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Evaluation of the antigen relatedness and efficacy of a single vaccination with different infectious bronchitis virus strains against a challenge with Malaysian variant and QX-like IBV strains

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ABSTRACT

Background: The predominant infectious bronchitis virus (IBV) strains detected in chickens in Malaysia are the Malaysian variant (MV) and QX-like, which are associated with respiratory distress, nephropathy, and high mortality. On the other hand, the antigenic relatedness and efficacy of IBV vaccines against these 2 field IBV strains are not well characterized.
Objectives: This study aimed to determine the antigen relatedness and efficacy of different IB vaccine strains against a challenge with MV and QX-like strains.
Methods: The antigen relatedness and the ability of different IB vaccine strains in conferring protection against MV and QX-like were assessed based on the clinical signs, macroscopic lesions, and ciliary activity.
Results: The MV strain IBS037A/2014 showed minor antigenic subtype differences with the vaccine virus Mass H120 and 4/91 strains but showed major antigenic subtype differences with the K2 strain. The Malaysian QX-like strain IBS130/2015 showed major antigenic subtype differences with the MV strain IBS037A/2014 and the vaccine strains except for K2. Chickens vaccinated once with Mass (H120) or with non-Mass (4/91 and K2) developed antibody responses with the highest antibody titer detected in the groups vaccinated with H120 and 4/91. The mean ciliary activities of the vaccinated chickens were between 56 to 59% and 48 to 52% in chickens challenged with IBS037A/2014 and IBS130/2015, respectively. The vaccinated and challenged birds showed mild to severe lesions in the lungs and kidneys.
Conclusions: Despite the minor antigenic subtype differences, a single inoculation with Mass or non-Mass vaccines could not protect against the MV IBS037A/2014 and QX-like IBS130/2015.

Keywords: Efficacy; infectious bronchitis virus; QX-like; variant

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Conflict of Interest

The authors declare no conflicts of interest.

Author Contributions

Conceptualization: Omar AR; Data curation: Ismail MI; Formal analysis: Ismail MI, Tan SW, Hair-Bejo M, Omar AR; Funding acquisition: Omar AR; Methodology: Ismail MI, Omar AR; Project administration: Omar AR; Resources: Omar AR; Supervision: Omar AR, Hair-Bejo M, Tan SW; Validation: Omar AR, Hair-Bejo M, Tan SW; Visualization: Omar AR; Writing - original draft: Ismail MI, Omar AR; Writing - review & editing: Omar AR.

INTRODUCTION

Infectious bronchitis (IB) is an acute, contagious disease affecting the respiratory, renal, and reproductive systems of chickens, causing a decrease in carcass weight, egg production, and death of the chickens, as well as significant economic losses worldwide [1]. The etiology of the disease is an infectious bronchitis virus (IBV), a highly prevalent coronavirus of chickens. IBVs primarily infect the respiratory tract of chickens, but some strains can cause lesions in the kidneys, oviduct, and other epithelial tissues [2].

In Malaysia, an IB case was first detected in 1967 [3]. Between 1967 and 1977, outbreaks of IB were due primarily to the Mass strains [3]. In the 1990's, a new IBV Malaysian variant strain MH5365/95 was detected, causing kidney disease, resulting in 20 to 30% mortality in the infected chickens [4]. In 2004, a new IBV variant was detected circulating in poultry farms and was reported as Malaysian IBV strain V9/04 [5]. Recently, a study carried out between 2014 and 2015 indicated the presence of Malaysian IBV QX-like and a local variant (MV) causing respiratory and kidney disease in broiler and layer chickens in several states in Malaysia [6]. A subsequent study indicated that the IBV QX-like is the predominant strain (47%) followed by the MV (13%) affecting poultry farms in Malaysia [7].

Besides biosecurity, the best strategy to control IB is by vaccination. On the other hand, the control of IB via vaccination is problematic because of the emergence of new variant IBV strains that are antigenic distinct. Moreover, vaccination with certain strains of IB failed to induce protection [8]. In most cases, poor protection of IB-vaccinated flocks is associated with the inability of the vaccine to provide adequate protection against the circulating field IBV.

In studies of chickens vaccinated with the Mass vaccine strain, the most commonly used IB vaccine could not provide adequate protection against the field IBV Belgian B1648 strain [9], Holland D1466, South Africa 890/80, Brazil 50/96 [10], Iranian QX-like [11], and Thai QX-like [12]. In another study, chickens vaccinated with the variant IB 4/91 vaccine showed poor protection against local IBV Holland D1466, South Africa 890/80, and Honduras 22/97 strains [10]. The main reason for the failure of the IB vaccine to provide protection is due primarily to antigenic differences at the S1 protein of the vaccine and the field strains of the viruses [13]. Furthermore, the diminished similarity of S1 protein can reduce the degree of cross-protection against homologous and heterologous IBV [14].

