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Avian Pathology

Immune protection conferred by three commonly used commercial live attenuated vaccines against the prevalent local strains of avian infectious bronchitis virus in southern China

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ABSTRACT. Live attenuated vaccines are critical in the control of avian infectious bronchitis. It is necessary to know the protection conferred by commonly used commercial live vaccines. In this study, specific pathogen-free chicks were vaccinated with the commercial live vaccines H120, 4/91 and LDT3-A. Blood samples were collected at weekly intervals for the detection of IBV-specific antibodies and quantification of $CD4^+$ and $CD8^+$ T lymphocytes. At 21 days post-inoculation the vaccinated birds were challenged with the IBV prevalent local strains GX-YL5, GX-GL11079 and GX-NN09032, respectively. Trachea and kidney samples were collected at 5 days post-challenge for the detection of the virus. The results showed that the H120 group exhibited medium antibody levels, the lowest percentages of $CD4^+$, $CD8^+$ T lymphocytes and the highest viral loads. The 4/91 group showed the lowest antibody levels, but the highest percentages of $CD4^+$, $CD8^+$ T lymphocytes and the lowest viral loads. The LDT3-A group showed the highest antibody levels, the medium percentages of $CD4^+$, $CD8^+$ T lymphocytes and the medium viral loads. The protection rates of H120, 4/91 and LDT3-A groups were 41.7–58.3%, 75.0–83.7% and 66.7–75.0%, respectively. The present study demonstrated that the vaccines H120, 4/91 and LDT3-A could stimulate the immunized chicks to produce different levels of humoral and cellular immunity to resist the infection of IBV, but couldn't provide complete protection against the prevalent local strains of IBV in southern China. Also, the vaccine 4/91 offered the best immune protection among the three vaccines.

KEY WORDS: commercial live vaccine, immune protection, infectious bronchitis virus, prevalent local strains, southern China

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Avian infectious bronchitis (IB), an acute, highly contagious disease, is caused by avian coronavirus infectious bronchitis virus (IBV), which results in severe economic losses to the poultry industry worldwide. An increasing number of new serotypes or variants of IBV, which were caused by gene mutation and recombination, have been identified [4, 9, 16, 17, 19]. Vaccination is the main measure for the prevention and control of IB, but since there are multiple IBV serotypes and different serotypes of IBVs, vaccinations result in incomplete or no cross-protection against the various types of IBV. Hence, it is important to choose suitable vaccines for the control of IBV in the field.

Live, attenuated and inactivated vaccines are commonly used to control IB at the present time. However, the effects of inactivated vaccines are uncertain and inactivated vaccines often fail to induce strong cellular immunity [23]. Although the live, attenuated vaccines can cause virus mutation and recombination, they can induce effective humoral and cellular immune responses as well as local immunity [3, 21]. In young birds, live, attenuated vaccines are especially used to achieve early protection and also to prime future layers and breeders, which will be boosted with the inactivated vaccines later [21]. Therefore, the live, attenuated vaccines are of importance in the prevention and control of IB in the field.

The live vaccine H120 has been the most commonly used commercial vaccine in China for many years. Besides the H120 vaccine, the 4/91 live vaccine is also commonly used in China [15]. LDT3-A live vaccine is a relatively new indigenous vaccine,

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which has been issued by the official authority in China since 2011. In spite of extensive vaccination with these three vaccines in chickens in China, the disease is still epidemic in vaccinated flocks [6, 25–27]. In addition, IBV strains prevalent in a specific geographic region are distinct and unique, although some have a more general distribution [1, 2]. Hence, it is extremely critical to know whether these live, attenuated vaccines confer protection against the prevalent local strains of IBV in particular regions or countries and whether the birds respond promptly to vaccination.

