cross with feather dystrophy. Then, the 5 IBV isolates were characterized by reverse transcription-polymerase chain reaction (RT-PCR) followed by DNA sequencing of partial S1 gene. The phylogenetic analysis showed that the 5 IBV isolates distinctly clustered into 3 groups. Group 1 consisting of IBV1/15, IBV2/15 and IBV3/15 shared more than 99% similarity with IBV genotype 793/B. Group 2 consisting of isolate IBV4/15 was closely related to genotype QX-like with the similarity of 98%. Interestingly, group 3 consisting of IBV5/15 had genetic distance ranged from 8% to 10% with other reported IBV genotypes such as 793/B, Massachusetts-type, Taiwan-type and QX-like viruses whilst IBV5/15 shared about 99% similarities with a nephropathogenic Malaysian IBV isolate.

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## 1 INTRODUCTION

Avian infectious bronchitis (IB) is a contagious, acute disease in chickens caused by Coronavirus. Chickens of all age are sensitive to IBV, and infected chickens showed clinical signs of tracheal rales, gasping, coughing, nasal discharge, sneezing and facial swelling (Cook, 2007). High mortality and poor growth rate have occurred in broiler flocks affected by nephropathogenic strains. In laying hen or breeder, the disease has seriously reduced egg production and quality. Infectious bronchitis virus is a single positive RNA strands virus and classified under genus gamma corona-

virus belonging to family Coronaviridae, order of Nidovirales (Cavanagh and Britton, 2008; Jackwood and Wit, 2013). The IBV genome expresses four structural proteins such as spike glycoprotein (S), small membrane envelope protein (E), membrane glycoprotein (M) and nucleoprotein (N). The spike glycoprotein of IBV undergoes post translational cleavage to form S1 and S2 subunits. Variation in S1 protein enables one strain of virus to avoid immune response induced by another strain of the same species (Cavanagh and Britton, 2008). In addition, S1 glycoprotein is the main protein that induces both serotype-specific antibody and neutralizing antibody that are able to confer protec-

tion against IB (Jackwood, 2013). The first isolation of IBV was detected in Massachusetts in the 1930s, and until now Mass serotype has appeared in almost countries in the world. Serological and molecular analysis of IBV strains from Italy isolated in 1997 showed the co-existence of four virus types (793/B, 624/I, B1648 and Mass), which were also identified in other European countries (Bochkov *et al.*, 2007). In China, QX serotype was first isolated in 1998 from chickens with proventriculitis (Yu *et al.*, 2001). Then, IB caused by QX strains rapidly spread to become the most widespread serotype of non-vaccine origin within a few years with various clinical manifestations. Currently, control of IB is problematic due to presence of multiple serotypes and variant strains of IBV circulating in the poultry farms. Virus isolation and reverse transcription-polymerase chain reaction (RT-PCR) identification are the most important factors contributing to the diagnosis of IB. In this study, virus isolation was performed by inoculating virus into specific pathogen free (SPF) embryonated eggs a few times for virus propagation and adaptation. RT-PCR followed by Sanger DNA sequencing was performed to detect and classify isolated IBVs.

## 2 MATERIALS AND METHODS

### 2.1 Tissue sampling from suspected chickens with IBV infection

Tissue samples including trachea, cecal tonsil and kidney from suspected chickens were collected in commercial chicken farms. The confirmation of positive IBV infection was performed by quantitative reverse transcription-polymerase chain reaction (qRT-PCR). Then, the positive samples were stored at -20°C until analysis.

### 2.2 Virus isolation

Virus isolation was carried out by inoculation of 9 day-old embryonated SPF eggs with 200µl of tissue homogenates. Then, the eggs were daily candled to check the status of embryos and mortality. Death embryos were discarded within 24 hours post inoculation. Allantoic fluid was harvested on day 3 of inoculation. The remaining eggs were further used for observing the typical embryonic lesions of IBV infection. The virus inoculation was performed in 4 passages for virus adaption and propagation.

