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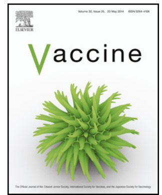

Successful cross-protective efficacy induced by heat-adapted live attenuated nephropathogenic infectious bronchitis virus derived from a natural recombinant strain

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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

Q2 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 EID_{50}/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 ($10^{4.0}$ EID_{50}/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 $10^{3.0}$ EID_{50}, while the other three groups were kept as non-immunized controls. Three weeks after immunization, all birds were challenged intraocularly with $10^{4.5}$ EID_{50} 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 EID_{50} 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

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