Southern China is the major poultry production region in China, especially for yellow chickens. Despite the widespread application of multiple vaccines in chickens, IB continues to be a major problem [6, 11, 17]. Our previous studies and those from other groups showed that there were multiple genotypes and serotypes of IBV isolates in southern China; most IBV isolates occurred from mutations and dominant epidemic strains which have existed in recent years [6, 11, 12, 17, 18, 20]. In China, a few previous studies reported the protection conferred by attenuated vaccines [13, 14, 24]. However, little information is available about the protection conferred by the vaccines of H120, 4/91 and LDT3-A against IBV prevalent local strains in southern China. No previous study has focused on the comparison of protection conferred by these three live attenuated vaccines. In the present study, three Guangxi IBV isolates, belonging to the three dominant serotypes in southern China, were selected to evaluate the protection conferred by the vaccines H120, 4/91 and LDT3-A. This data will provide a useful reference for choosing suitable vaccines and developing new vaccines to prevent and control IB in China.

MATERIALS AND METHODS

IBV vaccines and challenge viruses

Three live, attenuated vaccines, H120 (Guangdong Dahuanong Animal Health Products Co., Ltd., Guangzhou, China), 4/91 (MSD Animal Health (Shanghai) Trading Co., Ltd., Shanghai, China) and LDT3-A (QYH Biotech Co., Ltd., Beijing, China) were purchased commercially. Each vaccine was reconstituted according to the manufacturer's instructions. According to our previous description [12, 20], H120 (Massachusetts serotype) and 4/91 (793B serotype) vaccines were designated as serotype 3 and serotype 5, respectively. The serotype of LDT3-A vaccine was different from Mass-type vaccines and other vaccines used in China [13]. IBV local isolates GX-YL5, GX-GL11079 and GX-NN09032 belonging to serotype 1, 2 and 5, respectively, were isolated from Guangxi, China and were currently the representative dominant serotypes in southern China [10, 18]. GX-YL5, GX-GL11079 and GX-NN09032 strains were propagated in 10-day-old specific pathogen free (SPF) chicken embryos and the 50% tracheal organ culture infection dose (TOC-ID_{50}) was determined as previously described for the quantification of the viruses [12].

Chickens

The SPF white leghorn chickens used in this study were hatched in our facilities (Guangxi University, Nanning, China) from fertile SPF eggs (Beijing Merial Vital Laboratory Animal Technology Co., Ltd., Beijing, China). The birds were reared in separate isolators following animal welfare guidelines and under strict biosecurity measures. Animal experiments were approved by the Animal Care & Welfare Committee of Guangxi University (approval number GXU2016-011) and were performed in accordance with animal ethics guidelines and approved protocols.

Experimental design

Seven-day-old SPF birds were randomly divided into five groups, which were named as H120 group, 4/91 group, LDT3-A group, non-vaccinated group and blank control group, respectively. There were 12 birds in the blank control group and 36 birds in the other four groups. Birds in the groups of H120, 4/91 and LDT3-A were vaccinated by eye and nose drop with H120, 4/91 and LDT3-A attenuated vaccines, respectively, at the manufacturer's recommended dose. Birds in the non-vaccinated group were treated with sterile phosphate-buffered saline in the same manner and those in the blank control group received nothing. Blood samples were collected from 10 birds in each group prior to inoculation (0 day) and at 7, 14 and 21 post-inoculation (dpi) of the vaccines for detection of IBV-specific antibody and quantity analysis of $CD4^+$ and $CD8^+$ T lymphocytes. At 21 dpi birds in the H120, 4/91, LDT3-A and non-vaccinated groups were divided randomly into three groups of 12 birds each and challenged via eye and nose drop with 0.2 m*l* of 10^4 TOC-ID_{50} of IBV GX-YL5, GX-GL11079 and GX-NN09032 strains, respectively. Birds in the blank control group received no challenge virus. Clinical signs were observed and recorded daily. At 5 days post-challenge (dpc), the birds in each group were humanely killed and necropsied. The trachea and kidney samples were collected from each bird aseptically for the detection of viral loads. The dead birds were also necropsied to confirm the infection of IBV.

