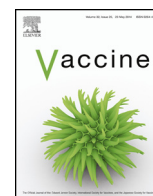




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Successful cross-protective efficacy induced by heat-adapted live attenuated nephropathogenic infectious bronchitis virus derived from a natural recombinant strain



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ABSTRACT

A natural recombinant nephropathogenic K40/09 strain of infectious bronchitis virus (IBV) was heat-adapted for possible future use as live attenuated vaccine. The K40/09 strain was selected during successive serial passages in specific-pathogen free (SPF) embryonated eggs at sub-optimal higher temperature (56 °C). Unlike the parental strain, the attenuated strain, designated K40/09 HP50, was found to be safe in 1-day-old SPF chicks, which showed neither mortality nor signs of morbidity, and rarely induced ciliostasis or histological changes in the trachea and kidney after intraocular and fine-spray administration. K40/09 HP50 provided almost complete protection against two distinct subgroups of a nephropathogenic strain (KM91-like and QX-like subgroup) and elicited the production of high titers of neutralizing antibody (neutralization index of 3.6). We conclude that the K40/09 HP50 vaccine virus is rapidly attenuated by heat adaptation and exhibits the desired level of attenuation, immunogenicity, and protective efficacy required for a live attenuated vaccine. These results indicate that the K40/09 vaccine could be helpful for the reduction of economic losses caused by recently emergent nephropathogenic IBV infection in many countries.

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1. Introduction

Infectious bronchitis virus (IBV) is a Gammacoronavirus that causes a highly contagious disease in chickens. The virus causes severe economic losses to the poultry industry worldwide because it can affect the upper respiratory and reproductive tracts, and some strains can cause nephritis in chickens [1]. It is well known that the primary problem in the control of infectious bronchitis is the ability of the virus to generate antigenic diversity by inaccuracy of the coronavirus RNA-dependent RNA polymerase and high frequency of homologous RNA recombination [2,3]. Many studies have shown that the degree of cross-protection tends to decrease among IBV serotypes and genotypes [4,5].

Despite intensive vaccination efforts using attenuated live and killed vaccines to prevent the disease, the emergence of new variant strains that do not serologically cross-react complicates disease

control and is an argument for vaccinating chickens with the type of IBV causing the disease [3,6]. However, producing a live IBV vaccine requires lengthy strategies that are time, cost, and labor intensive. Attenuation of IBV by multiple passages (over 100) in embryonated eggs can delay a new vaccine's clinical availability for several years. This drawback is compounded by the time required for verification of the vaccine to obtain licensing, as well as the current lack of available cell lines for vaccine production, which could accelerate vaccine production. Furthermore, there is no guarantee that the viral strain used to produce the vaccine will still be endemic at the time of vaccination [7–9].

Our previous study revealed that the QXIBV strain originating from China has been introduced in Korea and has formed a new cluster in the field [10]. This new cluster, represented by the K40/09 strain, is a natural recombinant strain between the Korean nephropathogenic strain KM91 and the QXIBV strain. In a previous challenge study, we characterized the Korean variant IBV K40/09 strain with regard to its immunogenicity and cross-protective efficacy against heterotype strains and its potential as a vaccine candidate [11].

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The development of a temperature-adapted vaccine has been attempted to increase safety and shorten the time of attenuation in several studies [12–14]. However, our experience shows that, besides desirable levels of attenuation and immunogenicity, other traits such as good growth properties should also be considered for live vaccine strains, and that this attenuation process may not work for all viruses. In the present study, we evaluated the safety and cross-protective efficacy of heat-adapted, live attenuated IBV derived from the K40/09 strain. The results of the present study might provide information for future IBV vaccine development and vaccination strategies.

2. Materials and methods

2.1. Viruses

The IBV strain K40/09, which was used for vaccine development, was isolated from a broiler farm in Korea. This variant strain belonged to the Korean new cluster 1, which originated from natural recombination between KM91 and QXIBV and showed a high level of cross-immunogenicity in a previous study [11]. Two challenge strains belonging to the KM91-like subgroup (KM91) and QX-like subgroup (K1277/03) were used to evaluate the cross-protective ability in chickens immunized with the heat-adapted IBV K40/09 strain (Table 1). All isolates were propagated in 10-day-old specific-pathogen free (SPF) embryonated chicken eggs (Hy-Vac, Adel, IA, USA) at 37 °C for 48 h. The allantoic fluid from eggs infected with each isolate was harvested and frozen at –70 °C until use.

