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Research Article

Development and immunoprotection assessment of novel vaccines for avian infectious bronchitis virus

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ABSTRACT

Infectious bronchitis (IB), a highly contagious acute respiratory disease affecting avian species, poses significant challenges to poultry production. The causative agent, infectious bronchitis virus (IBV), exhibits a high mutation rate, leading to limited cross-protection by existing vaccines. This necessitates the development of novel vaccines. This study, based on preliminary investigations conducted by our research team, identified six potential strains (PYG QX1, ZQF QX2, FQH QX3, LYZ QX4, XXX QX5, and CSL strains) for vaccine development. Previous pathogenicity test and serum cross-neutralization experiments conducted in this study have demonstrated that the FQH QX3 strain exhibited the weakest pathogenicity and the broadest spectrum of serum neutralization, while the CSL strain showed the highest pathogenicity and was the most challenging to neutralize, posing the greatest difficulty in prevention and control. Subsequently, we constructed and rescued recombinant vaccine candidates, H120-FQH QX3, and H120-CSL, expressing the S1 and N proteins of the FQH QX3 and CSL strains, respectively. Immunization protection experiments indicated that the H120-CSL recombinant vaccine candidate exhibited the most effective immune protection, making it a promising candidate for further study and evaluation as a recombinant vaccine. The *S1* and *N* genes of the CSL strain demonstrated strong immunogenicity, making them potential candidate antigen genes for future vaccine development.

INTRODUCTION

Infectious bronchitis (IB) is an acute and highly contagious respiratory disease caused by the infectious bronchitis virus (IBV). IBV primarily targets the respiratory, digestive, and reproductive systems of chickens (Cavanagh et al., 2005). Infected poultry can spread the virus into the environment through various vectors such as drinking water, feed, and feces, making chickens of different breeds and ages susceptible to IBV infection. Infected chickens exhibit stunted growth and are prone to secondary bacterial and mycoplasma infections (Cavanagh, 2007). Moreover, IBV can profoundly impact the reproductive system of hens, resulting in reduced egg production and diminished egg quality.

IBV belongs to genus *Gamma coronavirus* within the *Nidovirales* order, subfamily *Orthocoronavirinae*, and family *Coronaviridae*. IBV is a single-stranded, positive-sense enveloped RNA virus which contains approximately 27.6 Kb genome sequence. The 5′ end features a cap structure,

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while the 3′ end possesses a polyadenylate tail structure known as poly(A). There are over 10 discernible open reading frames (ORFs) within IBV (Jackwood et al., 2012). The length of the IBV ORF sequences varies among different genotypes. The 7.3 Kb ORFs located at the 3′ end of the viral genome primarily encode four structural proteins of IBV (Jackwood, 2012; Legnardi et al., 2020), including the spike protein (S protein), envelope protein (E protein), membrane protein (M protein), and nucleocapsid protein (N protein). Among these, the S protein is situated on the virus envelope's surface, forming surface filaments involved in receptor recognition, binding, and fusion processes between the virus and host cells. It plays a pivotal role in the virus's selection of colonization sites and immunogenicity (Jackwood et al., 2001). The S protein, being the primary glycosylated protein that induces neutralizing antibodies, is transcribed by host cell lysase and cleaved into N-terminal S1 and C-terminal S2 subunits (Promkuntod et al., 2015). The S1 subunit is responsible for virus attachment and houses the primary neutralizing epitope. IBV-neutralizing antibodies predominantly target the highly variable S1 subunit, with three antigenic sites corresponding to amino acid residues at positions 24–61, 132–149, and 291–398 of the S1 subunit, all of which are conformation-dependent neutralizing epitopes (Kant et al., 1992). Furthermore, the gene encoding the S1 subunit exhibits substantial variability, contributing significantly to the numerous genotypes and serotypes observed in IBV (Cook et al., 2012). The N protein is a highly conserved structural protein with significant homology across different IBV strains (Yu et al., 2010). During virus replication, the N protein is extensively synthesized, promoting the synthesis of viral mRNA (Masters, 2006). Additionally, it has been reported that the N protein is the most abundant viral-derived protein expressed during IBV infection and is involved in inducing high titer cross-reactivity antibodies and robust T cell responses (Tang et al., 2008).