A previous study confirmed the high amino acid variation of the S1 protein of the Malaysian QX-like and MV compared to several IBV strains [6]. The Malaysian QX-like and MV showed high amino acid differences in the S1 protein against Mass H120 (EU822341; 29%–30% variations), 793/B (KP118884; 21%–27% variations), D274 (X15832; 22%–29% variations), Italy-02 (AJ457137; 21%–27% variations), KM91 (JQ977698; 17%–23% variations), K2 (JQ920378; 17%–21% variations), and Malaysian MH5365/95 (EU086600; 11%–26% variations). The variations in the S1 protein may influence the cross-protection ability of IBV vaccine strains against the Malaysian QX-like and MV. Cavanagh [15] reported that differences as little as 5% in the S1 sequences could cause poor cross-protection against an IB challenge.

For effective vaccination, the live attenuated vaccines against IB are given to day-old chicks at a hatchery or later in the farm by either drinking water or spray. Boosters with inactivated vaccines are required for laying hens [16-18]. Although vaccination against IB is used widely, vaccination with 1 serotype does not ensure complete protection against different IB strains

[10]. Therefore, this study examined the antigenic relatedness between different IB vaccine strains and the Malaysian QX-like and MV strains as well as the efficacy of different IB vaccine strains in conferring protection against the Malaysian QX-like and MV in specific-pathogen-free (SPF) chickens.

MATERIALS AND METHODS

Ethical approval

All procedures involving experimental chickens were undertaken in strict accordance with the use of animals for experiments as reviewed by the Institutional Animal Care and Use Committee (IACUC) of Universiti Putra Malaysia, Malaysia (approval No.: UPM/IACUC/AUP-R074/2016).

Vaccine preparation

Four different IBV vaccine strains used in this study were the Mass H120 strain (Bioral H120, Merial, France), K2 strain (Himmvac Dalguban B+ Live Vaccine, KBNP Inc., Korea), and 4/91 strain (Nobilis IB 4-91, Intervet International B.V., The Netherlands). Each vaccine strain was prepared separately using the method recommended by the respective manufacturers.

Titration of virus

Two previous characterized strains, Malaysian QX-like strain IBS130/2015 (KU949743) and MV strain IBS037A/2014 (KU949737), were used as the challenge viruses [6]. The viruses were propagated and titrated in 10-day-old SPF embryonated chicken eggs (VALO BioMedia North America, USA) using the method described by Reed and Muench [19]. The virus titer was adjusted to $10^{5.0}$ EID_{50} per bird before being inoculated into the experimental chickens.

Production of IBV antisera

One-week-old SPF chicks were used for antisera production. Briefly, the chicks were assigned into 5 groups (n = 5 chicks per group) with each group inoculated via the oculonasal route with the Mass H120, K2, 4/91, IBS130/2015, and IBS037A/2014 strains and kept separated in different rooms. The chickens were given access to food and drinking water *ad libitum*. A group of chickens inoculated with saline was kept as a control. At 21 and 28 dpi, the birds were bled, and the serum from each bird was collected for antibody determination using an enzyme-linked immunosorbent assay (ELISA) before being used in the virus neutralization assay.

Virus neutralization assessment and antigenic relatedness estimation

All antisera were heated at 56°C for 30 min to inactivate the complement before performing the virus neutralization (VN) test. The VN test of β-method (varying antiserum, constant virus) was performed in 10-day-old SPF embryonated chicken eggs [20]. The neutralization indices and VN titer were assessed based on the formula described by Shimazaki et al. [21]. The antigenic relatedness between the IBV of the Malaysian QX-like and MV strains and the vaccine strains were determined using the relative value (r-value) according to the methods described by Archetti and Horsfall [22].

Vaccine efficacy trial

Specific-pathogen-free birds were assigned randomly to 2 experiments, with each experiment comprised of 60 birds. In Experiment 1, 4 groups of 5-day-old SPF chicks (n = 12 chicks per

group) were inoculated via the oculonasal route with Mass H120 (Group A), K2 (Group B) or 4/91 (Group C) according to the respective manufacturer's recommended vaccine dose. One group left unvaccinated served as the controls. The birds were bled before vaccination and at 7, 14, and 21 days post-vaccination (dpv). At 21 dpv, the birds were challenged with the MV strain IBS037A/2014 ($10^{5.0}EID_{50}$) via the oculonasal route. At 3, 5, and 7 days post-challenge (dpc), the birds were killed humanely, and the trachea and kidneys were examined for macroscopic lesions. The tracheal ciliary activity was evaluated at 5 dpc. In Experiment 2, a similar experimental design and procedures were carried as above (Mass H120, Group D; K2, Group E; 4/91, Group F) except that the birds were challenged with the QX-like strain IBS130/2015.

Detection of antibody responses

The serum samples were tested for the antibody level using an indirect ELISA antibody test kit CK119 (BioChek BV, The Netherlands) according to the manufacturer's instructions.

