

Molecular and pathogenicity of infectious bronchitis virus (Gammacoronavirus) in Japanese quail (*Coturnix japonica*)

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ABSTRACT Infectious bronchitis virus (IBV) infection is highly infectious respiratory disease in poultry industry with significant economic importance. The prevalence of IBV in quail industry in Malaysia was not well documented; therefore, its actual role in the epidemiology of the disease is relatively unknown. This study was to determine the susceptibility of Japanese quail, as one of the species in commercial poultry industry, toward IBV. In addition, it will also give a potential impact on the overall health management in the quail industry even though it had been established that quail are resistant to diseases affecting poultry. Moreover, to the best of our knowledge, it is the first experimental study on IBV inoculation in quail. In this experimental study, 20 quails were divided into 4 groups (n = 5 for group A, B, and C,

n = 5 for control group). The quails in group A, B, and C were infected via intraocular and intranasal routes with 0.2 mL of 10×5 EID₅₀ of the virus. Clinical signs, gross lesions, positive detection of virus, and trachea histopathological scoring were used to assess the susceptibility of these Japanese quails. The results have indicated mild ruffled feathers and watery feces in these inoculated birds. Trachea, lung, and kidney were subjected to one-step reverse transcription polymerase chain reaction for virus detection. The virus was found from trachea and lung samples, whereas it was absent from all kidney samples. Only 3 quails were found with gross lesions. There was a significant difference of tracheal lesion by 0.009 ± 0.845 ($P < 0.05$) within the treatment groups. In summary, Japanese quails might be susceptible to IBV.

Key words: Japanese quail, infectious bronchitis virus, RT-PCR, susceptibility, pathogenicity

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INTRODUCTION

Infectious bronchitis virus (IBV) case was first reported in North Dakota, USA, in 1931 as an acute, highly infectious respiratory disease of chicken (Sjaak de Wit et al., 2011). The distribution is currently worldwide. The family of Coronaviridae consists of 2 genera, coronavirus and torovirus. These viruses are linear, single stranded RNA viruses. However, only viruses of the Coronavirus genus have been reported to infect poultry.

Coronaviruses are enveloped, pleomorphic but usually spherical virus particles of 120 to 140 nm in diameter.

Avian IBV was the first coronavirus to be discovered under Gammacoronavirus, subfamily *coronaviridae* and order of *Nidovirale*. Infectious bronchitis virus synthesized 5 different sg mRNA and encoded for 4 structural proteins, S, E, M, and N and numbers of nonstructural proteins (nsp) that intersperse between other structural proteins (Lai and Cavanagh, 1997). The important antigenic and functional proteins known as spikes (S-protein) are in distinct, club-shaped projection (Sjaak de Wit et al., 2011). Molecular study showed that the S gene of IBV is responsible in determining the serotype or genotype of an IB virus or variant. Only small number amino acids changes in the S1 part of the spike can result in new variant as it is defined by laboratory tests (Sjaak de Wit et al., 2011). Infectious bronchitis virus has the S-protein as 2 subunit, amino