In 1996, a new genotype of IBV virus was first isolated from the glandular stomach of chickens in Shandong Province, China. The QX strain primarily glandular stomach tissue and was consequently named the QX-like type in China (Zhao et al., 2017). Subsequent research revealed that the QX strain exhibits broad tissue tropism and can cause lesions in the trachea, kidneys, and reproductive system of chickens. Since 1995, the QX-like type has been the predominant IBV genotype in China. From 2013 to 2021, the majority of IBV isolates in China were QX-like strains, with 50%–70% being of this type during that period (Han et al., 2011; Lian et al., 2021; Wu et al., 2022). After approximately three decades of transmission and epidemic spread, the QX strain has established persistent distribution and prevalence in numerous countries and regions including China, Asia, Europe, and Africa; QX-like type as one of the most important IBV genotypes globally while inflicting substantial economic losses on the global poultry industry (Bande et al., 2017; Franzo et al., 2017).

Based on our previous IBV molecular epidemiological investigation, through *S1* gene sequencing analysis, we found that five QX strains (PYG QX1, ZQF QX2, FQH QX3, LYZ QX4, and XXX QX5) and one CSL strain were repeatedly isolated from different detection time periods in various regions. These six strains have persistently circulated in poultry farms that have been vaccinated in multiple provinces, causing serious harm to the farms' profits and biosecurity control. Therefore, it is necessary to enhance research, prevention, and control of these 6 strains.

In this study, we conducted serum neutralization experiments using six strains (PYG QX1, ZQF QX2, FQH QX3, LYZ QX4, XXX QX5, and CSL strains) identified from previous work. According to the results of the serum cross-neutralization tests, it was found that among the six candidate strains, the antiserum from the FQH QX3 strain exhibited the broadest spectrum of neutralization, while sera from vaccines were the most difficult to neutralize the CSL strain. Consequently, we used the H120 strain as the vector which selected these two strains as the donor strains for antigen gene to construct recombinant vaccine candidates. The relative protection effects of these two recombinant vaccine candidates were evaluated in chicken models challenged with GY and CSL strains, with H120-CSL recombinant vaccine showing the highest protective efficacy. In summary, our work offers novel insights for the prevention and control of the currently prevalent IBV strains.

RESULTS

Serum neutralization characteristics of six candidate strains

The neutralizing characteristics of monospecific antisera from candidate strains (Supplementary Table S1) toward seven genotypic strains (Supplementary Table S2) were assessed in SPF chicken embryos using the constant virus with diluted serum method. The monospecific antisera of FQH QX3 and LYZ QX4 strains showed significant neutralizing effects. The antiserum from FQH QX3 strain effectively neutralized QX, LDT3, and TC07-2 strains, but had limited efficacy against Mass vaccine strains. On the other hand, the serum from LYZ QX4 strain effectively neutralized QX, LDT3, TW, and Mass vaccine strains, with lower activity against 4/91, HN08, and TC07-2 strains. Comparing average neutralization titers, the order of potency was FQH QX3 > LYZ QX4 > XXX QX5 > CSL > PYG QX1 > ZQF QX2. FQH QX3 antiserum had the highest average neutralization titer of 93.3 against seven genotypic strains, indicating broad spectrum neutralizing antigens (Table 1).

Among the seven genotypic strains, the antisera of the vaccine strains can effectively neutralize the LYZ QX4 strain, particularly the QX, 4/91, LDT3, and Mass types, with titers ≥128. In contrast, the FQH QX3 strain is effectively neutralized by serum from the QX and TC07-2 types, while antisera from other genotypes shows limited neutralizing effects. The HN08 antisera demonstrates a neutralizing titer of 128 against the CSL strain, indicating effective neutralization. However, other genotypes exhibit poor neutralization against the CSL strain (Table 2).

The cross-neutralization between these candidate strains were further evaluated. The average neutralizing titer of the FQH QX3 strain antiserum is the highest (107.33), demonstrating excellent neutralizing effects on five out of six screened strains, with a relatively weaker effect against the XXX QX5 strain. In contrast, the serum neutralizing titers against the XXX QX5 and CSL strains are notably higher for the CSL strain. However, they show lower neutralizing titers against the other screened strains. Additionally, the result also indicates that homologous serum can better neutralize homologous antigens (Table 3).

The antigenic correlation value was calculated based on heterologous and homologous serum neutralization results. The antigen correlation coefficients between PYG QX1 and ZQF QX2, FQH QX3, LYZ QX4, XXX QX5, and CSL strains were 45%, 99%, 35%, 60%, and 30%, respectively. PYG QX1 shares the same serum subtype (> 80%) with FQH QX3 and differs in subtype (25%–80%) from ZQF QX2, LYZ QX4, XXX QX5, and

Table 1
Neutralization results of the monospecific antisera of the six candidate strains with the seven genotypic strains.

