Identification and Characterization of Infectious Bronchitis Virus (IBV) in Indonesia (Identifikasi dan Karakterisasi Virus Infectious Bronchitis (IBV) di Indonesia)

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ABSTRACT

Infectious Bronchitis Virus (IBV) is an acute viral and highly infectious disease which attacks the respiratory system of chicken. The impact of economic losses is very large since it decreases the egg production and it causes the abnormal of egg shapes, decreased of body weight and death. In Indonesia, the IBV disease is still a serious problem in poultry regarding to many variants which arise from mutations of the IB virus. The decreasing possibility of the vaccination effectiveness due to incompatibility of strains used towards the virus circulated in the field. The variants of IB virus have not been well-documented in Indonesia since the lack of characterization of this virus. In this study, we characterize the IB virus isolates obtained from our local outbreaks in the field in order to determine the variant of IB virus in Indonesia and provide recommendations for the improvements of IB vaccine seed viruses which is adapted to IB virus circulating in the field. The IB virus characterized in this study shows close genetic relationship with the IB virus from Taiwan and China. However, it did not correlate with the local IB virus that has been found previously.

Keywords: Identification, Character, IBV, Indonesia

ABSTRAK

Infectious Bronchitis Virus (IBV) adalah penyakit virus sangat menular akut yang menyerang sistem pernapasan ayam. Dampak kerugian ekonomi yang sangat besar karena menurunkan produksi telur dan menyebabkan bentuk telur abnornal, penurunan berat badan dan kematian. Di Indonesia, penyakit IBV masih merupakan masalah serius pada ayam sehubungan dengan banyak varian yang timbul akibat mutasi dari virus IB. Ketidakcocokan antara strain dan vaksin digunakan terhadap virus yang beredar di lapangan megakibatkan penurunan efektivitas vaksinasi. Varian virus IB belum terdokumentasi dengan baik di Indonesia karena kurangnya penelitian yang mengkarakterisasi virus ini. Dalam studi ini, kami mengkarakterisasi virus IB yang diperoleh dari wabah di lapangan untuk menentukan varian dari virus IB di Indonesia dan memberikan rekomendasi untuk perbaikan bibit vaksin yang disesuaikan dengan virus IB yang beredar di lapangan. Hasil penelitian menunjukkan bahwa IB virus yang dikarakterisasi pada penelitian ini mempunyai hubungan genetik yang erat dengan virus IB dari Taiwan dan China. Namun, tidak berkorelasi dengan virus IB lokal yang telah ditemukan sebelumnya.

Kata Kunci : Identifikasi, Karakter, IBV, Indonesia

INTRODUCTION

Infectious Bronchitis (IB) is a contagious and acute respiratory disease in chickens caused by Infectious Bronchitis Virus (IBV) (Cavanagh 2007; Cook *et al.* 2012). The disease attacks the upper respiratory tract and uro genital tract in chickens. The disease is characterized by several respiratory symptoms such as gasping, coughing, sneezing, tracheal rales and nasal discharge. The impact of economic losses caused by this disease is quite significant since in some cases, respiratory stress can occur in young chickens while in laying hens the respiratory stress decreased egg production and the quality of the egg shell. In some virus strains, it causes damage for kidney and resulting in high mortality (Butcher *et al*. 1998; Cavanagh & Naqi 2003).

Infectious bronchitis virus belongs to Coronavirus family, positive single stranded DNA, the genome length approximately 27.6 Kb (Boursnell *et al.* 1987). The viral genome contains information for four structural proteins, namely spike glycoprotein (S), membrane glycoprotein (M), small membrane or envelope glycoprotein (BC tau E) and nucleocapsid (N). Spike glycoprotein is associated with the neutralization virus, serotype specificity and cell attachment and it is separated in post translational which become subunits N terminal S-1 and C terminals S-2 (Koch *et al* 1990; Niesters *et al.* 1989; Cavanagh & Naqi 2003). The antigenic differences among the IBV serotypes are associated with

structural variations of spike glycoprotein (S), a *peplomeric structure* on the surface of the virus. Subunit S-1 shows higher sequence of variability than the subunit S-2 (Binns *et al.* 1986) and the sequence of the S-1 is known as hyper variable region (HVR) -1 and HVR-2.