Clinical signs observation

The clinical signs were observed daily and scored visually based on the scoring method described by Avellaneda et al. [23]. The severity of the clinical signs was scored as follows: 1 = no clinical signs, 2 = lacrimation and slight head movement, 3 = lacrimation, presence of nasal exudates, depression, or 4 = lacrimation, presence of nasal exudates, depression, swollen heads. The scores were pooled to determine the final score by calculating the average.

Macroscopic lesions examination

The trachea and kidneys were examined for gross lesions based on the scoring method described by Avellaneda et al. [23]. The lesion scores were described as follows: trachea (0 = no lesion, 1 = slight increase of mucin, 2 = large increase of mucin, 3 = large increase of mucin and mucosal congestion), and kidney (0 = no lesion, 1 = swelling, visible urate [under microscope], 2 = swelling, visible urate, 3 = swelling with large amount of urate deposition in kidney).

Ciliary activity evaluation

The tracheas from 3 randomly selected chickens from each challenged and control group at 5 dpc were removed and assessed for ciliary activity. Each tracheal ring was analyzed microscopically to estimate the ciliary movement using optical microscopy at × 200 magnification (Leica Microsystems, Germany). The ciliary movement was scored according to Cook et al. [10] as follows: 0, all cilia beating; 1, 75% beating; 2, 50% beating; 3, 25% beating; 4, 0% beating (100% ciliostasis). The mean ciliary movement score was calculated for each group. The maximum score for each trachea was 40 (for the 10 rings/trachea examined).

Protection score estimation

The protection scores were estimated using the formula described by Jackwood et al. [8] as follows: protection score = 100 – [(total of an individual score for the group/number of individuals in the group × 20) × 100]. A bird was considered protected if 50% or more ciliary activity was retained, with the maximum obtainable protection score being 100%. A higher protection score reaching 100% indicated a higher level of protection provided by the vaccine.

Statistical analysis

ELISA antibody titers data were analyzed using 1-way ANOVA followed by post-hoc Tukey's HSD tests (IBM SPSS 12, USA). The clinical signs, ciliary activity, and macroscopic gross lesions data were analyzed using the Kruskal-Wallis H test (IBM SPSS 12) to obtain statistical analysis among the groups. The p values < 0.05 were considered significant.

Table 1. The r-values (%) based on neutralization index of homologous and heterologous reactions between infectious bronchitis virus of IBS037A/2014, IBS130/2015, H120, K2 and 4/91 strains

Virus strains	Antiserum				
	IBS 037A/2014	IBS 130/2015	H120	K2	4/91
IBS 037A/2014	-	13	53	26	51
IBS 130/2015	-	-	13	42	14
H120	-	-	-	ND	ND
K2	-	-	-	-	ND
4/91	-	-	-	-	-

ND, not determined.
Antigenic relatedness: r-value = 70%–100%, antigenic identity (same serotype); 33%–70%, minor subtype difference; 11%–32%, major subtype difference; 0%–10%, serotype difference.

RESULTS

Neutralization index

The relative value (r-value) of the neutralization test was used to estimate the antigenic relatedness between the different IB vaccines and the 2 challenge IB viruses (**Table 1**). The r-values for the MV strain IBS037A/2014 and Mass H120, K2, and 4/91 were 53%, 26%, and 51%, respectively, indicating that IBS037A/2014 has a minor antigenic subtype difference with Mass H120 and 4/91 strain. On the other hand, the r-value between IBS037A/2014 and IBS130/2015 was only 13%, indicating no antigenic relatedness between the 2 virus strains. In the case of the QX-like challenge virus, IBS130/2015 showed major antigenic differences with Mass H120 (13%) and 4/91 (14%), indicating that the virus is a distinct strain. In contrast, the r-value between IBS130/2015 and K2 was 42%, indicating that IBS130/2015 has minor antigenic subtype differences with the K2 strain.

Experiment 1: Efficacy of different IBV vaccines following a challenge with the MV IBV strain in SPF chickens

Antibody response

Chickens vaccinated with the commercial vaccines showed antibody response at 7 to 21 dpv (**Table 2**). On the other hand, only chickens vaccinated with Mass H120 strain developed consistent antibody. Nevertheless, positive antibody titers were detected in all the vaccinated chickens before challenge with the highest antibody titer was detected in the Mass H120 group followed by 4/91 and K2 groups at 21 dpv.