2.2. Chickens

SPF white leghorn chickens (Nam-Deog Sanitek, Korea) were maintained in positive pressure high-efficiency particulate air-filtered stainless steel isolation cabinets (Three Shine, Korea). The food and water were provided *ad libitum* under constant illumination within a biosafety level 2 laboratory. All study procedures and animal care activities were conducted in accordance with the national and institutional guidelines for the care and use of laboratory animals.

2.3. Attenuation

Total 12 mL allantoic fluid (K40/09 IBV, 10^7 EID₅₀/mL) that propagated in embryonated chicken eggs were incubated at 56 °C and 1 mL aliquots were removed every 5 min for 60 min. Each aliquot was inoculated via the chorioallantoic sac (100 µL/egg) into five embryonated eggs (10- or 11-day-old), respectively and was incubated for 6 days. The allantoic fluid was harvested from eggs confirmed embryonic lesions of curling and stunting; those with the highest viral load as quantified by real-time reverse transcription polymerase chain reaction (qRT-PCR) were selected [15] and used for subsequent serial passages, which were repeated fifty times. The titer of three viruses designated K40/09 (parental strain, no heat-adapted passages), K40/09 HP40 (forty heat-adapted passages), and K40/09 HP50 (fifty heat-adapted passages) was determined in 10-day-old embryonated eggs and was calculated by the method of Reed and Muench [16].

2.4. Safety study in SPF chicks

One hundred and forty SPF 1-day-old chicks were divided into 7 groups, with 20 chicks in each group. A 10× dose ($10^{4.0}$ EID₅₀/bird) of three IBV strains (K40/09, K40/09 HP40, and K40/09 HP50) was inoculated by the eyedrop or fine-spray method (Three Shine, droplet size = 50 µm) at 1 day of age, while those in the control

group were inoculated with phosphate-buffered saline (Table 2). To determine the pathologic characteristics of IBV, 10 birds were observed twice daily for clinical signs for 14 days. At 5 days after inoculation, 10 chickens were sacrificed and used to score ciliostasis and histological lesions. Tracheas and kidneys were collected, fixed with 10% neutral-buffered formalin, and routinely processed in paraffin, after which 5-µm sections were cut for hematoxylin and eosin staining for histological studies. The tracheal lesion scores included epithelium deciliation, proliferation, degeneration, exudate, congestion, and hemorrhage. The renal lesion scores included epithelial degeneration, tubulonephrosis, interstitial nephritis, and regeneration. Lesions were scored as follows: 0 for normal, 1 for extensively focal lesions, 2 for multifocal lesions, and 3 for diffuse lesions.

2.5. Cross-protection study

A total of 120 3-week-old SPF chickens were divided into 12 groups of 10 chickens each. Nine groups were immunized intraocularly with K40/09, K40/09 HP40, or K40/09 HP50 strain at $10^{3.0}$ EID₅₀, while the other three groups were kept as non-immunized controls. Three weeks after immunization, all birds were challenged intraocularly with $10^{4.5}$ EID₅₀ of three strains belonging to KM91-like subgroup (KM91), QX-like subgroup (K1277/03), or new genetic cluster (K40/09) (Table 3). Five days after challenge, the challenge virus was re-isolated from the tracheas and kidneys of birds by inoculating 9- to 11-day-old embryonated SPF chicken eggs. After 48 hr of incubation, allantoic fluids were harvested and the presence of challenge virus was examined using the dot-immunoblot assay [17].