Antigen	Antiserum					
	PYG QX1	ZQF QX2	FQH QX3	LYZ QX4	XXX QX5	CSL
QX	32	85	>128	>128	128	102
4/91	54	32	95	38	49	64
LDT3	28	31	>128	>128	>128	>128
HN08	32	35	89	50	74	>128
TW	31	<8	64	>128	29	41
TC07-2	107	128	>128	32	93	22
Mass	128	32	21	128	25	19
Average potency	58.9	50.1	93.3	90.3	75.1	72.0

Note: A serum with a neutralization titer > 128 can protect 50% of chicken embryos from disease after being diluted by 2^8 in a double gradient dilution. A titer < 8 indicates that the serum, when neutralized in equal amounts with the antigen after an 8-fold dilution, fails to protect 50% of chicken embryos. A serum with a neutralization titer ≥ 128 effectively protects 50% of chicken embryos. A titer ≥ 64 provides better protection, while a titer ≤ 64 indicates poor neutralization and protection efficacy.

Table 2
Neutralization results of the monospecific antisera of the seven genotypic strains with the six candidate strains.

Antigen	Antiserum							Average potency
	QX	4/91	LDT3	HN08	TW	TC07-2	Mass	
PYG QX1	19	107	>128	45	40	>128	49	79.3
ZQF QX2	32	32	>128	16	28	32	126	61.0
FQH QX3	95	45	40	14	17	75	32	50.2
LYZ QX4	>128	128	>128	75	81	102	>128	114.8
XXX QX5	45	>128	128	86	28	89	102	96.3
CSL	16	47	32	128	64	12	35	47.1

Note: As in Table 1.

Table 3
Cross-neutralization reactions among IBV candidate strains (serum-neutralization titers).

Antigen	Antiserum					
	PYG QX1	ZQF QX2	FQH QX3	LYZ QX4	XXX QX5	CSL
PYG QX1	128	26	126	47	128	45
ZQF QX2	>128	>128	>128	128	16	8
FQH QX3	125	81	125	63	128	20
LYZ QX4	32	64	126	95	11	64
XXX QX5	46	39	50	10	>128	86
CSL	32	38	89	50	74	>128
Average potency	81.8	62.7	107.3	65.5	80.8	58.5

Note: As in Table 1.

CSL. For ZQF QX2, the coefficients with FQH QX3, LYZ QX4, XXX QX5, and CSL were 80%, 82%, 20%, and 14%, respectively, indicating shared serum subtype ($\geq$ 80%) with FQH QX3 and LYZ QX4 but different serotypes ($<$ 25%) from XXX QX5 and CSL. FQH QX3 has coefficients of 82%, 63%, and 33% with LYZ QX4, XXX QX5, and CSL, sharing the same serum subtype ($>$ 80%) with LYZ QX4 but differing in subtypes (25%–80%) from XXX QX5 and CSL. LYZ QX4 shows 10% and 51% coefficients with XXX QX5 and CSL, indicating different serotypes and subtypes from XXX QX5 and CSL. The correlation coefficient between XXX QX5 and CSL is 62%, implying shared serum but different subtypes (Table 4).

Construction of recombinant vaccines

FQH QX3 strain and CSL strain were chosen as the antigen donor strains for the development of genetic engineering vaccines. Leveraging the reverse genetic system and operational techniques were established for the H120 strain within our laboratory. The viral recombination and rescue processes were shown in Fig. 1. We successfully engineered and rescued two candidate strains: H120-FQH QX3 and H120-CSL recombinant vaccines. The genetic stability of the two recombinant strains was confirmed by RT-PCR amplification and sequencing analysis. Analysis of sequencing results showed that the nucleotide and amino acid sequence similarities of *S1* gene and *N* gene of recombinant vaccine candidates and donor strains were 100% (Fig. 2A). The expression of N protein was successfully detected in the lysates of CK cells infected with recombinant strains H120-FQH QX3 and H120-CSL with a molecular weight of approximately 45 kDa, as identified by Western blot (results for S1 protein are not shown here because no relevant antibody to IBV S1 protein is available) (Fig. 2B). The growth characteristics of the two recombinant vaccine candidates and the H120 strain were evaluated in SPF chicken embryos. As shown in Fig. 2C, both recombinant strains grew at a lower rate than the H120 strain at all time points, with the H120-FQH QX3 strain being the slowest, however, both recombinant vaccine candidates and the H120 strain reached maximal tit at 36 h post-infection (hpi).

Table 4
Antigen correlation among candidate strains (%).