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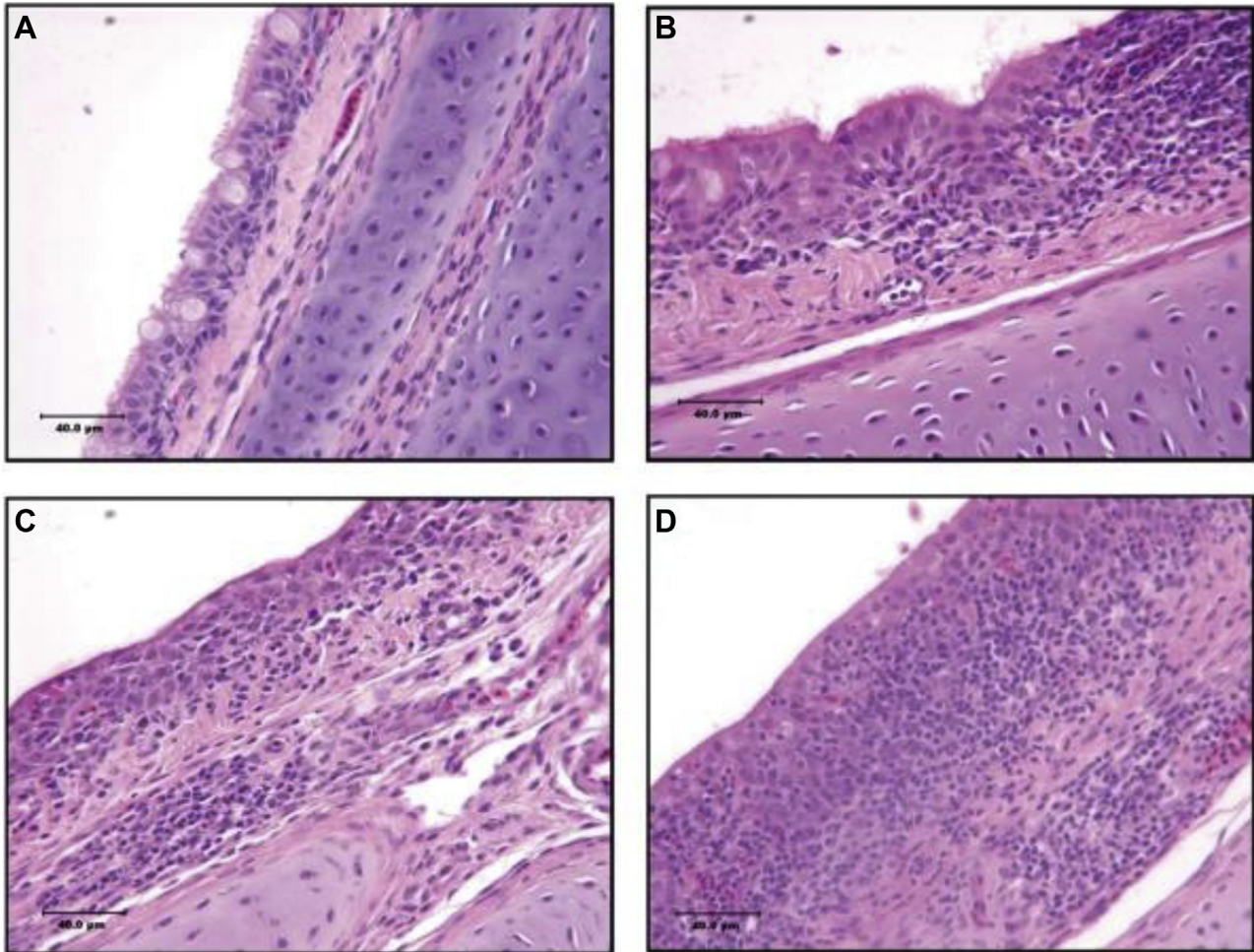


Figure 1. Tracheal histopathological scoring reference. A: No lesion, score 1. B: Mild epithelial hyperplasia and mild subepithelial lymphoid infiltration with occasional germinal centers, and mucous glands were distorted and elongated, score 2. C: Moderate epithelial hyperplasia with loss of cilia and moderate subepithelial lymphoid infiltration was seen, mucous glands were decreased in size, and there was edema of the lamina propria, score 3. D: Extensive epithelial hyperplasia and subepithelial lymphoid infiltration, the superficial epithelial layer was flattened and had a squamous appearance, mucous glands were not detectable, score 4 (Grgic et al., 2008).

terminal S1, and carboxyl terminal S2. S1 is responsible for attachment of virus to cells. S2 is responsible for membrane fusion (Cavanagh, 2007).