2.6. Neutralizing index

Sera from chickens of all groups in the efficacy studies were collected at 3 weeks after immunization and inactivated at 56 °C for 30 min. The viral neutralization test was performed as previously described [18]. Briefly, the viruses used for immunization were 10-fold serially diluted before mixing with an equal volume of inactivated serum sample. The virus-serum mixtures were incubated for 1 h at 37 °C prior to inoculating 10-day-old embryonated SPF eggs. All specimens were inoculated into the allantoic cavity of 9- to 11-day-old SPF chicken embryonated eggs (Hy-Vac). After 48 h of incubation, the eggs were chilled, and the allantoic fluids were harvested and tested using the dot-immunoblot assay [17]. At the end of the experimental period, the EID₅₀ values of the inoculated viruses were determined and the neutralizing index (NI) was calculated.

2.7. Statistical analysis

The mean ciliostasis and histopathologic lesion scores were analyzed using a two-tailed *t*-test and re-isolation rate of challenge virus among the groups was analyzed using one-tailed Fisher's exact test. A *p*-value of <0.05 was considered statistically significant.

3. Results

3.1. Safety study of heat-adapted K40/09

The K40/09 parental virus was pathogenic to 1-day-old chicks, inducing 40% mortality as well as respiratory signs and nephritis regardless of the administration method; however, K40/09 HP40 and K40/09 HP50 did not induce any clinical signs or mortality

Table 1
Viruses used in this study.

IBV isolate	Genotype	Usage	Accession number
KM91	Korean II subgroup 1	Challenge strain	FJ807946
K1277/03	Korean II subgroup 2 (QX-like)	Challenge strain	FJ807930
K40/09	Korean New genetic cluster 1	Challenge strain (Parent strain)	HM486957
K40/09 HP ^a 40	Korean New genetic cluster 1	Vaccine strain (Heat adapted 40 times)	NS ^b
K40/09 HP50	Korean New genetic cluster 1	Vaccine strain (Heat adapted 50 times)	NS

^a Heat passage.^b NS = not submitted.**Table 2**
Safety test of K40/09 HP50 compared with the K40/09 parent and K40/09 HP40 in 1-day-old SPF chicks.

Administration method ^a	Treatment	Mortality (%)	Mean ciliostasis scores ^b	Histopathologic lesion scores ^c			
				Upper trachea	Middle trachea	Lower trachea	Kidney
Control	PBS	0/10 (0)	0.1	0	0	0	0.3
Intraocularly	K40/09 (Parent)	4/10 (40)	2.6 ^{***}	1.3 [*]	1.5 [*]	1.2 [*]	2.8 ^{***}
	K40/09 HP40	0/10 (0)	1.6	0.4	0.4	0.2	2.9 ^{***}
	K40/09 HP50	0/10 (0)	1.2	0.2	0.3	0.1	0.9
Fine spray	K40/09 (Parent)	4/10 (40)	3.3 ^{†††}	1.9 [†]	2.6 ^{†††}	1.9 [†]	2.8 ^{†††}
	K40/09 HP40	0/10 (0)	3.2 ^{†††}	2 [†]	1.7 [†]	1.2	2.2 ^{†††}
	K40/09 HP50	0/10 (0)	1.8	0.9	0.7	0.9	0.5

^a One-day-old SPF chicks were inoculated with IBV ($10^{4.0}$ EID₅₀/bird) by eyedrop or fine spray (droplet size = 50 μm).^b On day 5 post-vaccination, tracheas were removed from five chickens in each group. Ten trachea rings per chick were prepared (three upper, four middle, and three lower). The rings were examined under low-power magnification and ciliary activity was scored as follows: 0, no ciliostasis; 1, 25% ciliostasis; 2, 50% ciliostasis; 3, 75% ciliostasis; 4, 100% ciliostasis.^c Histopathology of the trachea and kidney at 5 days post-vaccination with IBV is reported as histopathologic lesion scores. 0, normal; 1, extensively focal lesions, 2, multifocal lesions; 3, diffuse lesions.^{*} $p < 0.05$, by two-tailed t -test, compared to the K40/09 HP50 intraocularly vaccinated group.^{***} $p < 0.001$, by two-tailed t -test, compared to the K40/09 HP50 intraocularly vaccinated group.[†] $p < 0.05$, by two-tailed t -test, compared to the K40/09 HP50 vaccinated group by fine spray method.^{†††} $p < 0.001$, by two-tailed t -test, compared to the K40/09 HP50 vaccinated group by fine spray method.**Table 3**
Cross-protective effects in 3-week-old SPF chickens immunized with K40/09 parent, K40/09 HP40 and K40/09 HP50 against challenge with three distinct subgroups of nephropathogenic IBV strain.