Clinical signs observation

No abnormal clinical signs were observed in all the vaccinated groups at 1 dpc. In comparison, birds in the unvaccinated-challenged group showed mild (score 1.1) to moderate

Table 2. Experiment 1 on enzyme-linked immunosorbent assay antibody titer in specific-pathogen-free chickens after inoculation with infectious bronchitis vaccines at 7, 14 and 21 dpv

Groups (vaccine strain)	Antibody titer at different dpv		
	7	14	21
A (H120)	$303.21 \pm 40.93^{A,C,a,c,d,e}$	$1,441.00 \pm 529.61^{A,a,b,d,e}$	$1,774.75 \pm 111.87^{C,a,b,c,d,e}$
B (K2)	$733.71 \pm 116.02^{A,C,a,b,c,d,e}$	947.00 ± 386.52^{A}	$1,219.13 \pm 87.79^{C,a,b,c}$
C (4/91)	$1,080.71 \pm 62.24^{C,a,b,c,d,e}$	$1,011.86 \pm 44.48^{B,b,e}$	$1,393.38 \pm 262.88^{B,C,a,b,e}$
Control	$325.90 \pm 49.69^{a,c,d,e}$	399.90 ± 81.47^{a}	$367.00 \pm 75.60^{a,c,e}$

Data are expressed as mean ± SEM. Cut-off antibody titer is at 834. The mean antibody titer for pre-vaccination is at 179.60 ± 11.31.
dpv, day post-vaccination.
^{A,B,C}Capital letters represent significant differences of antibody titers between day post-vaccination time points at $p < 0.05$; ^{a,b,c,d,e}Small letters represent significant differences of antibody titers between groups at $p < 0.05$.

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Table 3. Clinical signs score of specific-pathogen-free chicken after inoculation with infectious bronchitis vaccines and challenged with IBS037A/2014 at different time points

Groups (vaccine strain)[A]	dpc[B]						
	1	2	3	4	5	6	7
UC	1.0 ± 0.0	1.1 ± 0.4	2.1 ± 0.4	2.6 ± 0.7	3.0 ± 0.0[a,b]	3.0 ± 0.0[a,b]	3.0 ± 0.0[a,b]
A (H120)	1.0 ± 0.0	1.3 ± 0.5	2.0 ± 0.0	2.3 ± 0.5	2.4 ± 0.5[a,b]	2.3 ± 0.5[a,b]	2.3 ± 0.5[a,b]
B (K2)	1.0 ± 0.0	1.1 ± 0.3	2.0 ± 0.0	2.5 ± 0.5	2.4 ± 0.5[a]	2.7 ± 0.5	2.7 ± 0.5
C (4/91)	1.0 ± 0.0	1.3 ± 0.5	2.0 ± 0.0	2.3 ± 0.5	2.2 ± 0.4[a]	2.2 ± 0.4[a]	2.2 ± 0.4[a]

dpc, day post-challenged; UC, unvaccinated-challenged.
[A]Unvaccinated-unchallenged group showed average clinical signs score at 1.0 ± 0.0 from 1 to 7 dpc; [B]Score range from 1 (normal), 2 (mild), 3 (moderate) to 4 (severe); [a,b]Small letters represent significant differences of antibody titers between groups at $P < 0.05$.

Table 4. Macroscopic lesion scores in trachea and kidney of specific-pathogen-free chicken after inoculation with infectious bronchitis vaccines and challenged with IBS037A/2014 at different time points

Groups (vaccine strain)	Day post-challenged					
	Trachea[A]			Kidney[B]		
	3	5	7	3	5	7
UC	1.0 ± 0.0	2.0 ± 0.0[a,b]	2.0 ± 0.0[a]	1.0 ± 0.0	1.0 ± 0.0	1.0 ± 0.0
A (H120)	1.3 ± 0.5	1.0 ± 0.0[a,b]	1.3 ± 0.5[a]	1.3 ± 0.5	1.0 ± 0.0	1.0 ± 0.0
B (K2)	1.0 ± 0.0	1.3 ± 0.5[a]	1.0 ± 0.0[a]	1.3 ± 0.5	1.0 ± 0.0	1.0 ± 0.0
C (4/91)	1.0 ± 0.0	1.0 ± 0.0[a]	1.0 ± 0.0[a]	1.0 ± 0.0	1.0 ± 0.0	1.0 ± 0.0
UU	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0

UC, unvaccinated-challenged; UU, unvaccinated-unchallenged.
[A]Trachea lesion scores: 0 = no lesion, 1 (mild) = slight increase of mucin, 2 (moderate) = large increase of mucin, 3 (severe) = large increase of mucin and mucosal congestion; [B]Kidney lesion scores: 0 = no lesion, 1 (mild) = swelling, visible urate (under microscope), 2 (moderate) = swelling, visible urate, 3 (severe) = swelling with large amount of urate deposition in kidney; [a,b]Small letters represent significant differences of antibody titers between groups at $p < 0.05$.

clinical signs (score 3.0) between 2 and 7 dpc (**Table 3**). All chickens in groups A, B, and C showed mild clinical signs, score range from 1.1 to 1.3 with the highest score in group C (score 1.3). On the other hand, between 3 to 7 dpc, all vaccinated-challenged groups showed mild to moderate clinical signs with a score range from 2.0 to 2.7. The highest clinical signs were observed in group B (score 2.7) at 6–7 dpc. Overall, a significant difference ($P = 0.00$) in clinical signs scores were detected between the vaccinated-challenged and unvaccinated groups between 3 and 7 dpc.