### 2.3 RNA extraction and RT-PCR

Viral RNA was extracted from 400µl allantoic fluid from infected embryonated SPF eggs using Trizol<sup>®</sup> Reagent (Invitrogen, USA) as described by manufacturer's instructions. RT-PCR was carried

out to amplify partial S1 gene of IBV. The forward primer XCE1 (5'-CACTGGTAATTTTTCAGATGG-3') and reverse primer XCE2 (5'-CTCTATAAACACCCTTACA-3') for targeted on the partial of S1 gene were used (Adzhar *et al.*, 2007). First strand cDNA was synthesized using MMLV reverse transcriptase 1st-strand cDNA synthesis kit (Epicentre<sup>®</sup> USA). In brief, a 12.5 µl total reaction volume including 5.5 µl of RNase-free water, 5 µl of extracted RNA sample, 2 µl specific reverse primer was prepared in 1.5 ml Eppendorf tube and kept on ice. Then, the tube was incubated at 65°C for 2 minutes in thermocycler with heated lid. After incubation, the mixture was chilled on ice for 1 minute and added 2 µl of MMLV RT 10x reaction buffer, 2 µl of 100 mM DTT, 2 µl of dNTP Premix, 0.5 µl of RiboGuardRNase inhibitor and 1 µl of MMLV reverse transcriptase up to total volume of 20 µl. The reaction was incubated at 37°C for 60 minutes followed by heating at 85°C for 5 minutes and chilled on ice for at least 1 minute. The synthesized cDNA was immediately used for PCR amplification.

The cDNA was used to synthesize a double strand DNA using KAPA HiFiHotStartReadyMix kit (KAPA Biosystems, USA) according to the manufacturer's instructions. In brief, a 22 µl of master mix including 7.5 µl of nuclease free water, 12.5 µl of 2x KAPA HiFiHotStartReadyMix, 1 µl of 10 µM forward and reverse primer were combined with 3 µl of cDNA to reach a total of 25 µl reaction. Subsequently, PCRs were performed with oligonucleotide pairs using following protocol: 1 cycle of initial denaturation at 95°C for 3 minutes, a sequence of 35 cycles followed by denaturation at 98°C for 20 seconds, annealing at 59°C for 20 seconds, extension at 72°C for 1minute, and 1 cycle of final extension 72°C for 5 minutes. The PCR products were detected on 1.5 % (w/v) agarose gel electrophoresis, and the gel picture was captured by using a UV light trans-illuminator (Bio-Rad, USA).

### 2.4 Sequencing and phylogenetic analysis

PCR products were purified using Mega quick-spin<sup>™</sup> (Intron Biotechnology, Korea). The purified DNA was sequenced by using Sanger sequencing. The evolutionary relationship of partial S1 gene of the IBV isolates was compared with other IBV isolates of different genotypes/serotypes retrieved from GenBank NCBI. Sequencing of partial S1 genes were aligned with ClustalW multiple alignment method using BioEdit 7.2.0 software. Evolutionary and phylogenetic analyses were conducted using MEGA 6 software. The phylogenetic trees were constructed using the Maximum Likelihood