IBV strain immunized ^a	Genogroup of challenge virus	IBV strain of challenge virus ^b	No. of challenge virus isolated/no. of challenged ^c			
			Trachea Control	Vaccinated	Kidney Control	Vaccinated
K40/09 (Parent)	Korean II subgroup 1	KM91	10/10	0/10 ^{***}	10/10	2/10 ^{***}
	Korean II subgroup 2 (QX-like)	K1277/03	10/10	0/10 ^{***}	10/10	1/10 ^{***}
	Korean new cluster 1	K40/09	10/10	0/10 ^{***}	10/10	0/10 ^{***}
K40/09 HP40	Korean II subgroup 1	KM91	10/10	0/10 ^{***}	9/10	0/10 ^{***}
	Korean II subgroup 2 (QX-like)	K1277/03	10/10	0/10 ^{***}	10/10	0/10 ^{***}
	Korean new cluster 1	K40/09	10/10	0/10 ^{***}	10/10	1/10 ^{***}
K40/09 HP50	Korean II subgroup 1	KM91	10/10	0/10 ^{***}	9/10	0/10 ^{***}
	Korean II subgroup 2 (QX-like)	K1277/03	10/10	1/10 ^{***}	10/10	0/10 ^{***}
	Korean new cluster 1	K40/09	10/10	0/10 ^{***}	10/10	0/10 ^{***}

^a Three-week-old chickens were immunized with IBV K40/09 parent, K40/09 HP40 or K40/09 HP50 ($10^{3.0}$ EID₅₀/bird) via the intraocular route.^b At 3 weeks post-immunization, all birds were challenged with $10^{4.5}$ EID₅₀ of three challenge strains via the intraocular route.^c Five days after challenge, protection was evaluated by the absence of challenge virus in the trachea and kidney.^{***} $p < 0.001$, by Fisher's exact test, compared to non-vaccinated control group.

in 1-day-old chicks (Table 2). In this study, we observed different results for ciliostasis and histological lesions according to the vaccination method. When administered intraocularly, the mean ciliostasis histopathologic lesion scores for tracheas of birds inoculated with K40/09 HP40 and K40/09 HP50 were lower than those for birds inoculated with K40/09. However, when administered by fine spray, only K40/09 HP50 showed low ciliostasis and histopathologic lesion scores. In the kidneys, K40/09 HP50 did not induce any histopathologic lesions, but chickens administered K40/09 and K40/09 HP40 IBV exhibited definite epithelial degeneration, tubulonephrosis, and interstitial nephritis when compared with the non-vaccinated control.

3.2. Cross-protective efficacy of heat-adapted K40/09

As shown in Table 3, chickens immunized with K40/09, K40/09 HP40, or K40/09 HP50 were challenged with three nephropathogenic strains belonging to the KM91-like subgroup (KM91), the QX-like subgroup (K1277/03), and the new genetic cluster (K40/09), respectively. All vaccination groups showed complete protection of the respiratory tract and kidney against three nephropathogenic strains ($p < 0.001$) compared with the non-vaccinated control group, and there were no significant differences between vaccination groups. Sera from immunized birds were collected at 3 weeks post-immunization and the NI of the

experimental group was examined. The infectious bronchitis vaccine is defined as effective if the NI of immunized groups exceeds 2.0 and the NI of the non-immunized control group is <1.0 [18,19]. The NI of chickens immunized by K40/09, K40/09 HP40, and K40/09 HP50 was 4.0, 3.8, and 3.6, respectively, whereas that of the non-immunized control was 0.3 (data not shown).