Macroscopic lesions examination

In the trachea, lesions, such as an increase in mucin and mucosal congestion, were observed. In the kidneys, however, lesions, such as kidney swelling and urate depositions, were observed in the infected birds (**Table 4**). Mild to moderate tracheal lesions in the vaccinated groups were observed between 3 and 7 dpc with the highest scores of 1.3 for group A at 3 dpc, 1.3 for group B at 5 dpc, and 1.0 for group C at 3–7 dpc. On the other hand, the tracheal lesions in the unvaccinated-challenged group were higher than the vaccinated groups with the highest score of 2.0 at 5–7 dpc. Mild to moderate kidney lesions with a score of 1.3 were observed in groups A and B at 3 dpc and a score of 1.0 for group C at 3–7 dpc.

Ciliary activity and protection score estimation

Measurements of the ciliary activity were carried out to determine the vaccine-induced protection (**Table 5**). A bird was considered protected if 50% or more ciliary activity was retained, with the maximum obtainable protection score being 100%. Although all vaccinated birds showed a mean ciliary activity of more than 50%, the vaccinated chickens were not protected against a challenge with IBS037A/2014. Moreover, the tracheal tissue samples of chickens in groups A, B, and C showed positive results by a reverse transcriptase-polymerase chain reaction (RT-PCR), indicating the presence of the challenge virus (data not shown).

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Table 5. Ciliary activity and protection % of specific-pathogen-free chickens inoculated with different infectious bronchitis virus vaccines and challenged with IBS037A/2014

Groups (vaccine strain)	Mean ciliary activity (%)[A]	Number of birds with 90% ciliary activity[B]	Protection (%)[C]
UC	16.7	0/3	0
A (Mass H120)	58.3	0/3	0
B (K2)	58.9	0/3	0
C (4/91)	56.7	0/3	0
UU	97.8	3/3	100

UC, unvaccinated-challenged; UU, unvaccinated-unchallenged.
[A]Mean ciliary activity per group calculated from ciliostasis scores for 10 tracheal rings per individual bird in each group using formula 100 – [(total of individual score for the group/number of individuals in the group × 20) × 100]; [B]Ciliary activity assessed according to Jackwood et al. [8]; [C]The vaccine is considered effective when > 80% of the birds in a group were protected from ciliostasis.

Experiment 2: Efficacy of different IBV vaccines following a challenge with QX-like IBV strain in SPF chickens

Antibody response

Chickens vaccinated with the commercial vaccines developed antibody responses at 7 to 21 dpv (**Table 6**). The results showed that the chickens vaccinated with the vaccine strain could mount a consistent increment of antibody titers. Positive antibody titers were detected in the vaccinated chickens before the challenge. The highest antibody titer was detected in the 4/91 group followed in order by the Mass H120 and K2 groups at 21 dpv.

Clinical signs observation

The clinical signs scores were normal in all the vaccinated groups at 1 dpc, while the unvaccinated-challenged group showed mild (score 1.7) to moderate clinical signs (score 3.0) between 3 and 7 dpc (**Table 7**). Birds in group D had mild clinical signs (score 1.2 to 1.5) between 2 and 4 dpc, but became moderate (score 2.5 to 3.0) between 5 dpc and 7 dpc. Group E showed mild clinical signs (score 1.4) at 2 to 3 dpc then turned to moderate clinical signs (score 2.0 to 2.6) between 4 and 7 dpc. Group F showed mild clinical signs (score 1.2 to

Table 6. Experiment 2 of enzyme-linked immunosorbent assay antibody titer in specific-pathogen-free chickens after inoculation with infectious bronchitis vaccines at 7, 14 and 21 dpv

Groups (vaccine strain)	Antibody titer at different dpv		
	7	14	21
D (Mass H120)	$333.33 \pm 70.67^{C,a,d}$	$854.47 \pm 257.85^{B,a}$	$1{,}590.86 \pm 289.53^{B,C,a,b}$
E (K2)	$283.33 \pm 150.42^{C,c,d}$	$499.25 \pm 141.40^{B,b}$	$1{,}195.50 \pm 149.01^{B,C}$
F (4/91)	$454.67 \pm 223.76^{C,d}$	534.75 ± 35.93^{B}	$1{,}821.79 \pm 246.87^{B,C,a,c}$
Control	$110.67 \pm 30.13^{a,d}$	$162.20 \pm 34.82^{a,b}$	$199.30 \pm 63.06^{a,b,c}$

Data are expressed as mean ± SEM. Cut-off antibody titer is at 834. The mean antibody titer for pre-vaccination is at 242.90 ± 31.88.
dpv, day post-vaccination.
[A,B,C]Capital letters represent significant differences of antibody titers between day post-vaccination time points at $p < 0.05$; [a,b,c,d]Small letters represent significant differences of antibody titers between groups at $p < 0.05$.