Infectious bronchitis virus infection is characterized by increased oculo-nasal secretion and excessive mucous in the trachea together with reduction of weight gain and feed efficiency (Grgic et al., 2008). Infectious bronchitis virus also disrupts kidney and reproductive tract, resulting in renal dysfunction and decreased egg production, respectively. Primarily, this disease occurred in young

chicken, but it is susceptible toward chickens of all age (Cavanagh, 2007). With the experimental inoculation of IBV in quail, the potential susceptibility of the local domesticated quails against IBV can be determined. This new finding will improve the perceptions toward quail disease management and control, particularly in the tropics.

MATERIAL AND METHODS

Animals

Twenty Japanese quails at 20 D old were bought from a closed system quail farm. Twenty-days-old quail were

Table 1. The cycling protocol used in RT-PCR assay during amplification of IBV.

Stage	Temperature and time	No. of cycle
Reverse transcription	48°C for 20 min	1 cycle
AMV RT inactivation and cDNA denaturation	95°C for 2 min	1 cycle
Denaturation	95°C for 20 s	40 cycles PCR amplification
Annealing	52.8°C for 40 s	
Extension	72°C for 15 s	
Final extension	72°C for 10 min	1 cycle
Incubate	12°C	Infinity

Abbreviation: IBV, infectious bronchitis virus.

Table 2. Primers sequence used in one-step reverse transcription PCR.

Primer	Primer sequence 5'-3'	Expected size of PCR product
Forward (N104 F)	CCT GAT GGT AAT TTC CGT TG	335 bp
Reverse (N101 R)	CTC ATT CAT CTT GTC ATC ACC	

Table 3. The result of IBV detection by RT-PCR from organ sample of 20 quails after virus inoculation.

Groups	No. of animals	Lungs	Trachea	Kidney
A 2 d.p.i	5	-	+	-
B 4 d.p.i	5	+	+	-
C 6 d.p.i	5	+	+	-
Control group	5	+	+	-

used according to a study by (Circella et al., 2007). Infectious bronchitis virus syndromes were first observed in 3-week-old quail bird that were still housed in cages. The quails were randomly divided into 4 groups, which were 3 experimental groups and 1 control group. The birds were kept in confined cages and were supplied with feed and water ad libitum.

The quails were kept in commercial battery cages with dimension of 1 m in length \times 0.5 m in width \times 0.5 m height. The cage floor was covered with fine wire mesh. The birds were kept in room with temperature maintained at 22°C to 25°C, which is considered ideal for quail. The relative humidity was maintained in between 50 and 70% by using ventilation fan. Lighting regimes varied for commercial producers but are typically designed with 16 h of light per day (Cheng et al., 2010). No enrichment was set up in the cage.

The experimental group and control group were separated in different rooms. Observation of the birds constantly started with the control group, using new protective clothing for each group to prevent cross-contamination. During the postmortem and tissue samplings, the same order was followed, and new sterile forceps and scissors were used for each group. During subsequent procedures, all precautions were taken to avoid contamination between samples.

Method of handling the quails was according to the laboratory animal guidelines. Furthermore, the study protocol was approved by the Institutional Animal Care and Use Committee at the Faculty of Veterinary Medicine, UPM (AUP-U046/2018).

Pathogenicity Study

The quails were randomly divided into 4 groups with 5 quails in each group. Considering this is the first experimental study of inoculating IBV toward quail, only 5 quails were used for each group to study the development of the infection. This was to avoid the use of inappropriate high number of birds for the experiment. The groups are

based on infection period: Group A, 2 d of postinfection (d.p.i); Group B, 4 d.p.i; Group C, 6 d.p.i; and 1 control group. Each quail was tagged with a group alphabet and number of quail in the group for identification.

Blood sample was taken from representative birds of each group via wing venipuncture for virus screening before virus inoculation.

At the age of 23 D, the quails were inoculated with 0.2 mL of 10^5 EID₅₀/mL IBS130/2015 QX-like IBV via intraocular and intranasal route. The amount of virus was divided equally between the routes.