Detection of IBV-specific antibody

A portion of the blood samples collected above were used to detect the IBV-specific antibody using a commercial enzyme-linked immunosorbent assay (ELISA) kit (IDEXX Laboratory, Inc., Westbrook, ME, U.S.A.) following the manufacturer's instructions.

Quantification of $CD4^+$ and $CD8^+$ T lymphocytes in peripheral blood

Peripheral blood mononuclear cells were isolated from another portion of the blood samples collected above and then used to analyze the percentages of the $CD4^+$ and $CD8^+$ T lymphocytes by flow cytometry. 1×10^6 cells were stained with Mouse Anti-Chicken CD4-PE and CD8a-FITC antibodies (Wuhan AmyJet Scientific Inc., Wuhan, China). All staining reactions were conducted following the manufacturers' protocols. Then the fluorescence positive cells were analyzed by a BD Accuri™ C6 Flow Cytometer (Becton, Dickinson and Co., NJ, U.S.A.).

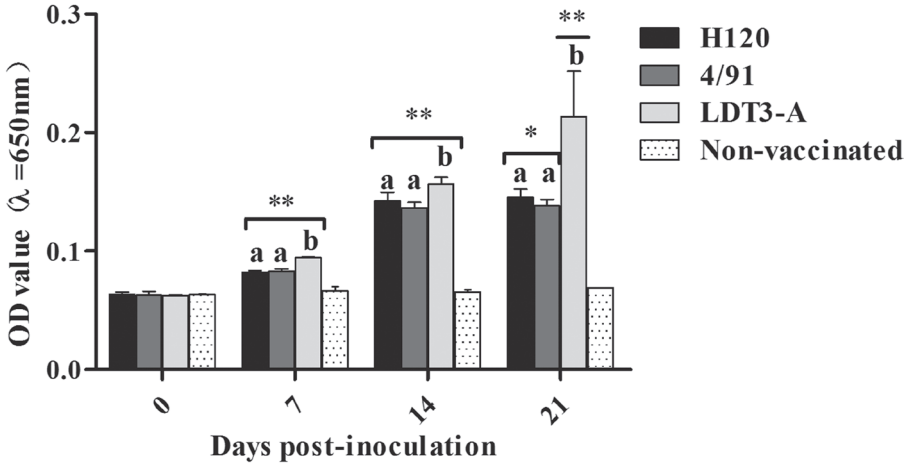

Fig. 1. IBV-specific antibody levels in the serum of vaccinated birds at 0, 7, 14 and 21 dpi and measured by indirect ELISA. Optical densities were read at 650 nm. Values are expressed as mean optical density ± standard deviation (mean ± SD). **The IBV-specific antibody levels of vaccinated groups significantly (P<0.01) higher than those of non-vaccinated group. *The antibody levels of vaccinated groups significantly (P<0.05) higher than those of non-vaccinated group. The different letters indicate significant difference between vaccinated groups (P<0.05). The same letters indicate no significant difference between vaccinated groups (P>0.05). n=10/group.

Detection of viral loads in trachea and kidney

Viral RNA was extracted from trachea and kidney samples of each bird at 5 dpc using EasyPure RNA Purification Kit (TransGen Biotech, Beijing, China). First-strand cDNA was synthesized using random hexamers (TaKaRa, Tokyo, Japan). The IBV viral loads in trachea and kidney samples were detected by quantitative reverse transcriptase polymerase chain reaction (qRT-PCR) targeting the nucleocapsid (*N*) gene according to our previous description [8].

Statistical analysis

All data were shown as mean values ± standard error (mean ± SE). Statistical analysis was performed using SPSS version 18.0 (SPSS Inc., Chicago, IL, U.S.A.), and significant differences (P<0.05) were determined by the Student's *t*-test. *P* values less than 0.05 (P<0.05) were regarded as significant and those less than 0.01 (P<0.01) were regarded as highly significant.