Table 7. Clinical signs score of specific-pathogen-free chicken after inoculation with infectious bronchitis vaccines and challenged with IBS130/2015 at different time points

Groups (vaccine strain)[A]	dpc[B]						
	1	2	3	4	5	6	7
UC	1.0 ± 0.0	$1.7 \pm 0.5^{a,b}$	$2.0 \pm 0.0^{a,b,c}$	$2.4 \pm 0.5^{a,b}$	2.7 ± 0.8	3.1 ± 0.4	3.0 ± 0.0
D (H120)	1.0 ± 0.0	$1.2 \pm 0.4^{a,b}$	$1.2 \pm 0.4^{a,b}$	$1.5 \pm 0.5^{a,b}$	2.5 ± 0.5	3.0 ± 0.0	3.0 ± 0.0
E (K2)	1.0 ± 0.0	1.4 ± 0.5	$1.4 \pm 0.5^{a,c}$	$2.0 \pm 0.0^{a,c}$	2.3 ± 0.5	2.6 ± 0.5	2.4 ± 0.5
F (4/91)	1.0 ± 0.0	1.2 ± 0.4^{a}	1.2 ± 0.4^{a}	$1.2 \pm 0.4^{a,c}$	1.8 ± 0.9	2.5 ± 0.5	2.5 ± 0.6

dpc, day-post challenged; UC, unvaccinated-challenged.
[A]Unvaccinated-unchallenged group showed average clinical signs score at 1.0 ± 0.0 from 1 to 7 dpc; [B]Score range from 1 (normal), 2 (mild), 3 (moderate) to 4 (severe); [a,b,c]Small letters represent significant differences of antibody titers between groups at $p < 0.05$.

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Table 8. Macroscopic lesion scores in trachea and kidney of specific-pathogen-free chicken after inoculation with infectious bronchitis vaccines and challenged with IBS130/2015 at different time points

Groups (vaccine strain)	Day post-challenged					
	Trachea[A]			Kidney[B]		
	3	5	7	3	5	7
UC	1.0 ± 0.0	3.0 ± 0.0[a]	2.2 ± 0.4[a]	1.0 ± 0.0	3.0 ± 0.0[a]	2.8 ± 0.4[a]
D (H120)	1.0 ± 0.0	2.0 ± 0.0[a]	2.0 ± 0.0[b]	1.0 ± 0.0	1.8 ± 0.8[a]	2.0 ± 0.0[a]
E (K2)	1.0 ± 0.0	2.0 ± 0.0[a]	1.2 ± 0.8	1.0 ± 0.0	1.2 ± 0.8[a]	2.0 ± 0.0[a]
F (4/91)	1.0 ± 0.0	2.2 ± 0.4[a]	1.2 ± 0.8	1.0 ± 0.0	2.0 ± 0.0[a]	2.0 ± 0.0[a]
UU	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0

UC, unvaccinated-challenged; UU, unvaccinated-unchallenged.
[A]Trachea lesion scores: 0 = no lesion, 1 (mild) = slight increase of mucin, 2 (moderate) = large increase of mucin, 3 (severe) = large increase of mucin and mucosal congestion; [B]Kidney lesion scores: 0 = no lesion, 1 (mild) = swelling, visible urate (under microscope), 2 (moderate) = swelling, visible urate, 3 (severe) = swelling with large amount of urate deposition in kidney; [a,b]Small letters represent significant differences of antibody titers between groups at $p < 0.05$.

1.8) between 2 and 5 dpc and then turned to moderate (score 2.5) at 6 to 7 dpc. A significant difference ($P = 0.02$) in the clinical signs scores were detected between the vaccinated and unvaccinated groups between 1 and 7 dpc. No clinical signs were observed in the unvaccinated-unchallenged group. In addition, the tracheal tissue samples of the chickens in groups D, E, and F showed positive results by RT-PCR, indicating the presence of the challenge virus (data not shown).

Macroscopic lesions examination

Mild to severe tracheal lesions were observed between 3 and 7 dpc in the vaccinated and unvaccinated-challenged groups with the highest tracheal lesions score of 2.2 being observed in group F at 5 dpc (**Table 8**). Moderate tracheal lesions were observed with the highest score of 2.0 for group D at 5–7 dpc, 2.0 for group E at 5 dpc, and 2.2 for group F at 5 dpc. Severe tracheal lesions were observed in the unvaccinated-challenged group, with a high score of 3.0 at 5 dpc. Moderate kidney lesions were observed in vaccinated birds with a score of 2.0 for group D at 7 dpc, 2.0 for group E at 7 dpc, and 2.0 for group F at 5–7 dpc. On the other hand, severe kidney lesions were observed in the unvaccinated-challenged group, with a high score of 3.0 at 5 dpc.

Ciliary activity and protection score estimation

The mean ciliary activity of all the vaccinated-challenged group were less than 50% (**Table 9**). Similar to the results of experiment 1, however, none of the vaccinated groups were protected when challenged with IBS130/2015.