Clinical signs were observed from the quails, which includes oculo-nasal discharge, coughing, sneezing, loss in appetite, abnormal resting posture, laborious breathing, and ruffled feathers. The clinical signs were scored twice daily according to the following formula: 0 = no signs; 1 = mild signs; and 2 = severe signs. Mild gasping, coughing, or depressions were considered mild signs. Severe gasping, coughing, or depression or both accompanied by ruffled feathers were determined as severe signs (Grgic et al., 2008)

The quails were sacrificed via cervical dislocation according to the infection group. Trachea were obtained from each quail for observation of the gross lesion. The gross lesion also scored according to the criteria of McMartin. Selected tissue such as lungs and kidneys were also obtained for observation. The organs were fixed in 10% buffered formalin for at least 48 h, then embedded in paraffin, sectioned, and stained with hematoxylin and eosin (Prophet et al., 1994)

The histological slides were evaluated in “blinded” fashion. Five examiners had absolutely no information on the infection grouping. The slides were examined using image analyser (Moticam Pro 285A, Motic, Europe) at 20 \times 10 magnification. The lesions were scored based on the modified protocol of Alvarado et al. (2003). Score of 1 indicates no lesion. Score of 2 indicates mild epithelial hyperplasia and mild subepithelial lymphoid infiltration with occasional germinal centers with distorted and elongated mucous glands. For a score of 3, moderate epithelial hyperplasia with loss of cilia and moderate subepithelial lymphoid infiltration was observed with a decrease in size of mucous glands and edema of the lamina propria. A score of 4 represents an extensive epithelial hyperplasia, subepithelial lymphoid infiltration with flattened superficial epithelial layer, and an a squamous appearance with absence of mucous glands. These microscopic changes of the trachea for the scoring system are presented in Figure 1.

Table 4. The histology score result of trachea sample done by 5 examiners according to Grgic et al., 2008.

Animals	Examiner 1				Examiner 2				Examiner 3				Examiner 4				Examiner 5			
	A	B	C	D	A	B	C	D	A	B	C	D	A	B	C	D	A	B	C	D
Quail 1	2.4	2.4	1	1	1	2	1.6	1	1.4	1.4	1.2	1	1.8	2	2	1	2	2	1	1
Quail 2	2	2.4	1.4	1	1	2.6	2.4	1	2.2	3	1	1	2	2	1.6	1	2.6	3.4	1	1
Quail 3	1	2.4	1	1	2	1.8	1.6	1	1.8	1.6	1.4	1	2.2	2	2	1	2.6	1.6	1.4	1
Quail 4	2	1.6	1.4	1	2.4	1.8	1.6	1	2	1.4	1.4	1	2	2	1.6	1	1.8	1.6	1.2	1
Quail 5	2	2	1.4	1	2	2	1.4	1	2	2	1.4	1	2	2	1.4	1	2	2	1.4	1

Group A: 2 d.p.i, group B: 4 d.p.i, group C: 6 d.p.i, group D: control group.

Table 5. The mean score of trachea histology examination of each quail from 4 examiners.

Animals	Group A 2 d.p.i	Group B 4 d.p.i	Group C 6 d.p.i	Group D, control
	Mean score			
Quail 1	1.72	1.96	1.36	1
Quail 2	1.96	2.68	1.48	1
Quail 3	1.92	1.88	1.48	1
Quail 4	2.04	1.68	1.4	1
Quail 5	2	2	1.4	1

Extraction of RNA From Serum Sample

Blood sample obtained was collected and centrifuged using Centrifuge 5415 D, Eppendorf, Germany at 2,000 rpm for 5 min. The upper aqueous layer was the serum transferred into 1.5 mL collection tube (Eppendorf tube). The serum sample was kept in -20°C for RNA extraction later.