4. Discussion

Current IBV vaccine strains do not cross-protect against new viral strains because of the extensive genetic diversity and high mutation rate of IBV [20]. Therefore, the best protective effect that can be achieved would be only against strains of the same serotype or genotype [3]. However, the development of vaccines against the new IBV variants is not generally an option owing to the high cost and time required for their final approval. Moreover, the use of multiple strains of live vaccines should be practiced with caution owing to concerns regarding the formation of variants of viruses by recombination with field strains resulting from the spread of vaccine strains [21]. Thus, rapid development of a live IBV vaccine that provides broad cross-protection would be a highly relevant and practical method in IBV control. In this study, we aimed to develop a novel live attenuated IBV vaccine with broad-spectrum protective capability using a heat-attenuation method in a considerably short time.

In this report, the natural recombinant nephropathogenic IBV strain K40/09 was heat-adapted for possible future use as a live attenuated vaccine. The initial selection of the K40/09 virus for this purpose was guided by the fact that this strain has a favored vaccine candidate background that contains two genetically characterized nephropathogenic strains (KM91 and QXIBV) as a recombinant strain [22] and showed significantly high levels of immunogenicity and protective ability against many different types of IBV [11]. Our approach to heat-adapting IBV differed in two ways from previous attempts [13]. Differences included the viral strain (nephropathogenic IBV strain) and the number of passages at high temperature (up to 50 times). In general, current methods for IBV attenuation include serial passage of virus in chicken embryos over 50 times, and the nephropathogenic IBV strain seemed particularly refractory to attenuation [7,8]. Although the mechanism of heat attenuation is unknown, this is the first study to attenuate a nephropathogenic IBV strain by heat adaptation. Furthermore, based on our data, it is likely that adaptation at progressively higher temperatures would provide rapid attenuation of IBV and selective pressure for cumulative mutations that confer less virulence for chickens.

The susceptibility of the vaccine strain to high temperature is closely associated with IBV transmission and outbreak in the field. The goal of vaccination by drinking water or spray is to immunize the highest percentage of the poultry in a flock at a given time. However, in summer, uniformity of immunization can be further decreased by a loss of IBV vaccine titer caused by relatively high temperatures during vaccination. A persistent rolling reaction among birds in a flock can occur if many birds are missed or receive a low dose, resulting in disease outbreak. When the virus was incubated at 56 °C, the heat resistance time of K40/09 parent was 120 min, but K40/09 HP50 virus survived up to 450 min (data not shown). The results presented here suggest that use of K40/09 HP50 subpopulations resistant to heat inactivation could be helpful for reducing vaccine failure rates through the uniform administration of the vaccine.

Spray vaccination is one of the most common vaccination practices performed worldwide in the poultry industry [4], as it triggers local immunity in the upper respiratory tract and also stimulates general humoral immune responses [23,24]. However, fine droplets (50–80 µm in size) containing the vaccine virus might be inhaled too deeply into the respiratory tract (lung and air sac) tissues,

resulting in disease with an excessive post-vaccination reaction in young chicks. Thus, the use of a coarse spray with a droplet size greater than 100 µm is generally suitable for hatchery vaccination. The safety of K40/09 HP50 was demonstrated by administering the vaccine to 1-day-old SPF chicks via spray. Based on their safety profiles, the non- and low-heat-passaged viruses (K40/09 and K40/09 HP40) produced a milder clinical disease response with mild histopathological lesions of tracheas and kidneys when administered by fine spray, but the K40/09 HP50 virus was no longer pathogenic for 1-day-old chicks, as demonstrated by lower ciliostasis and histopathologic lesion scores. Considering the fact that spray vaccination was applied using fine spray (50-µm droplets) in our safety study, K40/09 HP50 is considered to be very safe and useful for hatchery spray vaccination.