Strain	PYG QX1	ZQF QX2	FQH QX3	LYZ QX4	XXX QX5	CSL
PYG QX1	100					
ZQF QX2	45	100				
FQH QX3	99	80	100			
LYZ QX4	35	82	82	100		
XXX QX5	60	20	63	10	100	
CSL	30	14	33	51	62	100

Safety evaluation of recombinant vaccine candidates

We first determined the safety of those two candidate vaccines in one-day-old chicken models by administering the vaccine at a 10-fold dose (Supplementary Table S3). Following immunization and prior to autopsy, none of the chickens in the immunized groups displayed symptoms such as respiratory distress, tracheal rales, depression, or impaired movement, and there were no fatalities. During autopsy, two chickens in the H120-FQH QX3 group and one in the H120-CSL group exhibited mild myogastritis symptoms in their stomachs (Fig. 3A). In contrast, the remaining chickens in the control group and the two immunized groups were asymptomatic, resulting in a 0% incidence rate (note that gastromyositis symptoms were not factored into the incidence rate calculation).

At 5 days post-vaccination (dpv), the outcomes of the tracheal cilia swing arrest test revealed that the mean scores of both recombinant vaccine candidate groups were elevated relative to the control group, albeit these variances did not reach statistical significance (Fig. 3B). Furthermore, at 10 dpv, no notable disparities in body weight were detected among the H120-FQH QX3 and H120-CSL immunized groups and the control group (Fig. 3C).

After 10 days post-immunization, five tissue samples from the trachea, lungs, kidneys, spleen, and duodenum were randomly selected from each group for histopathological examination, with three chickens per group analyzed. No pathological alterations, such as mucosal hyperplasia, inflammatory cell infiltration, submucosal edema, or villus shedding, were observed in the tissue sections of the immunized groups. These findings were consistent with the histopathological results of the control group. Therefore, it can be inferred that neither of the recombinant vaccine candidates caused any tissue damage in the chickens (Fig. 3D).

Evaluation of immune efficacy of recombinant vaccine candidates

The GY virulent strain, which shares the same serotype as the FQH QX3 strain (Supplementary Table S4 and S5), and CSL strain were selected to establish the challenging models in 14-day-old SPF chickens (Supplementary Table S6). The result revealed that the incidence rates of the GY strain in the three challenge dose groups ($10^{4.16}EID_{50}$, $10^{5.16}EID_{50}$, and $10^{6.16}EID_{50}$) were 90%, 90%, and 100%, respectively, with corresponding mortality rates of 10%, 40%, and 50%. For the CSL strain, the incidence rates in the two groups ($10^{5.0}EID_{50}$, $10^{6.0}EID_{50}$) were 80% and 90%, with a mortality rate of 10%. In contrast, the blank group showed 0% incidence and mortality rates. Based on autopsy results (Supplementary Table S8) and the requirements of the vaccine challenge protection test model, the challenge doses for the GY and CSL strains were determined to be $10^{4.16}EID_{50}$/0.1 mL and $10^{6.0}EID_{50}$/0.1 mL, respectively.