The RNA extraction was carried out using a QIAamp Viral RNA Mini Kit (250) (QIAGEN, Hilden, Germany) following manufacturer's instruction. The RNA extracted from the serum was kept in -20°C for later use.

Extraction of RNA From Organ Sample

Organs that were taken from the infected quails were trachea, lungs, and kidney. Each organ samples were pooled according to their group of infection. RNA extraction from the organ was done using TRIzol RNA Isolation Reagents (Invitrogen, Carlsbad, CA) as described by the manufacturer. The extracted RNA was kept in RNase free water at -20°C for later use.

Detection of RNA

Detection of IBV antigen from serum samples and organ samples was conducted using MyTaq One-Step RT-PCR Kit (Bioline, London, UK). The protocol used is provided in Table 1.

A pair of primers was designed based from the conserved region of the nucleocapsid (N) gene of IBV. Among the different IBV genes, N gene is the most conserved after untranslated region (Zwaagstra et al., 1992). The sequence and the expected size of PCR product are shown in Table 2. The chosen primers were also compared with other coronaviruses sequences from GenBank to confirm the sequences were conserved to IBV sequences only.

Table 6. Kruskal-Wallis test result showing significant difference of histological result between all 3 experimental groups.

Group	Mean rank	P-value
Group A 2 d.p.i	10.60	0.009
Group B 4 d.p.i	10.40	
Group C 6 d.p.i	3.00	

Table 7. Mann-Whitney test result showing significant difference of histological result between group A 2 d.p.i and group C 6 d.p.i.

Variable	Mean rank		z statistic	P-value
	Group A 2 d.p.i	Group C 6 d.p.i		
1	8.00	3.00	-2.627	0.009

Statistical Analysis

All scores obtained from clinical signs observation and gross lesion exposed to infection throughout the study period was analyzed using Kruskal-Wallis test. Following a statistically significant result, Mann-Whitney test was used for pair-wise comparison between treatment groups. A P -value < 0.05 was interpreted as a statistically significant result.

RESULT AND DISCUSSION

According to RT-PCR of serum sample for screening purpose before virus inoculation, 12 out 15 serum samples were found to be positive for IBV antigen (Figure 2). This shows quail is potentially susceptible to IBV infection. To further strengthen the arguments, detection of IBV antigen were also positive in organ samples particularly on the trachea (Figure 3). This finding is consistent with another study that indicated that out of the majority of positive organ samples detected with IBV antigen, 50% were from trachea samples (Terregino, et al., 2008). Lung sample was found positive from group B 4 d.p.i and group C 6 d.p.i. However, all kidney samples were tested negative (Table 3). A previous study reported that cecal tonsils and kidney were persistently having continuous excretion of IBV at usually below the detection levels of tests or true reactivation after true latency. Terregino also reported that only in 1 case that the IBV antigen was successfully isolated from kidney. Infectious bronchitis virus was detected from both trachea and kidney at 7th and 14th d.p.i. (Pohuang et al., 2011).

From the observation throughout the study period, no significant respiratory clinical signs were observed. Besides, ruffled feathers were observed from 1 d.p.i and subsided by 3 d.p.i. Watery feces started to be observed on 3 d.p.i and persisted throughout the experiment. Presence of ruffled feathers and watery feces were among the signs for nephritic form of IB (Ignjatovic and Sapats, 2000).

For the histology examination of trachea, tracheal lesions were present. Tracheal lesions were mild at 2 d.p.i

Table 8. Mann-Whitney test result showing significant difference of histological result between group B 2 d.p.i. and group C 4 d.p.i.