In Indonesia, IBV have spreaded in Java with a prevalence rate about 40-60% (Darminto 1995). IB vaccines are widely used in breeding farm, laying and broiler which generally contain IB virus serotype Masschusetts (Mass) and some of them contain serotype Connecticut (Conn). The data indicates that the current vaccine is not effective against IB attack in the field. The ineffectiveness of existing IB vaccines is mainly caused by the differences between virus vaccine serotypes and IB virus which causes outbreaks in the field (Darminto 1992). Mutations in the genome will produce a new variant IB virus (Moore *et al.* 1998). Isolation and identification of IBV isolates are important to do since the vaccine serotype isolates were selected based on the location where the vaccine was given in order to provide better immunity. Besides, the identification of new IBV serotype strains or IB variants based on geography aspect should be made in order to support the latest vaccine development program, including in Indonesia. Identification of IB virus variants in Indonesia has not been done, so this study will be conducted for identification and characterization of AI virus circulating in poultry in Indonesia.

MATERIALS AND METHODS

In this study, we conducted the molecular identification of IB virus which derived from previous study namely IB / WJ.2010 with (virus titer content (VCT) 6.44 EID_{50}), IB / L8.1996 (5.17 EID_{50}) and IB / JB.1990 (5.3 EID_{50}). The virus was grown in the allantoic fluid of egg chicken embryos (10 days old) of Specific Pathogen Free (SPF) and was incubated for 72 hours at 37 ° C. The allantoic fluid containing the IB virus was extracted using QIAmp RNA Mini Kit (Qiagen) with according to the manual instructions. The extracted of RNA is stored at minus -70° C until used. The RNA viral is used as a template for testing IB virus by RT-PCR. The primers used to amplify the gene S1 is SX1 (CACCTAGAGGTTTGT / CTA / TGCAT) and SX2 (TCCACCTCTATAAACACCC/TTT) according to Jones *et al.* (2005). It is also used primer F-XCE1 + (CACTGGTAATTTTTCAGATGG), r-XCE2-(CTC TATAAACACCCTTACA) according to Adzhar *et al.* (1997).

The RT-PCR result in amplicons are cut exactly on the band size and purified by using QIAquick Gel Extraction Kit (Qiagen) and followed by DNA sequencing. Sequencing is conducted by a kit BidDye Terminator V3.1. Cycle seq kit (Applied Biosystems) on Genetyc Analyzer 3130 (Applied Biosystems) machine. The DNA Electropherogram sequencing results are verified and edited by using Bioedit software Version 7 (http://www.mbio.ncsu.edu/ BioEdit). The nucleotide sequences results are compiled, aligned, compared and analyzed by gene sequence data S1 of IB virus which are stored in GenBank (NCBI). The phylogenetic construction trees are conducted by using MEGA 5.2 software. (http://www.megasoftware.net).

Fifty chickens specific pathogen free (SPF) aged 28 days were divided into 5 groups which were infected by local virus isolates IB; that is group one with the virus IB IB / WJ.2010, group 2 with IB virus / L8.1996, group 3 with IB virus /JB.1990, group 4 with virus I.37 and group 5 with PTS-3 virus. The I.37 and PTS-3 were local isolates. The I.37 virus based on previous study is similar with IBV Connecticut (Darminto 1992; Dharmayanti *et al.* 2003) and PTS-3 virus similar to N2 / 62 (Darminto 1992). Serum were taken from each group that were collected after 3 weeks of post-infection.

The IB virus / WJ.2010, IB / L8.1996 and IB / JB.1990 were cross-reacted I.37 and PTS-3. Each serum is tested against each virus by serum neutralization test on SPF chicken eggs aged of 9-11 days. SN test with β-procedure (virus serum variables constant) is described by Marius *et al.* (1982) and Picault *et al.* (1986). The serum was diluted by twofold serial in PBS pH 7.2 containing antibiotics 200 IU penicillin and 200 ug streptomycin per mL. Each virus with concentration 100 EID_{50} per 0.05 ml was diluted in PBS containing antibiotics. Serum and virus were mixed and allowed to stand for 30 minutes at room temperature. Aliquots of 100 µl from each mixture were then inoculated into a set of (5 eggs) SPF chicken eggs aged of 9-11 days per diluted serum and 5 uninfected eggs as control, then incubated at 37° C for nine days with at candling every day. Neutralization is shown by the highest dilution serum which still provides protection to the embryo of lesions and deaths due to IB virus. SN titer

was calculated by the Reed & Muench Method (1938) and expressed in log2.