Adjusting the balance between attenuation and immunogenicity is a critical factor in developing live vaccines due to the possibility of over-attenuation, which leads to poor protection with a loss in immunogenicity [25]. Although the K40/09 parental strain was chosen based on its cross-protective ability [8], it is not clear whether the fully attenuated K40/09 HP50 still induced cross-protection. In efficacy studies, all viruses (K40/09, K40/09 HP40, and K40/09 HP50) provided almost complete protection for tracheas and kidneys against nephropathogenic strains of Korean group II (QXIBV-like and KM91 strains) with high neutralizing-antibody titers. These findings indicate that K40/09 HP50 retained its immunogenicity after serial passage. Nephropathogenic strains of different serotypes are a major problem worldwide; therefore, it has been proposed that the polyvalent vaccine formulation expanded the level of cross-protection against nephropathogenic IBV strains over that achieved by individual vaccine strains [26]. However, the use of multiple strains of live vaccines may contribute to the variation and recombination of IBV, and engender even more complex IBV epidemics. Conversely, our results suggest that single administration of K40/09 HP50 is markedly effective and economical due to its broad-spectrum protection against nephropathogenic IBV strains, including QXIBV, which are worldwide epizootic strains [11].

Given the tremendous ability of coronaviruses to evolve, it is not surprising that genetic diversity through point mutation and recombination might lead to the emergence of new variant viruses and cause new diseases by host switching in animals and humans [27–29]. These characteristics of coronaviruses create difficulties in the design of disease control strategies; therefore, vaccine candidates that offer broad-spectrum protection and methodologies that shorten the time of live vaccine development are needed. The results of the present study show that heat adaptation could be a very useful method to enhance the safety of IBV, and the heat-adapted K40/09 HP50 virus exhibits a fine balance between attenuation and immunogenicity, and exhibits cross-protective efficacy. These results further suggest that the heat-adapted K40/09 HP50 virus can help to reduce economic losses caused by newly evolving nephropathogenic IBV strains and improve IB vaccine effectiveness in many countries where IBV infection is a major problem.

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References

- [1] Jackwood MW. Review of infectious bronchitis virus around the world. *Avian Dis* 2012;56:634–41.
- [2] Casais R, Dove B, Cavanagh D, Britton P. Recombinant avian infectious bronchitis virus expressing a heterologous spike gene demonstrates that the spike protein is a determinant of cell tropism. *J Virol* 2003;77:9084–9.