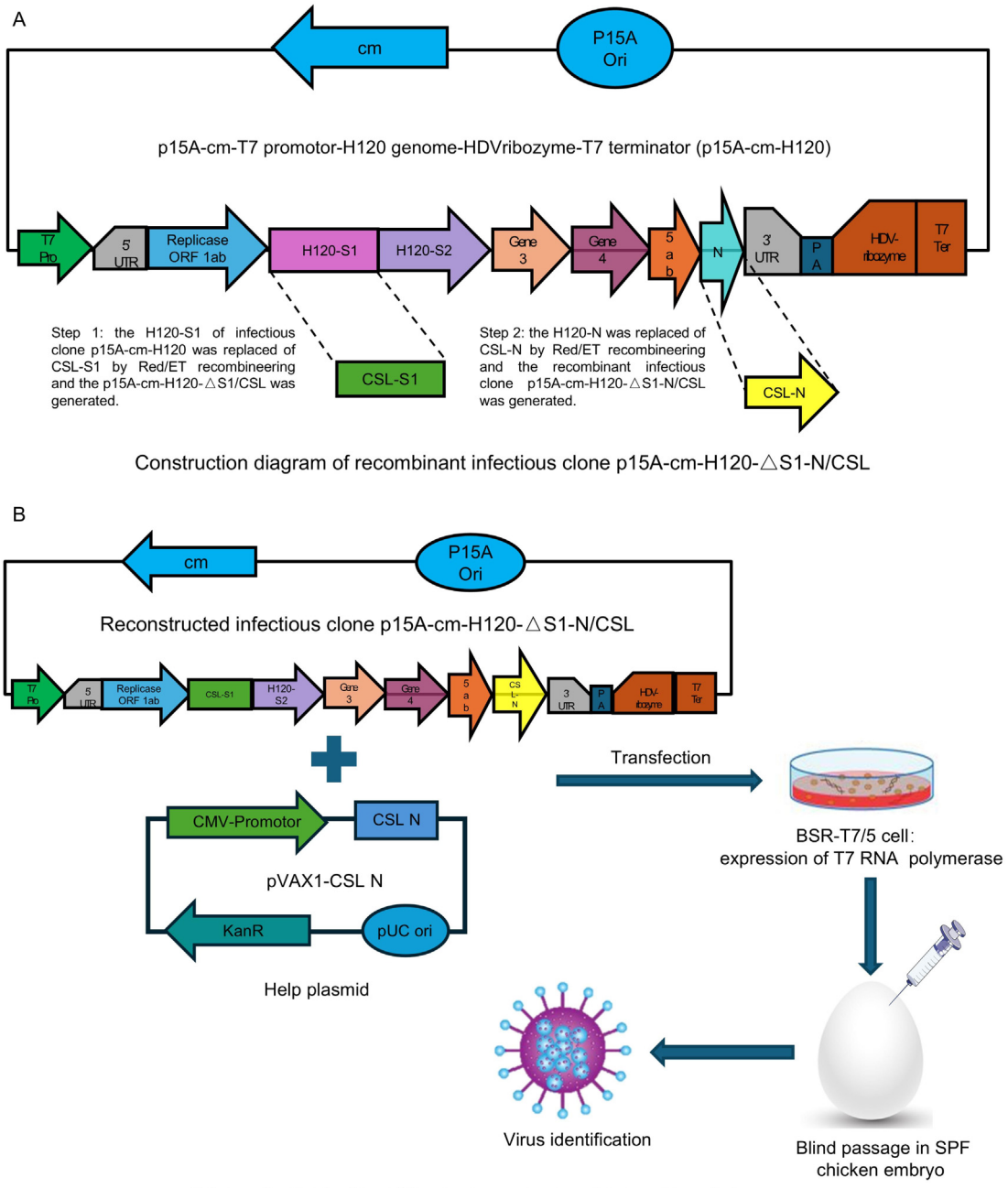
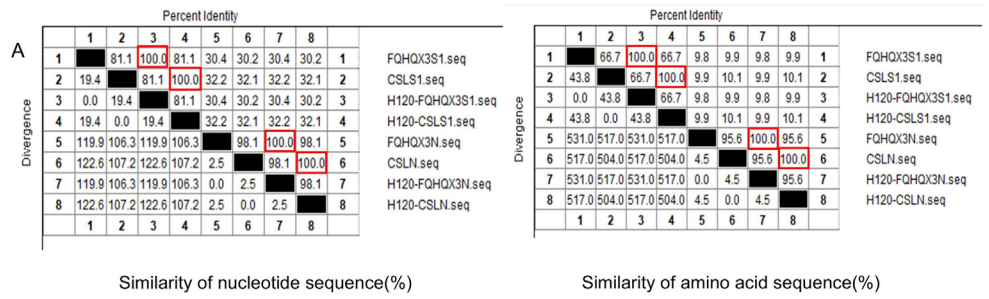
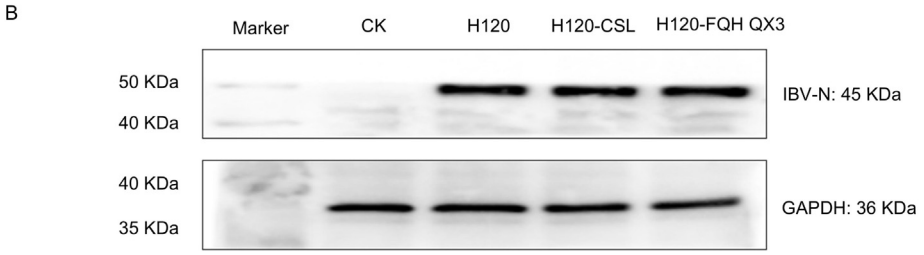


Fig. 1. Schematic diagram of the construction and rescue process of the vaccine candidate strain. **A** Construction diagram of recombinant infectious clone p15A-cm-H120-ΔS1-N/CSL. The construction process of the recombinant vaccine candidate H120-CSL strain was as follows: Gene replacement was carried out on the infectious clone p15A-cm-H120 using a two-step recombination method. The process of constructing the recombinant vaccine candidate H120-FQH QX3 strain is the same as that of the H120-CSL strain. **B** Rescue and identification of recombinant virus: The recombinant infectious clone p15A-cm-H120-ΔS1-N/CSL and auxiliary plasmid pVAX1-CSL N were co-transfected into BSR-T7/5 cells. Subsequently, the virus was blindly passaged through SPF chicken embryos for three generations at 48 hours post-transfection. Successful rescue of the virus was confirmed by RT-PCR analysis and observation of pathological changes in chicken embryos. The rescued virus was further propagated in chicken embryos, and its stability and antigen gene expression were identified using RT-PCR, Western-Blotting, etc. The suitable recombinant virus strain was selected and preserved for subsequent vaccine evaluation trials.