Variable	Mean rank		z statistic	P-value
	Group B 2 d.p.i	Group C 4 d.p.i		
1	8.00	3.00	-2.627	0.009

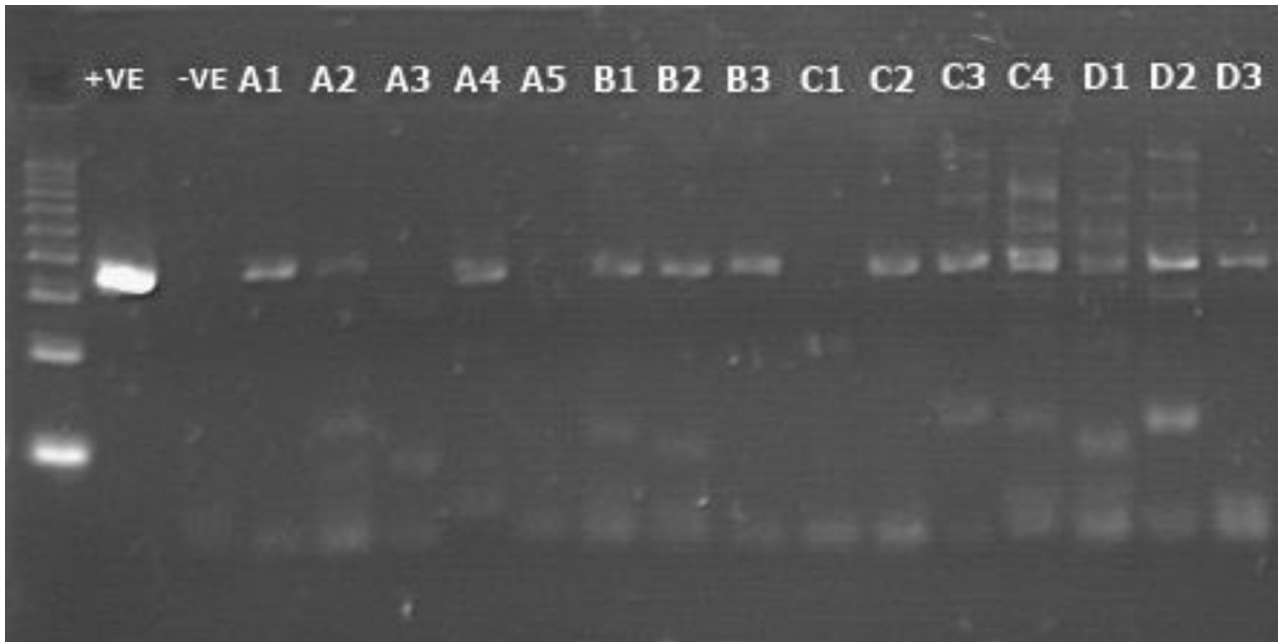


Figure 2. The RT-PCR result from serum samples from representative quails for each group for screening purpose prior to virus inoculation.

but becoming mild to moderate at 4 d.p.i and recovering at 6 d.p.i. (Table 4).

At 2 d.p.i., mild epithelial hyperplasia with lymphoid infiltration were observed. At 4 d.p.i, the tracheal lesion becomes moderate with moderate epithelial hyperplasia with loss of cilia and moderate lymphoid infiltration. At 6 d.p.i, the tracheal lesion seems to be recovering with very mild to no lesion could be observed from the quails in group C (Figures 4 and 5 and Table 5).

According to Kruskal-Wallis test result, there was significant difference of 0.009 ± 0.845 (P -value < 0.05) between the groups (Table 6). The Mann-Whitney test result shows that there was significant difference between group A 2 d.p.i and group C 6 d.p.i with 0.009 ± 0.845 (P -value < 0.05) (Table 7), and between group B 4 d.p.i and group C 6 d.p.i with 0.009 ± 0.845 (P -value) (Table 8). These findings were in agreement with another study that reported the highest concentration of IBV is in the