RESULTS

Antibody responses to IBV following vaccination

The birds in all the three vaccinated groups produced antibodies against IBV at 7dpi and the antibody levels continued to rise from 7 to 21 dpi, especially in the LDT3-A group (Fig. 1). The antibody levels of vaccinated groups were significantly higher (P<0.05 or P<0.01) than those of non-vaccinated groups from 7 to 21 dpi. At 21 dpi, the LDT3-A group showed the highest antibody level (P<0.01), while the H120 group showed a medium level and the 4/91 group presented the lowest level (P<0.05).

$CD4^+$ and $CD8^+$ T lymphocytes in peripheral blood of vaccinated chickens

The percentages of $CD4^+$ and $CD8^+$ T lymphocytes in all the three vaccinated groups showed different upward trends and were significantly higher (P<0.05 or P<0.01) than those of the non-vaccinated groups within 21 dpi (Fig. 2). At 7 dpi, the 4/91 and H120 groups showed the highest $CD4^+$ and $CD8^+$ T lymphocyte percentages, respectively. At 14 dpi, the H120 and 4/91 groups had the highest (P<0.05) $CD4^+$ and $CD8^+$ T lymphocyte percentages, respectively. At 21 dpi, the percentages of $CD4^+$ and $CD8^+$ T lymphocytes in the 4/91 group were the highest, and those in the H120 group were the lowest.

Clinical signs and gross lesions in birds challenged with IBV

At 2–5 dpc, there were birds in the vaccinated groups exhibited gasping, coughing, sneezing, tracheal rale and nasal discharge and some looked depressed with anorexia, ruffled feathers, dry claw and wet droppings. Some even exhibited severe respiratory distress and eventually died. And all the birds in the non-vaccinated group showed much more severe clinical signs than those in the vaccinated groups. In the necropsy at 5 dpc, the kidneys of the birds challenged with GX-YL5 strain were swollen and pale, and showed a white sludge of urate deposition, and some birds showed trachitis with exudates. The birds challenged with GX-GL11079 and GX-NN09032 strains exhibited more exudates in the trachea. No clinical signs or gross lesions were observed in any birds in the blank control group.