One-day-old chickens were administered with indicated dose of combined ND and IB commercial vaccine or recombinant vaccines at day one and challenged with GY and CSL strains at day 14 (Fig. 4A). At 6 dpv, chickens immunized with combined ND and IB commercial vaccine showed signs of delayed movement and depression. Subsequently, on the 8th, 9th, and 10th dpv, one chicken in commercial vaccine group challenged with CSL strain (Group B) succumbed each day. Additionally, at one day post-challenge (dpc) (15 days old), a weak chicken died in commercial vaccine group challenged with GY strain (Group A). By 4 dpc, one chicken died in the CSL strain challenge positive control group (Group H), while the remaining chickens showed delayed movement and mental depression without respiratory distress symptoms. The GY strain positive control group (Group G) exhibited open-mouth/neck breathing at 5 dpc, with one chicken succumbing at 6 dpc. Remarkably, no clinical symptoms were observed in the other groups.

At 13 dpv, the average weight gain of Group A and Group B, which were immunized with combined ND and IB commercial vaccine, was the lowest, with an average weight gain of 88.14 g and 85.67 g, respectively.





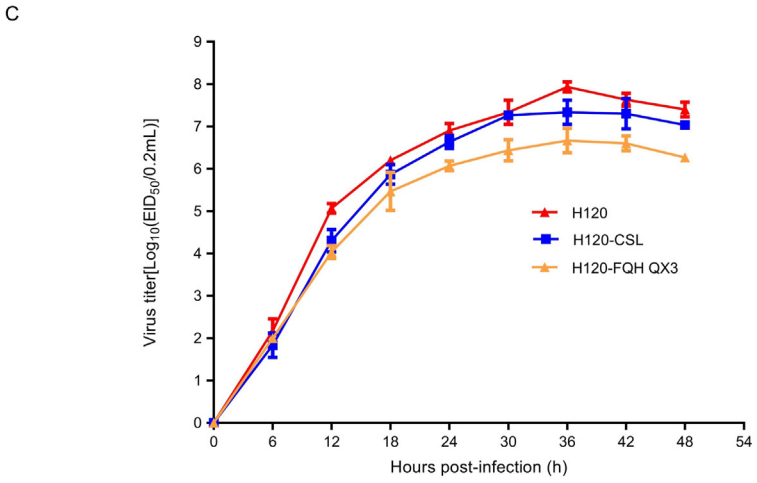


Fig. 2. Recombinant vaccine candidate strains virus rescue assay. Two recombinant strains, H120-FQH QX3 and H120-CSL, which were successfully rescued, were inoculated into 9-day-old SPF chicken embryos at a dose of 100 EID_{50}/0.1 mL respectively and blindly transmitted for 5 generations for the recombinant virus rescue assay. **A** Sequence analysis results of *S1* and *N* genes of two recombinant vaccine candidates and donor gene strains. The viral RNA of H120-FQH QX3 F5, H120-CSL F5, FQH QX3 and CSL were extracted, and the *S1* and *N* genes were amplified by RT-PCR, and the nucleotide and amino acid sequences were compared. **B** Western-blotting assay of two recombinant vaccine candidates and vector strain H120 N protein. H120-FQH QX3 F5, H120-CSL F5, and H120 were infected with CK cells, and the total proteins were harvested for Western-blotting assay 24 h after infection. **C** Growth curves of H120-FQH QX3 and H120-CSL and H120 on SPF chicken embryos. H120-FQH QX3 F5, H120-CSL F5 and H120 strains were inoculated into 10-day-old SPF chicken embryos via the allantoic cavity at a dose of 100 EID_{50}/embryo. The allantoic fluid was harvested at 6h, 12h, 18h, 24h, 30h, 36h, 42h and 48h after inoculation, and virus titers (EID_{50}/0.2mL) were measured by Reed-Muench method at different time points to plot the growth curves.