The analysis of serotype variation of local isolate virus IB was done by using the formula r values based on serum titer SN from pair of IB virus and anti-serum IB of homologous and heterologous. The formula $R = 100 \sqrt{(r1 \times r2)}$, in this case r1 or r2 is heterologous serum titers which is divided by homologous serum titers (Archetti & Horsfall 1950), and then modified according to Lashgari & Newman (1983). IB virus is stated as two different serotypes if it has a value of $R<20$, otherwise it is the same serotypes if the value of $R \geq 50$ and grouped dubious if it has a value of R between 20-50.

RESULTS

RT-PCR and Phylogenetic

The results of IB virus S1 gene amplification with primers SX shows the amplicon about 400-500 basepairs (bp). From five isolates of IB virus used in this study, the reference virus of IB M1 and IB H120 can be amplified according to the target, which indicates that the primer can be used to amplify the IBV. The IB / WJ.2010, IB / L8.1996 and IB / JB.1990 shows the same results according to the IB reference virus whereas XCE primers shows the amplification results approximately 400-500 base pairs (bp).

Phylogenetic analysis showed that IB / WJ.2010 and IB / JB.1990 genetically adjacent to the gene level of S1 gene. Both of these isolates one group with IB virus CK / CH / LSD / 051 (NCBI data) originating from China, while the IB L8.1996 isolates showed genetic closeness to the virus strain S1 glycoprotein of Taiwan (Figure 1). Results of this analysis showed that all three IB isolates analyzed in this study did not show the proximity to isolate M41 or H120 is widely used as a vaccine seed IB circulating in Indonesia. The study also shows that there is some genetic variation IB virus in Indonesia in line Dharmayanti *et al.* (2003) that also showed finding genetic variation of IB virus circulating in Indonesia.

Cross reactions test of local isolates IB virus

Serum neutralization titers of each serum combination, homologous and heterologous viruses are presented in Table 1. In the homologous serum condition showed the high titer (3.7 to 6.6 log 2). The third isolates (WJ / 2010, L8 / 1998, JB / 1990) showed low reaction against the previous IB isolates (I.37 and PTS-3) with SN titer <2 log 2. The IBV WJ / 2010 reacted with IB isolate JB / 1990 with SN titer 2,5log2, whereas the IB isolates L8 / 1996 shows a low reaction (SN titer <2log2). The SN test results showed isolates WJ IB / 2010, L8 / 1998, JB / 1990 are not neutralized by serum I.37 and PTS-3, which isolates I.37 based on previous research is similar to Conn IB virus (Darminto 1992; Dharmayanti *et al*. 2003), while isolates PTS-3 is similar to virus IB N2 / 62 (Darminto 1992), so virus in this study (WJ / 2010, L8 / 1998, JB / 1990) it can be stated as variant of IBV.

The analysis of three serotypes of isolates viruses were tested by cross neutralization in SPF embryonated chicken eggs and stated as *r value* shown in Table 2. The pair of homologous virus has the value *r 61*, whereas the heterologous virus varies between 10-18. Isolates WJ / 2010 and JB / 1990 has a close relationship with serotype *r value 61*, while isolates L8 / 1996, I.37 and PTS-3 does not have the same relationship. In previous study (Wadey & Faragher 1981) it is stated that the pair of homologous virus has varied *value* $r \geq 50$, whereas the heterologous virus <20.

DISCUSSION

In Indonesia, the IB virus was first isolated by Ronoharjo (1977), and a few years later by Darminto *et al*. (1985). Those isolates are known belongs to four groups of serotypes, where a serotypes group which closes to the Mass, a serotype group which closes to Connecticut (Conn) and two groups of serotypes that have a close relationship with IB virus strains from Australia (Darminto 1992). Until now IB disease cases on commercial chicken farms are still common occured in vaccinated IB chicken flocks. According to Darminto (1995) this is due to insufficient IB vaccine protection used for heterologous IB virus serotypes. Box *et al.* (1988) stated that IB vaccination can cause immune response to IB virus attacks. Infected chickens will naturally get immune to the IB virus attacks which are homologous, but it is widely varied for the resistance against IB virus strains which are heterologous. According to Cavanagh & Naqi (1997) Circulation of IB virus in the field showed various serotypes of IB virus which attempt to get polyvalent poultry vaccination (containing more than one serotype or