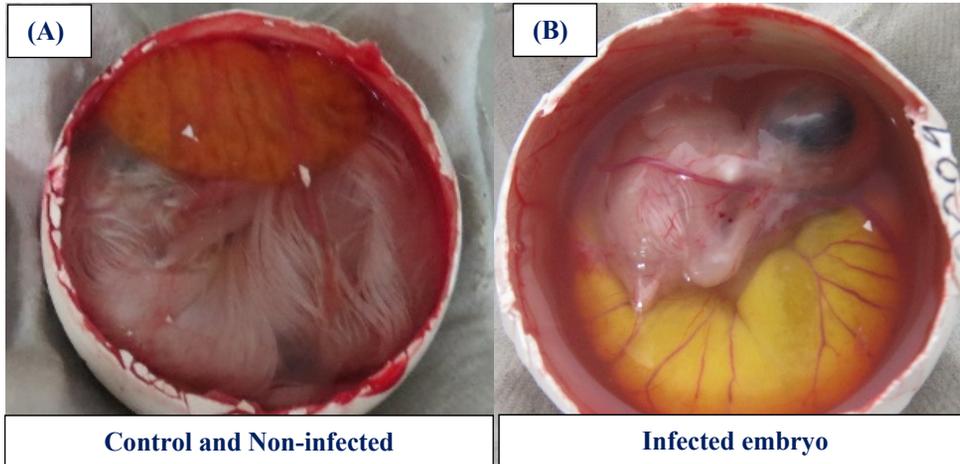
method based on the Tamura 3-parameter model with 1000 bootstrap replicates.

**3 RESULTS AND DISCUSSION**

**3.1 Virus isolation**

A total of 5 isolates isolated from commercial

chicken farms include IBV1/15, IBV2/15, IBV3/15, IBV4/15 and IBV5/15. The IBV infected embryos showed typical lesions like stunted and curled embryos with feather dystrophy from 4<sup>th</sup> passage to 6<sup>th</sup> passage (Figure 1).

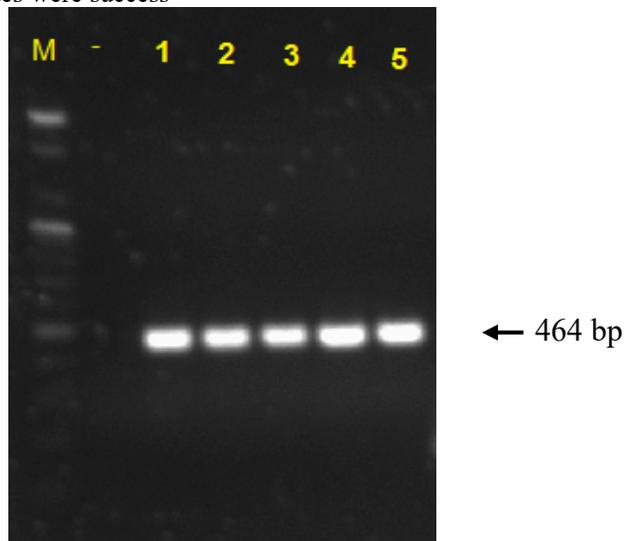


**Fig. 1: The 16-day-old embryo infected with IBV in the 5<sup>th</sup> passage: (A) The control and non-infected embryo was in normal condition. (B) The infected embryo showed signs of curling and stunting with feet deformed and compressed over the head with the thickened amnion and feather dystrophy**

**3.2 Conventional PCR results**

The partial S1 gene of 5 IBV isolates were success

fully amplified and the agarose gel picture showed the expected band of 464 bp (Figure 2).

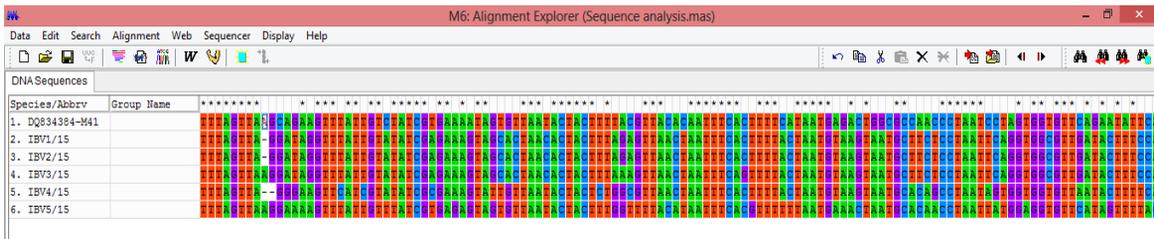


**Fig. 2: M: RT-PCR products of S1 gene of IBV isolates. M: DNA marker (100bp), 1: IBV1/15, 2: IBV2/15, 3: IBV3/15, 4: IBV4/15, 5: IBV5/15**

**3.3 Sequence analysis**

Figure 3 revealed that the deletion of 1 and 2 nucleotides detected at position 755 in IBV1 and

IBV2 isolates, and at position 755-756 in IBV4 isolate in S gene, respectively (reference M41, Massachusetts serotype).