In contrast, the average weight gain of the other groups was significantly higher than the commercial vaccine group ($P < 0.001$) (Fig. 4B). These results suggest that the commercial vaccine negatively impacts chicken growth and weight gain.

At 10 dpc, chickens in the GY strain challenge group (Group A) had the lowest average weight gain, significantly lower than the two recombinant vaccine candidate strains ($P < 0.001$). In the CSL challenge group, the average weight gain of the three immune challenge groups (Group B, D and F) and the blank control group (Group I) was higher than that of the CSL strain challenge positive control group (Group H). Specifically, H120-FQH QX3 vaccine group challenged with CSL strain (Group D) showed a significantly higher average weight gain than Group H ($P < 0.05$), and H120-CSL vaccine group challenged with CSL strain (Group F) had a significantly higher average weight gain than Group H ($P < 0.01$). These results indicate that the recombinant vaccine candidate strains effectively protect chickens against infection by GY and CSL strain, while demonstrating no adverse impact on weight gain or growth performance post-immunization (Fig. 4C and D).

The viral load of vaccine strains at day five after administration were evaluated by quantitative real-time RT-PCR. The results showed that there was no significant difference in viral load between the H120-FQH QX3, H120-CSL, and the combined ND and IB commercial vaccine group in tissues such as lung, kidney, duodenum, proventriculus, and ventriculus at 5 dpv (Fig. 5A). However, the vaccine viral load of the H120-FQH