Table 9. Ciliary activity and protection % of specific-pathogen-free chickens inoculated with different infectious bronchitis virus vaccines and challenged with IBS130/2015

Groups (vaccine strain)	Mean ciliary activity (%)[A]	Number of birds with 90% ciliary activity[B]	Protection %[C]
UC	14.2	0/3	0
D (H120)	49.4	0/3	0
E (K2)	48.3	0/3	0
F (4/91)	49.4	0/3	0
UU	97.8	3/3	100

UC, unvaccinated-challenged; UU, unvaccinated-unchallenged.
[A]Mean ciliary activity per group calculated from ciliostasis scores for 10 tracheal rings per individual bird in each group using formula 100 – [(total of individual score for the group/number of individuals in the group × 20) × 100]; [B]Ciliary activity assessed according to Jackwood et al. [8]; [C]The vaccine is considered effective when > 80% of the birds in a group were protected from ciliostasis.

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DISCUSSION

Currently, both Mass and variant IBV vaccine strains have been used to control IB in commercial poultry farms in Malaysia. On the other hand, limited studies have been carried out to determine the antigenic relatedness and efficacy of Mass and non-Mass (variant) vaccine strains in conferring protection against the Malaysian field IBV strains. In addition to the vaccine strains, studies have indicated that 2 distinct strains, the MV and QX-like, are the prevalent field IBV affecting commercial poultry farms in Malaysia [6,7]. Based on genotype classification analysis [24], the Malaysian QX-like is grouped with the QX-like viruses from other countries as genotype GI-19. In contrast, the MV strain is a unique strain closely related to a previously isolated nephropathogenic strain, MH5365/95 (data not shown). In this study, the efficacy of single inoculation with the Mass strain (H120) or with different variant strains (4/91 or K2) was evaluated in SPF chickens following a challenge with MV (IBS37A/2014) and QX-like (IBS130/2015) at 21 dpv.

An antigenic relatedness study indicated that the MV strain IBS037A/2014 has a minor subtype difference with the H120 and 4/91 strains but antigenically different with IBS130/2015. On the other hand, the Malaysian QX-like IBS130/2015 has no antigenic relatedness with IBS037A/2014, H120, and 4/91 strains but has a minor subtype difference with the K2 strain. This result is based on serologic r-value analysis that provides quantitative information on the antigenic relatedness of different virus strains using a mathematical calculation to predict the variation in the antigenic composition of the strains against the specific immune serum used [22]. An analysis of the serologic antigenic differences of IBVs is important because different serotypes of IBVs generally do not cross-protect, which is the main reason for the failure of the IB vaccine to provide protection [13]. The antigenically different IBVs usually induce poor cross-protective immunity. On the other hand, some IBV strains can provide cross-protection against other serotypes [10]. In many studies, these IBV strains are identified as protectotype strains [11,25-27].

Following a single IB vaccination, the detected ELISA antibody titers were not consistent at different days post-vaccination. On the other hand, all the vaccinated groups elicited antibody profiles at 21 dpv. The actual reasons of the different patterns in antibody titers in the vaccinated groups are unclear, but it could be influenced by various factors, such as host immune response [28], vaccine strain used [29], type of vaccination application method [30], and other local variables, such as temperature and feed quality [31]. Hence, the antibody titers alone are not a good indicator of a successful IB vaccination because the antibody titer does not always correlate with protection. The results of this study are supported by Roh et al. [32], who demonstrated a poor induction of antibody level detected by the Mass-type of ELISA antigen following vaccination with a live attenuated Ark-type IBV vaccine.

To evaluate the protection induced by the vaccine strains following the challenge with MV and QX-like IB, a few parameters, such as the clinical sign scores, macroscopic lesions examination, and ciliary activity, were measured. The same parameters were also used in a previous study to evaluate the protection of vaccinated and challenged chickens against homologous and heterologous IBV strains [8]. On the other hand, other studies reported that clinical signs usually vary and are challenging to quantify. Moreover, it is difficult to differentiate between vaccination or challenge groups [33]. In terms of practicality, direct observations, such as macroscopic lesions examination and ciliary activity estimation, are the most important parameters for a protection evaluation in many studies [8,33]. In

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addition, an indirect examination based on a VN assay is also helpful in differentiating the IBV serotypes [34].

The clinical signs and lesions in the trachea and kidneys, ranging from mild to severe, were observed in chickens following a challenge with IBS037A/2014 and IBS130/2015. Generally, the clinical signs and lesions were far more severe in the unvaccinated chickens than the vaccinated chickens. Depression, lacrimation, coughing, and ruffled feathers are the common signs observed. Sometimes gasping, sneezing, and the presence of nasal exudates also can be detected. Furthermore, the tissue samples collected from these birds tested positive to IBV based on RT-PCR. These findings were in line with other studies with a single vaccination using IB vaccine virus strains, such as H120 and CR88 challenged with TN20/00 [25], H120, Ma5 and 793/B challenged with M41 and QX-like strains [26], and H120 and Conn IB vaccines challenged with Thai QX-like [12]. On the other hand, less severe clinical signs and lesions were recorded when the chickens were given the Mass-type vaccine and booster with variant IB vaccine strains [27,35]. Protection studies indicated that the homologous IBV vaccine strains, either Mass-type or variant strains, usually induce better protection against an IBV challenge [10]. On the other hand, an assessment of cross-protection of some IBV vaccine combinations against circulating IBV strains of different serotypes could be an alternative approach to IB control [27]. A combination of the Mass-type (H120) and 4/91 vaccine strains, to some extent, could provide adequate protection against the heterologous IBV field variant strain [35]. These could be explained by the combination of different IBV vaccine strains broadening the protection by increasing the cellular and local immune responses [18]. This has also been postulated as the mechanism behind some of the protectotype IB strains in inducing a robust immunity against challenges with different serotypes of IBV.