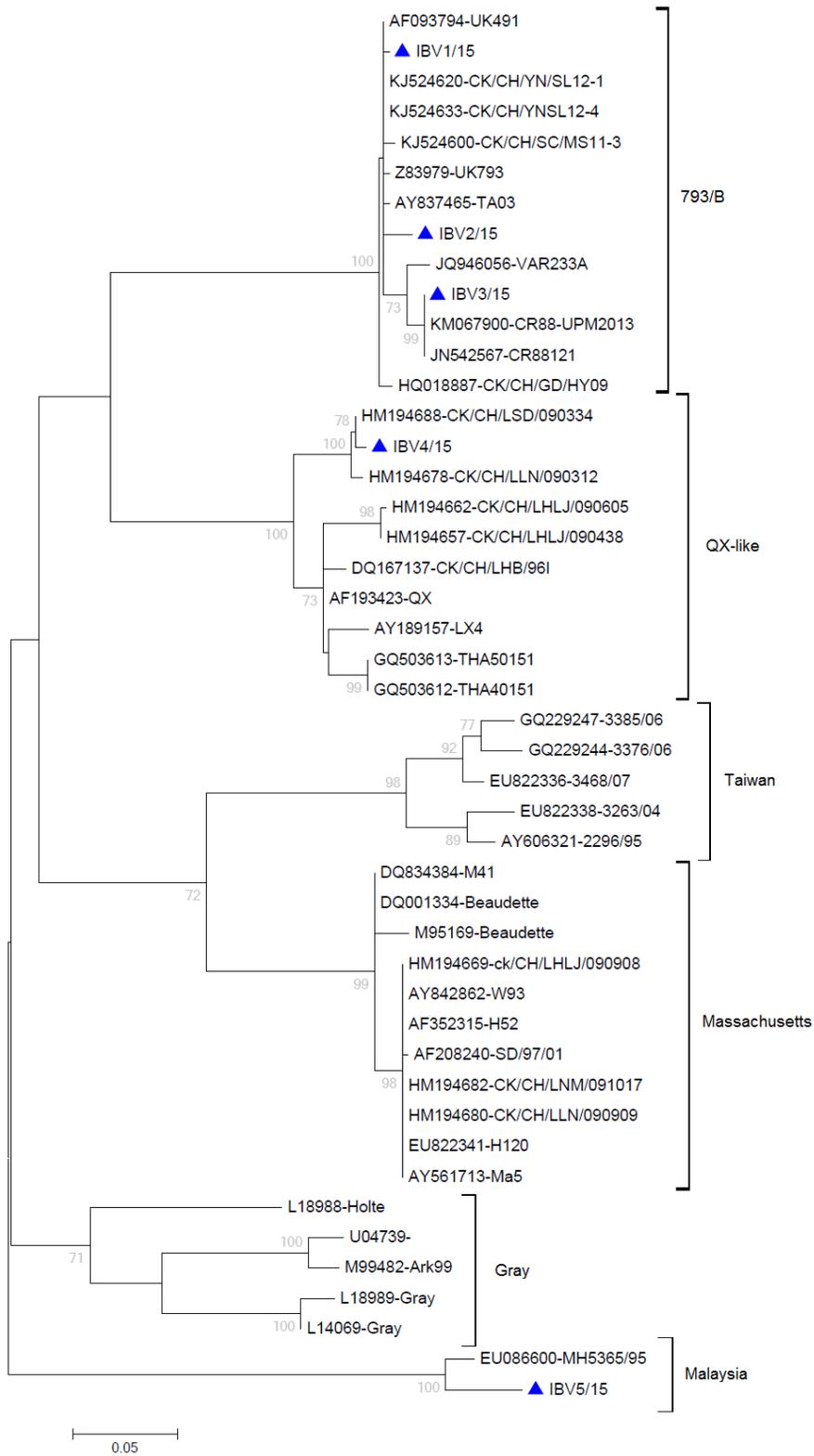


**Fig. 3: Comparison the nucleotide sequences between IBV isolates and M41 (Massachusetts serotype)**

**3.4 Phylogenetic analysis and pairwise comparison**

Published IBV sequences of Massachusetts genotype [M41 (DQ834384), Beaudette (DQ001334), Beaudette (M95169), CK/CH/LHLJ/090908 (HM194669), W93 (AY842862), H52 (AF352315), SD/97/01 (AF208240), CK/CH/LNM/091017 (HM194682), CK/CH/LLN090909 (HM194680), H120 (EU822341), Ma5 (AY561713)], QX-like [CK/CH/LSD/090334 (HM194688), CK/CH/LLN/090312 (HM194678), CK/CH/LHLJ/090605 (HM194662), CK/CH/LHLJ/090438 (HM194657), CK/CH/LHB/96I (DQ167137), QX (AF193423), LX4 (AY189157), THA50151 (GQ503613), THA40151 (GQ503612)], 793/B [UK491(AF093794), CK/CH/YN/SL12-1 (KJ524620), CK/CH/YN/SL12-4 (KJ524633), CK/CH/SC/MS11-3 (KJ524600), UK793 (Z83979), TA03 (AY837465), VAR233A (JQ946056), CR88-UPM2013 (KM067900), CR88121 (JN542567), CK/CH/GD/HY09 (HQ018887)], Taiwan [3385/06 (GQ229247), 3376/06 (GQ229244), 3468/07 (EU822336), 3263/04 (EU822338), 2296/95 (AY606321)], Gray

[Holte (L18988), Gray (U04739), Ark99 (M99482), Gray (L18989), Gray (L14069)] and Malaysia [MH5365/95 (EU086600)] available in the GenBank were used to determine genetic characteristics and molecular epidemiology of the IBV isolates in commercial chicken farms. The