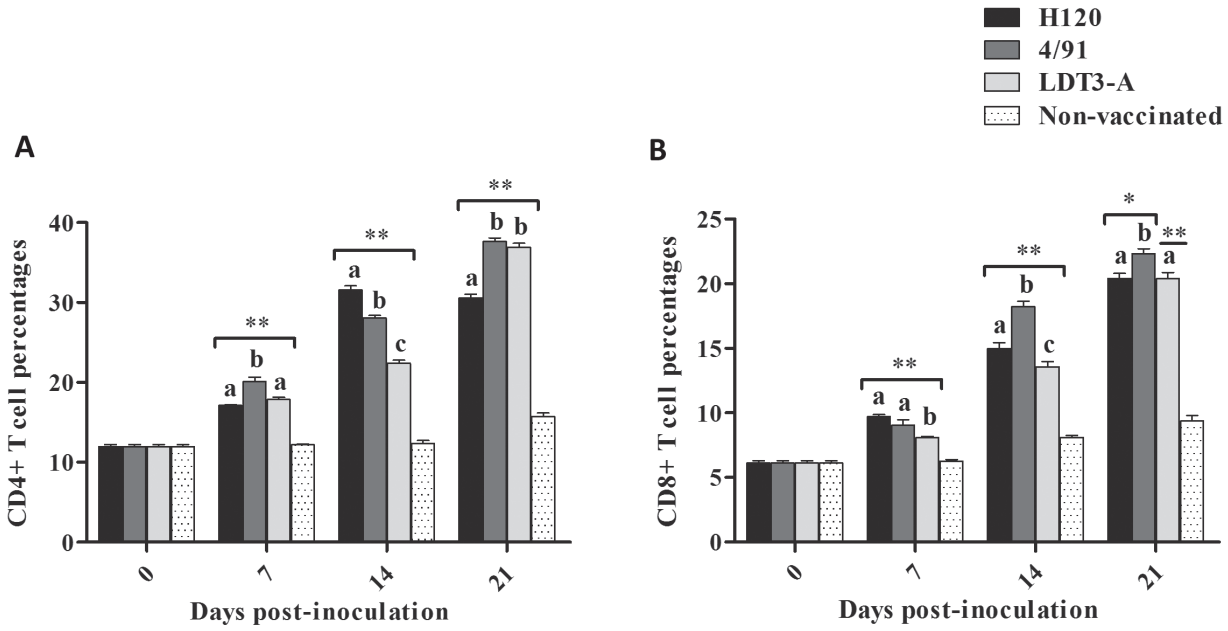

Fig. 2. Percentages of peripheral $CD4^+$ and $CD8^+$ T lymphocytes in vaccinated chickens. Peripheral blood mononuclear cells were collected at 0, 7, 14 and 21 dpi and analyzed by flow cytometry (A) $CD4^+$ T lymphocyte; (B) $CD8^+$ T lymphocyte. Error bars indicate the means ± SE per group. **The $CD4^+$ and $CD8^+$ T lymphocyte percentages of vaccinated groups significantly (P<0.01) higher than those of non-vaccinated group. *The $CD4^+$ and $CD8^+$ T lymphocyte percentages of vaccinated groups significantly (P<0.05) higher than those of non-vaccinated group. The different letters indicate significant difference between vaccinated groups (P<0.05). The same letters indicate no significant difference between vaccinated groups (P>0.05). n=10/group.

Table 1. Mortality, morbidity and protection rates of different vaccinated groups challenged with GX-YL5, GX-GL11079 and GX-NN09032 strains of IBV

Groups[a)] (serotype)	Challenge strains[b)] (serotype)	Mortality[c)] (%)	Morbidity[d)] (%)	Protection rates[e)] (%)
H120 (serotype 3)	GX-YL5 (serotype 1)	41.7 (5/12)	58.3 (7/12)	41.7
	GX-GL11079 (serotype 2)	16.7 (2/12)	41.7 (5/12)	58.3
	GX-NN09032 (serotype 5)	0 (0/12)	41.7 (5/12)	58.3
4/91 (serotype 5)	GX-YL5 (serotype 1)	0 (0/12)	16.7 (2/12)	83.7
	GX-GL11079 (serotype 2)	0 (0/12)	25.0 (3/12)	75.0
	GX-NN09032 (serotype 5)	0 (0/12)	16.7 (2/12)	83.7
LDT3-A (unknown)	GX-YL5 (serotype 1)	8.3 (1/12)	33.3 (4/12)	66.7
	GX-GL11079 (serotype 2)	0 (0/12)	25.0 (3/12)	75.0
	GX-NN09032 (serotype 5)	0 (0/12)	25.0 (3/12)	75.0
Non-vaccinated	GX-YL5 (serotype 1)	58.3 (7/12)	100 (12/12)	0
	GX-GL11079 (serotype 2)	25.0 (3/12)	100 (12/12)	0
	GX-NN09032 (serotype 5)	0 (0/12)	100 (12/12)	0
Blank control	-	0 (0/12)	0 (0/12)	-

a) Vaccination at 7 days of age. b) Challenge at 21 dpi. c) Mortality was determined by dividing the number of dead chickens by the total number of chickens. d) Morbidity was determined by dividing the number of affected chickens by the total number of chickens. e) Protection rate was determined by dividing the number of unaffected chickens by the total number of chickens.

The immune protection rates of each group against the challenge

The morbidity, mortality and protective rate of the experiment groups at 5 dpc were summarized in Table 1. The results showed that the protection rates of H120, 4/91 and LDT3-A vaccines against the infection of GX-YL5, GX-GL11079 and GX-NN09032 strains were 41.7–58.3%, 75.0–83.7% and 66.7–75.0%, respectively. The protection rate of the 4/91 group against the challenge of GX-NN09032 strain was the highest (83.7%). As expected, no protection was observed in the non-vaccinated group. No morbidity and mortality were observed in any birds in the blank control group.