- [3] Cavanagh D. Coronavirus avian infectious bronchitis virus. *Vet Res* 2007;38:281–97.
- [4] Ignjatovic J, Sapats S. Avian infectious bronchitis virus. *Rev Sci Tech* 2000;19:493–508.
- [5] Gelb Jr J, Weisman Y, Ladman BS, Meir R. S1 gene characteristics and efficacy of vaccination against infectious bronchitis virus field isolates from the United States and Israel (1996 to 2000). *Avian Pathol* 2005;34:194–203.
- [6] Cavanagh D. Coronaviruses in poultry and other birds. *Avian Pathol* 2005;34:439–48.
- [7] Lee HJ, Youn HN, Kwon JS, Lee YJ, Kim JH, Lee JB, et al. Characterization of a novel live attenuated infectious bronchitis virus vaccine candidate derived from a Korean nephropathogenic strain. *Vaccine* 2010;28:2887–94.
- [8] Feng K, Xue Y, Wang J, Chen W, Chen F, Bi Y, et al. Development and efficacy of a novel live-attenuated QX-like nephropathogenic infectious bronchitis virus vaccine in China. *Vaccine* 2015;33:1113–20.
- [9] Macdonald JW, Randall CJ, McMartin DA, Dagless MD. Immunity following vaccination with the H120 strain of infectious bronchitis virus via the drinking water. *Avian Pathol* 1981;10:295–301.
- [10] Lim TH, Lee HJ, Lee DH, Lee YN, Park JK, Youn HN, et al. An emerging recombinant cluster of nephropathogenic strains of avian infectious bronchitis virus in Korea. *Infect Genet Evol* 2011;11:678–85.
- [11] Kim BY, Lee DH, Jang JH, Lim TH, Choi SW, Youn HN, et al. Cross-protective immune responses elicited by a Korean variant of infectious bronchitis virus. *Avian Dis* 2013;57:667–70.
- [12] Gelb Jr J, Lunt RL, Metz AL, Fries PA. Attenuation of avian infectious bronchitis virus by cold-adaptation. *Avian Dis* 1991;35:847–53.
- [13] Jackwood MW, Hilt DA, Sellers HS, Williams SM, Lasher HN. Rapid heat-treatment attenuation of infectious bronchitis virus. *Avian Pathol* 2010;39:227–33.
- [14] Lee KH, Seo SU, Song JM, Lee CM, Kim HA, Seong BL. Characterization of live influenza vaccine donor strain derived from cold-adaptation of X-31 virus. *Vaccine* 2006;24:1966–74.
- [15] Callison SA, Hilt DA, Boynton TO, Sample BF, Robison R, Swayne DE, et al. Development and evaluation of a real-time Taqman RT-PCR assay for the detection of infectious bronchitis virus from infected chickens. *J Virol Methods* 2006;138:60–5.
- [16] Reed LJM. A simple method of estimating fifty per cent endpoints. *Am J Epidemiol* 1938;27:493–7.
- [17] Song CS, Kim JH, Lee YJ, Kim SJ, Izumiya Y, Tohya Y, et al. Detection and classification of infectious bronchitis viruses isolated in Korea by dot-immunoblotting assay using monoclonal antibodies. *Avian Dis* 1998;42:92–100.
- [18] Gelb Jr J, Wolff JB, Moran CA. Variant serotypes of infectious bronchitis virus isolated from commercial layer and broiler chickens. *Avian Dis* 1991;35:82–7.
- [19] Choi KS, Lee EK, Jeon WJ, Park MJ, Kim JW, Kwon JH. Pathogenicity and antigenicity of a new variant of Korean nephropathogenic infectious bronchitis virus. *J Vet Sci* 2009;10:357–9.
- [20] Lim TH, Kim MS, Jang JH, Lee DH, Park JK, Youn HN, et al. Live attenuated nephropathogenic infectious bronchitis virus vaccine provides broad cross protection against new variant strains. *Poult Sci* 2012;91:89–94.
- [21] Farsang A, Ros C, Renstrom LH, Baule C, Soos T, Belak S. Molecular epidemiology of infectious bronchitis virus in Sweden indicating the involvement of a vaccine strain. *Avian Pathol* 2002;31:229–36.
- [22] Lim TH, Lee HJ, Lee DH, Lee YN, Park JK, Youn HN, et al. An emerging recombinant cluster of nephropathogenic strains of avian infectious bronchitis virus in Korea. *Infect Genet Evol* 2011;11:678–85 (journal of molecular epidemiology and evolutionary genetics in infectious diseases).
- [23] Ibrahim AL, Lai MC, Aini I. Spray vaccination with an improved F Newcastle disease vaccine. A comparison of efficacy with the B1 and La Sota vaccines. *Br Vet J* 1983;139:213–9.
- [24] Samberg Y, Hornstein K, Cuperstein E, Gottfried R. Spray vaccination of chickens with an experimental vaccine against Newcastle disease. *Avian Pathol* 1977;6:251–8.
- [25] Tarpey I, Orbell SJ, Britton P, Casais R, Hodgson T, Lin F, et al. Safety and efficacy of an infectious bronchitis virus used for chicken embryo vaccination. *Vaccine* 2006;24:6830–8.
- [26] Terregino C, Toffan A, Beato MS, De Nardi R, Vascellari M, Meini A, et al. Pathogenicity of a QX strain of infectious bronchitis virus in specific pathogen free and commercial broiler chickens, and evaluation of protection induced by a vaccination programme based on the Ma5 and 4/91 serotypes. *Avian Pathol* 2008;37:487–93.
- [27] Jackwood MW, Boynton TO, Hilt DA, McKinley ET, Kissinger JC, Paterson AH, et al. Emergence of a group 3 coronavirus through recombination. *Virology* 2010;398:98–108.
- [28] Decaro N, Mari V, Elia G, Addie DD, Camero M, Lucente MS, et al. Recombinant canine coronaviruses in dogs, Europe. *Emerg Infect Dis* 2010;16:41–7.
- [29] Gralinski LE, Baric RS. Molecular pathology of emerging coronavirus infections. *J Pathol* 2015;235:185–95.