The unvaccinated challenged groups demonstrated clinical signs and trachea lesions, as well as increased mucin secretion and swollen kidneys with urate deposition, as reported in a previous study [36]. All the vaccinated groups challenged with IBS037A/2014 demonstrated mild to moderate trachea and kidney damage between 3 and 7 dpc. On the other hand, the vaccinated chickens challenged with IBS130/2015 demonstrated mild to moderate tracheal lesions, whereas moderate kidney damage was observed in all the vaccinated groups between 5 and 7 dpc. Unlike an evaluation of the clinical signs and lesions, studies have shown that a tracheal ciliary activity examination is an efficient method to assess the protection against an IBV challenge [8,10,37]. The ciliary activity examination for assessing the damage to the chicken caused by IBV is fast and easy to perform [30]. By estimating the score or percentage of the surface of the tracheal epithelium that is showing ciliary movement, the protection can be assessed in IBV post-challenge birds [8]. The recommended time for assessing a protection post-challenged assessment is between 4 and 7 dpc [37]. Therefore, most vaccine efficacy studies assess protection from 5 to 7 dpc [38]. In this study, the tracheal ciliary activity of the vaccinated groups challenged with the Malaysian QX-like IBS130/2015 (48% to 52%) is slightly lower than the vaccinated groups challenged with the MV strain IBS037A/2014 (56% to 59%). On the other hand, the percentage mean ciliary activity failed to confer protection against the respective challenged viruses, which is probably due to the antigenic variations between the tested vaccines and challenge virus strains.

Several studies have shown that some IBV vaccine strains can provide protection after a single vaccination [12,26]. Therefore, in this study, the birds were vaccinated once with a single heterologous IB vaccine strain to evaluate the efficacy of the vaccine against challenge

virus of Malaysian QX-like IBS130/2015 and MV IBS037A/2014. The results from single-dose heterologous vaccination obtained showed that the vaccines used could not provide adequate protection against the challenge viruses. Currently, limited information is available on the common neutralizing epitopes found in different IBV serotypes, including protectotype strains that can confer protection. A previous study reported that a few changes in the S1 amino acid composition might produce a new IBV serotype, even though the remaining virus genome remained unchanged, indicating the importance of the S1 gene in determining the IBV serotype [14]. S1 also reported where the IBV antigenic determinants were located [2]. Therefore, if many antigens can be shared, this might suggest that a combination of currently available vaccines should provide robust protection against challenge IBVs belonging to different serotypes [10].

Several studies using IB booster vaccinations showed better protection against field IBV than a single vaccination alone [8,10]. For example, chickens primed with Mass M41 at 1-day-old, followed by a booster vaccination with IB-Var2 in 14-day-olds, showed better protection against field IBV Middle East IBV variant 2 (80%–100% protection) [26]. In another study, chickens primed with the Mass vaccine at 1-day-old followed by a booster vaccination with IBV 793/B showed effective protection against field M41 (90%–96% protection) and 793/B (93% protection) [27]. Chickens primed with Mass H120 at 1-day-old, followed by a booster vaccination with IB QX-like vaccine strain 1148 or 4/91, showed adequate protection against Thai QX-like (100% protection) [39]. Hence, improved protection was observed in the combination of 2 different serotypes IB vaccines, which was observed in the combined vaccination [10]. Moreover, another study showed that the sequential vaccination of different IBV vaccines could increase the broadness of the reacting antibody titers [40], which was proven by evaluating the cross-reactivity of sera collected from the prime and prime-boosted vaccinated chickens [38]. Therefore, this may explain the possibility of combined immunity between Mass and variant IBV strains against MV and QX-like. On the other hand, combination vaccinations of Mass and variant vaccine strains are not applied widely in Malaysia. This type of vaccination may be used in large commercial farms, but most small scale farmers usually prefer single vaccination due to the limitation of costs and human resources. Hence, vaccination with a Mass strain followed by a variant strain may be the answer to providing effective protection against a challenge with the MV and QX-like IB viruses.

In conclusion, although the Malaysian variant IBS037A/2014 and the H120 and 4/91 IB vaccine strains are grouped under 1 subtype, and the Malaysian QX-like strain IBS130/2015 and the K2 vaccine strain belong to another subtype group, single vaccination with the above vaccine cannot confer protection in SPF chickens.

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