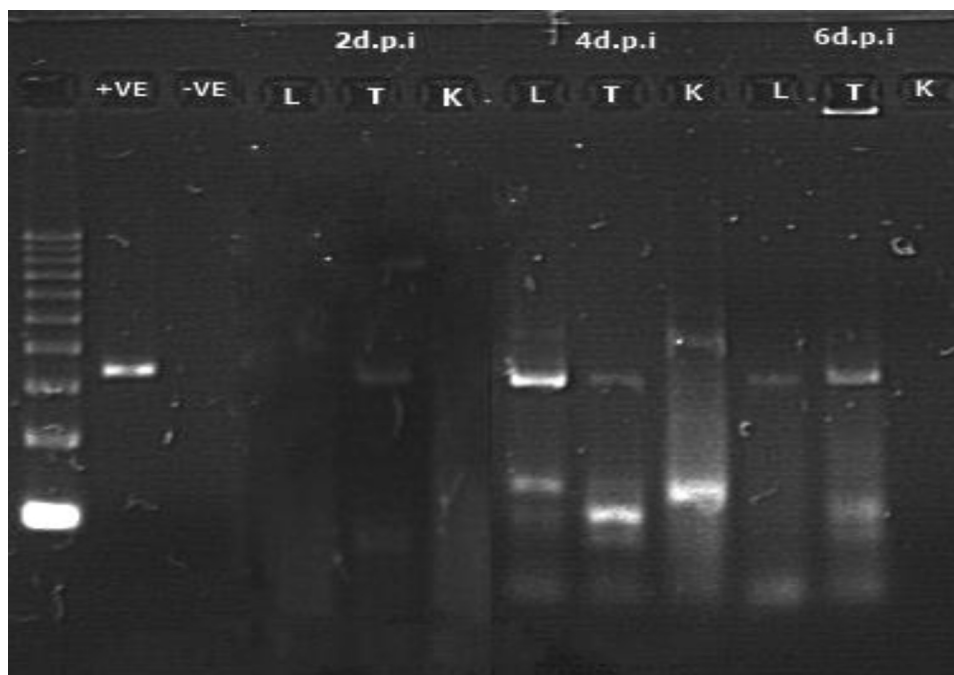


Figure 3. The figure showing RT-PCR result for IBV antigen detection from pooled organ samples from experimental groups. L: Lungs, T:trachea, K:kidney. IBV, infectious bronchitis virus.

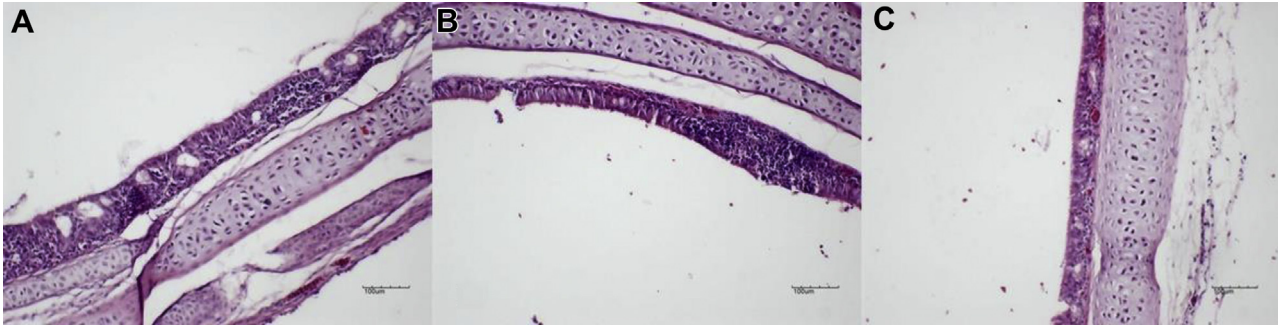


Figure 4. A: From group A 2 d.p.i showing mild epithelial hyperplasia with mild lymphoid infiltration. B: From group B 4 d.p.i showing moderate epithelial hyperplasia with loss of cilia and moderate lymphoid infiltration. C: From group C 6 d.p.i showing mild congestion of tracheal mucosa.

trachea on the first 3 to 5 D of postinfection. The virus titer then drops rapidly in the second wk p.i to below the detection level (De Wit, 2000). Another study also reported that severe lesions were found from 4 d.p.i, and the mucosal epithelium recovered completely by 21 d.p.i (Benyeda, et al., 2009). Even though QX-like is a nephropathogenic IBV strain, the virus replication first occurs in the trachea, which can cause histopathological lesions identical to those induced by respiratory strains (Ignjatovic and Sapats, 2000).