phylogenetic analysis based on the partial S1 gene showed the sequences distinguished into 3 groups (Figure 4). Group 1 with IBV1/15, IBV2/15 and IBV3/15 isolates clustered to 793/B genotype. The similarity of the IBV1/15, IBV2/15 and IBV3/15 isolates with selected IBV strain of 793/B genotype (UK793 (Z83979)) respectively was 99.32%, 99.62% and 99.21% (Table 1). In addition, group 2 with IBV4/15 isolate was genetically closed to QX-like IBV isolates with similarity of 98.26%. Interestingly, group 3 consisting of IBV5/15 isolate formed a distinct branch of the phylogenetic tree (Figure 4) and showed genetic distance ranged from 8% to 10% with other reported IBV genotypes/serotypes such as 793/B, Massachusetts-type, Taiwan-type and QX-like viruses whilst group 3 IBV isolate shared about 99% similarities with a nephropathogenic Malaysian IBV isolate MH5365/95 (EU086600) which was isolated in 1995.



**Fig. 4: Phylogenetic relationships of IBV isolates and selected reference strains based on partial S1 nucleotide sequences illustrated by maximum likelihood method based on the Tamura 3-parameter model with 1000 bootstrap replicates. The IBV isolates marked as filled triangles**

**Table 1: Comparison of nucleotide sequences of the partial S1 gene of 5 IBV isolates and selected IBV strains from different genotypes**