The viral loads in the trachea and kidney of birds challenged with IBV

The viral loads in trachea and kidney of vaccinated groups were significantly (P<0.01) lower than those of the non-vaccinated

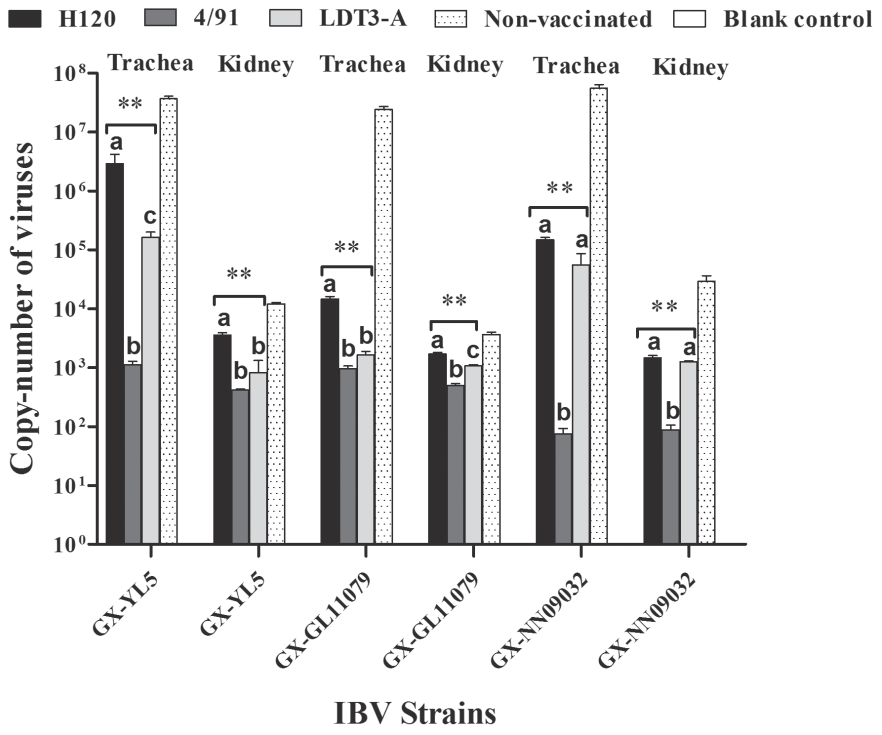

Fig. 3. Viral loads in trachea and kidney of challenged chickens at 5 dpc. The birds were vaccinated at 7 days of age with H120, 4/91 and LDT3-A vaccines, respectively and challenge with IBV GX-YL5, GX-GL11079 and GX-NN09032 strains, respectively at 21 dpi. IBV was detected by qRT-PCR. Data are expressed mean copy number ± SE. **The viral loads of vaccinated groups significantly (P<0.01) higher than those of non-vaccinated group. *The viral loads of vaccinated groups significantly (P<0.05) higher than those of non-vaccinated group. The different letters indicate significant difference between vaccinated groups (P<0.05). The same letters indicate no significant difference between vaccinated groups (P>0.05). n=12/group.

group at 5 dpc (Fig. 3). In all vaccinated groups, the viral loads in the trachea were higher than those in the kidney. The viral loads in the trachea and kidney of the vaccinated groups at every time points showed the same pattern, that was the H120 group was higher than that of the LDT3-A group and the LDT3-A group was higher than that of the 4/91 group. The viral loads in trachea and kidney of the 4/91 group challenged with the same serotype strain GX-NN09032 maintained a low level of less than 10^2 copies/μl and were significantly lower than those of the H120 group and the LDT3-A group at 5 dpc. None of the viruses was detected in the trachea or kidney samples from the blank control group.