From the necropsy, no significant finding was found from quails in group A 2 d.p.i. In group B 4 d.p.i., only a quail with bilateral focal congested lungs and 2 quails with bilateral generalized congested lungs from group C 6 d.p.i. were identified, respectively. All quails in control group were normal. The kidney was also in normal condition from all groups up to 6 d.p.i. A study reported that gross lesion of the kidney was only observed in dead chicken that had been inoculated with the QX-like isolate. No pathologic lesions were found from chickens that were humanely killed at 7 d.p.i and 14 d.p.i. (Pohuang et al., 2011).

IBS 130/2015 QX-like strain infection showing different pathogenicity in terms of tissue tropism in quail

compared with chicken where there were no obvious respiratory clinical signs, and development of the virus pathogenicity in selected organs was not as rapid as in chicken. Generally, avian coronavirus in quails was found in higher frequency in reproductive tracts (9%) and trachea (18.2%) compared with layer chickens (0% in reproductive tract and 9% in trachea). For the kidney pool sample, layer chicken still had a higher incidence of avian coronavirus compared with quail (Torres et al., 2013). The reported occurrence of coronavirus in quail was associated with enteric syndrome (Circella et al., 2007) and respiratory and reproductive problem (Torres et al., 2013). The pathogenicity of QX-like could be possibly related to the originality of the QX-like isolation (Pohuang et al., 2011)

This study has proven that quail is susceptible toward IBV infection even by experimental inoculation. From previous observational study sampling from farms, it was found that quail shows enteric syndrome in which enteritis was the prominent one (Circella et al., 2007).

According to Nain and Smits, 2011, "to further investigate functional immunity, using an infectious challenge may be used to better assess actual response of quails in the local settings when exposed to the studied pathogen" (Nain and Smits, 2011). This experimental study shows the pathogenicity of IBV in quail with the evidence supported by previous studies, can be used as reference for health management in quail toward IBV, such as the optimum age for vaccine inoculation, and the organs affected by the virus according to the d.p.i during the postmortem procedure.

For future studies, further experimental studies on the potential of cross infection of IBV from quail to chicken can be explored to seek any possibilities that this phenomenon might affect the production capability of rearing both quails and chickens together in the same farm.

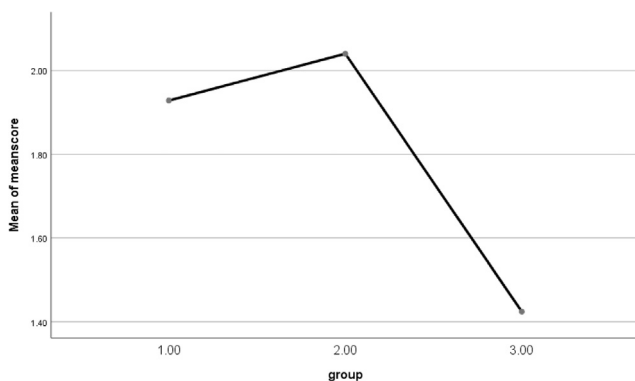


Figure 5. The graph of mean scores of tracheal histological lesions for each experimental group of birds infected with IBV throughout the study, it showed significant difference between experimental group. Group 1.00: Group A 2 d.p.i, Group 2.00: Group B 4 d.p.i, Group 3.00: Group C 6 d.p.i. IBV, infectious bronchitis virus.

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