Strain	1	2	3	4	5	6	7	8	9	10	11
1 IBV5/15											
2 IBV4/15	91.37										
3 IBV2/15	90.04	90.04									
4 IBV1/15	89.09	94.04	99.33								
5 IBV3/15	90.11	93.96	99.19	98.95							
6 AY189157-LX4	92.13	98.26	94.65	94.13	93.84						
7 DQ834384-M41	92.04	92.87	93.34	92.67	92.86	93.30					
8 EU086600-MH5365/95	98.62	91.68	90.97	90.10	90.18	92.55	92.92				
9 AY606321-2295/95	92.07	93.12	93.50	92.85	93.20	92.76	95.79	92.34			
10 Z83979-UK793	90.07	94.61	99.62	99.32	99.21	94.67	93.36	90.99	93.74		
11 L14069-GRAY	92.47	93.75	94.16	93.99	94.08	94.28	95.38	93.12	92.72	94.17	

**4 DISCUSSIONS**

Infectious bronchitis is a serious and popular disease circulating in most of the places around the world. High mortality and poor growth rate occurred in broiler flocks were affected by nephropathogenic strains. In addition, in laying hen or breeder, the disease seriously caused reduction of egg production and quality. These issues elucidated the severe economic effects from infectious bronchitis (IB) in chicken farms around the world. Detection of IB and understanding characterization of IBV play an important role to control the disease and to limit the effects caused the IB. In this study, after screening using qRT-PCR, positive samples for virus isolation were inoculated into embryonated specific pathogen free (SPF) eggs. There were 5 cases successfully isolated in SPF eggs. All isolates adapted to embryonated SPF eggs in passage 4 to 6 when most of infected embryos showed mortality of embryos and signs of stunting and curling with feather dystrophy (Figure 1). The result of virus isolation is consistent with published data in OIE (2013) and many published reports (Yu *et al.*, 2001; Zhou *et al.*, 2004; Jackwood and Wit, 2013;). These 5 IBV isolates were characterized by RT-PCR and then by sequencing of the partial S1 gene. According to the results, circulating IBV strains in commercial chicken farms (not shown) was classified into 3 genotypes such as 793/B, QX-like and Malaysian variant genotype. Isolated IBVs in group 1 was closely related to genotype 793/B with identity of over 99%. In addition, IBV4/15 (group 2) had very high similarity to QX-like genotype (98% identity) while isolate IBV5/15 (group 3) distinctly separated from other groups and reference genotypes excluding Malaysian IBV isolate (MH5365/95). These results illustrated that the vaccination in these chicken farms using Massachusetts-type live attenuated vaccines (source from commercial chicken farms) was not effective because the IB outbreak has continued to be a prob-

lem and the emergence of variant strains of 793/B and QX-like genotype that have occurred in these farms. In fact, in the present study, the low relationship between isolated IBVs and Massachusetts genotype (92% similarity) revealed the failure of vaccination programs to control IBV. These results are in agreement with the concept that the emergent strains can rapidly spread around the world and become established, while other unique IBV variants may continue to circulate among poultry in geographically isolated areas (Cavanagh *et al.*, 1992; Moore *et al.*, 1998). The evolution of IBV happens to be influenced by many factors including the use of diverse strains of live attenuated vaccines, the population density and the host immune status (Domingo *et al.*, 1985; Yan *et al.*, 2011). In addition, rapid replication, a high mutation rate, and genome recombination result in extensive genetic diversity and translate into many different types of the virus (Jenkins *et al.*, 2002; Duffy *et al.*, 2008). Moreover, spread of viruses could be due to the illegal import of chickens and wild birds (Liu *et al.*, 2005; Sun *et al.*, 2007; Pohjola *et al.*, 2014). Neighbour countries are also a risk factor to spread the IBVs. Further analyses such as pathogenicity and serotyping of these IBV isolates should be performed to give better understanding in characteristic of IBV isolates circulating in commercial chicken farms.

**5 CONCLUSIONS**

Five isolated IBVs circulating in commercial chicken farms were classified as QX-Like genotype, 793/B genotype and variant strain based on analysis of the sequences of partial S1 gene. The results have contributed to further studies of complete genome sequencing and vaccine development to control the IB.

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