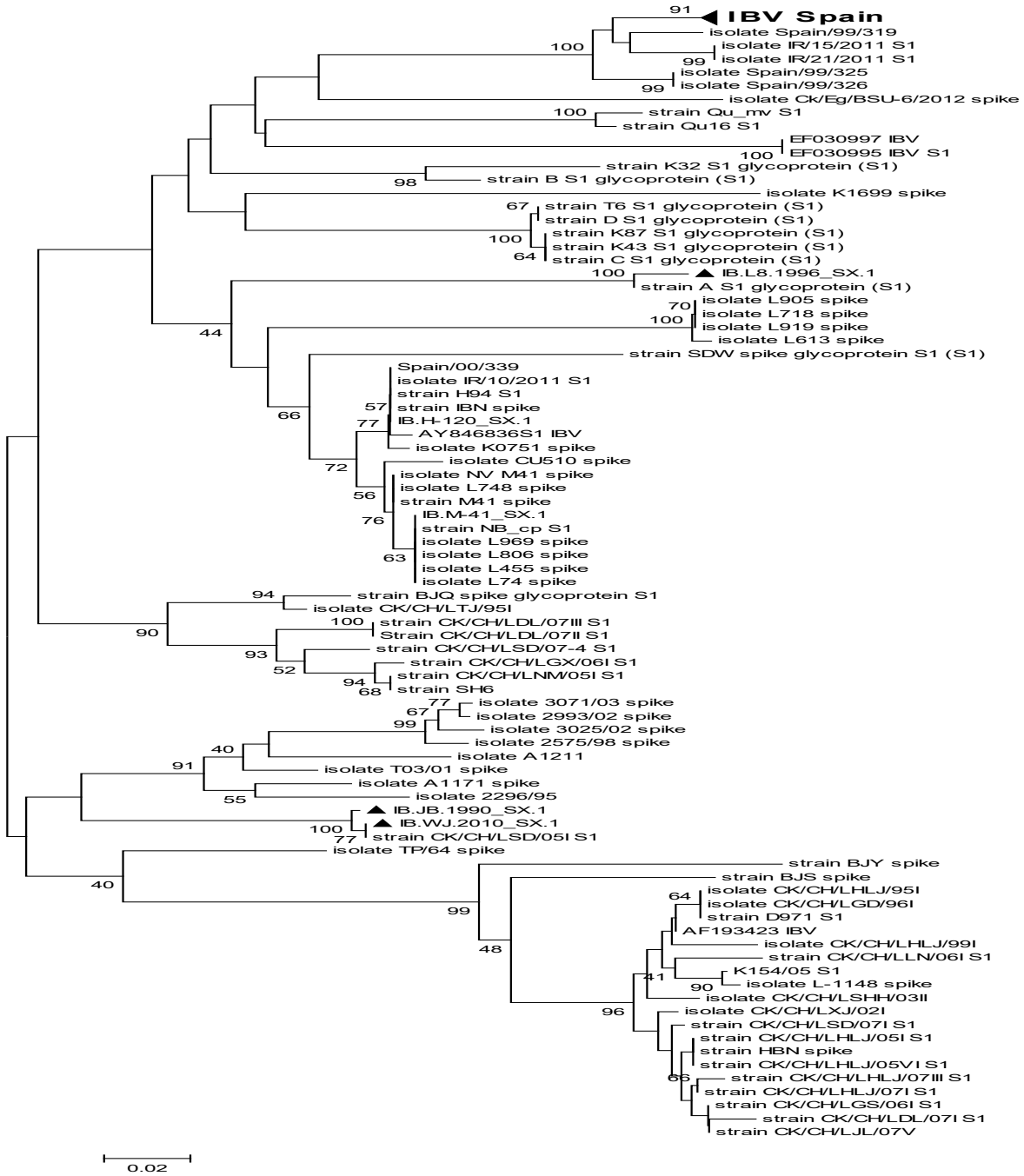

Figure 1. The phylogenetic tree of Si gene of IBV was generated in MEGA version 5.2 (www.megasoftware.net), using neighbor-joining analysis with 1,000 bootstrap replicates and the Kimura 2-parameter model.Viruses characterized in this study are indicated with a triangle mark.

subtype).