DISCUSSION

Live attenuated vaccines play a key role in the control of IB, but limited or no cross-protection confers between serotypes of IBVs. Therefore, it is extremely critical to choose the appropriate IBV vaccines in practice. Little is known about the protection conferred by the commonly used live attenuated vaccines H120, 4/91 and LDT3-A against IBV prevalent local strains in southern China. In the present study, significantly higher antibody levels and percentages of $CD4^+$, $CD8^+$ T lymphocytes in vaccinated groups compared to the non-vaccinated groups within 21 dpi indicated that the vaccines H120, 4/91 and LDT3-A could stimulate the immunized chickens to produce humoral and cellular immunity that potentially resist or eliminate the infection of IBV. However, at 5 dpc, the protection rates of H120, 4/91 and LDT3-A vaccines against the challenges of the three prevalent IBV isolates were 41.7–58.3%, 75–83.7% and 66.7–75.0%, respectively. Hence, none of the three attenuated vaccines could provide complete protection against all the current prevalent isolates, but vaccine 4/91 offered the best immune protection of the three and vaccine LDT3-A had better protection than the H120 vaccine.

A previous report showed that the best protection against challenge is achieved by a vaccine containing a homologous strain [7]. In this study, the attenuated vaccine H120 (serotype 3) provided poor protection against IBV GX-YL5 (serotype 1), GX-GL11079 (serotype 2) and GX-NN09032 (serotype 5) strains with the low protection rate of 41.7, 58.3 and 58.3%, respectively. The vaccine 4/91 (serotype 5) has provided lower protection rate against the challenge with GX-GL11079 (serotype 2, 75.0%) compared to GX-NN09032 (serotype 5, 83.7%). Interestingly, the vaccine 4/91 (serotype 5) provided the same protection rate (83.7%) against the challenge with isolate GX-YL5 (serotype 1) and GX-NN09032 (serotype 5), but the viral loads of trachea and kidney from GX-NN09032 challenged birds were always lower than those from GX-YL5 challenged birds. Therefore, our data indicated that the vaccine 4/91 had a greater ability to inhibit the replication and reduce the shedding of a homologous serotype virus than a

heterologous serotype virus; the vaccine H120 provided poor protection against the heterologous serotype viruses. These findings support the necessity of developing new vaccines whose antigenicity match the antigenic profile of current prevalent IBV variants in the region.

According to the requirements in China, the clinical protection offered by a compliant IB vaccine must be not less than 80% against a virulent challenge [13]. Previous studies demonstrated that the H120 vaccine couldn't provide sufficient protection against the field isolates in China [6, 13–15, 24]. The latest report showed that a novel recombinant virulent IBV strain was isolated from an H120- and 4/91-IBV-vaccinated flock in China [27]. In our study, the attenuated vaccine 4/91 could not provide complete protection against IBV GX-NN09032 strain even though they shared the same serotype [20]. The reason for this may be that a single vaccine dose of a monovalent vaccine could not provide sufficient immunity with complete protection to birds. Therefore, in an attempt to provide broader protection to chickens, the prime-boost vaccination program in combination with application of two different IBV vaccines ("live + live" or "live + inactivated") should be performed for the birds in southern China. In other countries, it has also been showed that vaccination with two antigenically distinct live-attenuated vaccines can achieved a broad cross-protection against multiple different IBV types [5, 21, 22]. Further investigation will be necessary to determine which combination of H120, 4/91 and LDT3-A vaccines could provide the most effective protection against the prevalent local strains in southern China.

In summary, the present study indicated that the commercial attenuated vaccines H120, 4/91 and LDT3-A could not provide complete protection against the prevalent isolates of IBV in southern China. The 4/91 vaccine demonstrated the best immune protection against the IBV strains prevalent in southern China, while LDT3-A vaccine was less protective and H120 vaccine provided the least protection. The results also stressed the necessity of developing new vaccines containing a homologous serotype strain and the prime-boost vaccination schedules in combination with application of two different IBV vaccine strains.

CONFLICT OF INTEREST. The authors declare no conflicts of interest.

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