All IB virus genome has been cloned and sequenced (Boursnell *et al.* 1987). DNA sequencing and genetic analysis is a fast and accurate method in determining, classifying and predicting serotypes IB virus including analysis of phylogenetic to know the evolution of the epidemiology of IB virus (Adzhar *et al.* 1997; Moore *et al.* 1998; Mase *et al.* 2004). In Indonesia, the IB virus genetic data is limited; the data has been published by Dharmayanti *et al.* (2003) about the discovery of several virus variants IB in Indonesia based on DNA sequencing. In this study we proved that we have already discovered variant IB virus in Indonesia by using DNA sequencing, namely IB virus / WJ.2010 and IB / JB.1990 is similar to IB virus China (CK / CH / LSD / 051) and

IB virus/L8 in 1996 which is similar to the Taiwanese IB virus. In neutralization serum test, it also shows that the three IB virus investigated in this study may relate to serotypes. It doesn't show cross local IB virus protection with Indonesia, I.37 and PTS-3. IB I.37 is an Indonesian local virus which is a variant of Connecticut while PTS-3 is a variant of the N2 / 62. IB vaccination generally used in Indonesia is serotype M41, H120, Conn vaccine or some local companies use local IB virus.

All three IB virus that we used in this study isolated from the poultry farm with egg production problem, any breathing disorder, abnormal egg shape and any damage symptoms to the kidneys. According to our study, there was no similarities of IB virus with QX strain. QX IB virus strain was first isolated in China in 1998 with clinical symptoms of diarrhea, proventriculitis, and loss of body weight in chickens aged 25-70 days (Wang *et al*. 1998). However, QX IB virus strains causes more nephritis rather than proventriculitis (Zhou *et al*. 2004; Beato *et al.* 2005; Bochkov *et al.* 2006; Park *et al.* 2005). There has never been official reports about the IB cases with symptoms of nephritis or isolation of virus strains IB QX in Indonesia, though some breeders informal reports have claimed the IB cases with nephritis symptoms are likely caused by a virus strain IB QX.

Until now, more than 20 serotypes virus have been widely known, with additional variants of serotypes virus which continue to arise and cause disease. IB disease situation in Indonesia also shows greater number of variants, consequently deciding serotype quickly and accurately is an important factor in controlling infection IB. The multiple serotypes usage are recognized as an effective vaccination program. Moreover, it is important to identify serotypes prevalent in order to determine potential *cross-protective* towards the available vaccines.

Table 1. Antibody titers serum of homologous and heterologous virus isolates for Infectious Bronchitis WJ/2010, L8/1996, JB/1990 with cross neutralization test in SPF chicken embryo

Serum	Local Isolate IB viruses				
	WJ/2010	**L8/1996**	**I.37**	**PTS-3**	**JB/1990**
WJ/2010	**4,9**	<2	<2	<2	2,5
L8/1996	<2	**3,7**	<2	<2	<2
I.37	<2	<2	**6,6**	<2	<2
PTS-3	<2	<2	<2	**5,8**	<2
JB/1990	3,31	<2	<2	<2	**4,5**

Remarks: neutralization titers (the highest serum dilution showing 50% protection for the embryo) is calculated by the Reed and Muench (1938) and stated as log2. Figures in bold indicate homologous serum titer

Table 2. The serological reaction relationship between isolates IB virus WJ/2010, L8/1996, JB /1990 and previous isolates I.37 and PTS-3 are stated as the value of r *(r values)*

Serum	Local Isolat IB Vi				
	WJ/2010	**L8/1996**	**I.37**	**PTS-3**	**JB/1990**
WJ/2010	**100**	16	13	11	**61**
L8/1996		**100**	14	12	18
I.37			**100**	12	10
PTS-3				**100**	13
JB/1990					**100**

Remarks: The value of r is calculation results of neutralization titer in Table 5 by Archetti & Horsfall (1950). R values in bold indicate IB viruses which have antigenic relationship

CONCLUSION

Infectious Bronchitis viruses which were characterized in this study shows close relationship with IB virus from Taiwan and China. However, it did not correlate with the local IB virus that has been found previously. The finding indicated that there many IBV variant in Indonesia and should be the vaccine will be update based on the virus circulation in the field.

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