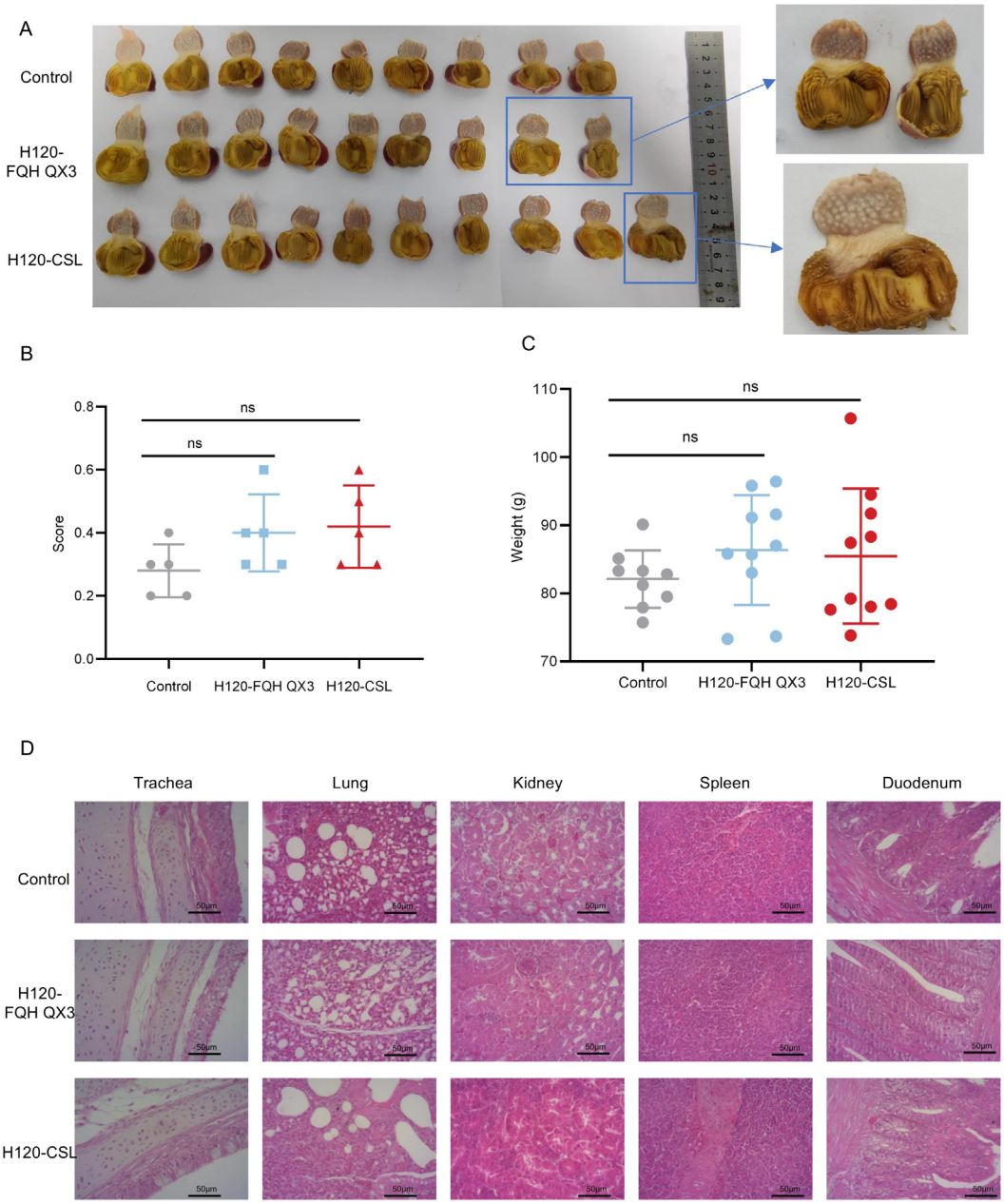


Fig. 3. Safety evaluation of recombinant vaccine candidates. One-day-old SPF chickens were randomly divided into two immunization groups and one control group, with 15 chickens in each group. At the first day of age, chickens in the immunization groups were nasally administered 10 times the dose of the recombinant vaccine ($10^{5.5}$ EID_{50}/0.1 mL/chicken), while chickens in the control group received normal saline. **A** The results of gizzard autopsy. The box marked shows mild gizzard erosion. **B** The score of ciliostasis in each group. If the average score of ciliostasis exceeds 2 points, it is considered as diseased, and if it is less than 2 points, it is considered as not diseased. **C** The weight status of each group. **D** The pathological sections of the trachea, lungs, kidneys, spleen, and duodenum in each group were observed under a 200 × microscope (200 ×). No abnormalities were found in all 5 tissues of the 3 groups. One-way analysis of variance (ANOVA) was used to identify the group differences, and the differences were of ciliostasis score and weight between three groups was not significant (ns means $P > 0.05$).

QX3 group colonized in the trachea was significantly higher than that of the H120-CSL and the commercial vaccine groups ($P < 0.01$). Comparison of viral load in different tissues for each vaccine revealed that the vaccine virus could colonize various tissues, with the highest viral load in the trachea and muscle stomach tissues, followed by glandular stomach and lung tissues, and the lowest viral load in the kidney and duodenum.

Following challenge with the GY strain, the viral load in the trachea, lung, duodenum, glandular stomach, and muscular stomach (chicken's gizzard-membrane) of chickens immunized with commercial vaccine, H120-FQH QX3 and H120-CSL groups (Groups A, C, E) were significantly lower than those in the GY-challenged control group (Group G) ($P < 0.001$). Similarly, after CSL strain was challenged, the same results were obtained of the three vaccinated-challenged groups (Groups B, D and F). The viral load in the trachea, glandular stomach and muscular stomach (chicken's gizzard-membrane) was significantly different (Fig. 5B and C).

Challenged with GY strain and CSL strain after three days, the viral load in the trachea and cloaca of the three vaccinated-challenged groups (Groups A, C, E) was significantly lower than that in the challenge control group (Group G) ($P < 0.001$), similar results were found in the three immunized groups (Groups B, D, and F) challenged with the CSL strain. While 10 dpc, the viral load in the genital cavity of the immune group and the challenge control had no significant difference (Fig. 5D and E).

A

Immunization, challenge procedures and grouping information of vaccine evaluation test

Group	Immune vaccines	Immunizing dose	Challenge strain	Challenge dose	Immune and challenge pathways	Quantity
A	Combined ND and IB commercial vaccine	One dose	GY	$10^{4.16}EID_{50}$/0.1 mL	Nasal drip	19
B	Combined ND and IB commercial vaccine	One dose	CSL	$10^{6.0}EID_{50}$/0.1 mL	Nasal drip	19
C	H120-FQH QX3	$10^{4.5}EID_{50}$/0.1 mL	GY	$10^{4.16}EID_{50}$/0.1 mL	Nasal drip	19
D	H120-FQH QX3	$10^{4.5}EID_{50}$/0.1 mL	CSL	$10^{6.0}EID_{50}$/0.1 mL	Nasal drip	19
E	H120-CSL	$10^{4.5}EID_{50}$/0.1 mL	GY	$10^{4.16}EID_{50}$/0.1 mL	Nasal drip	19
F	H120-CSL	$10^{4.5}EID_{50}$/0.1 mL	CSL	$10^{6.0}EID_{50}$/0.1 mL	Nasal drip	19
G	/	/	GY	$10^{4.16}EID_{50}$/0.1 mL	Nasal drip	16
H	/	/	CSL	$10^{6.0}EID_{50}$/0.1 mL	Nasal drip	16
I	Normal saline	0.1mL	Normal saline	0.1mL	Nasal drip	19

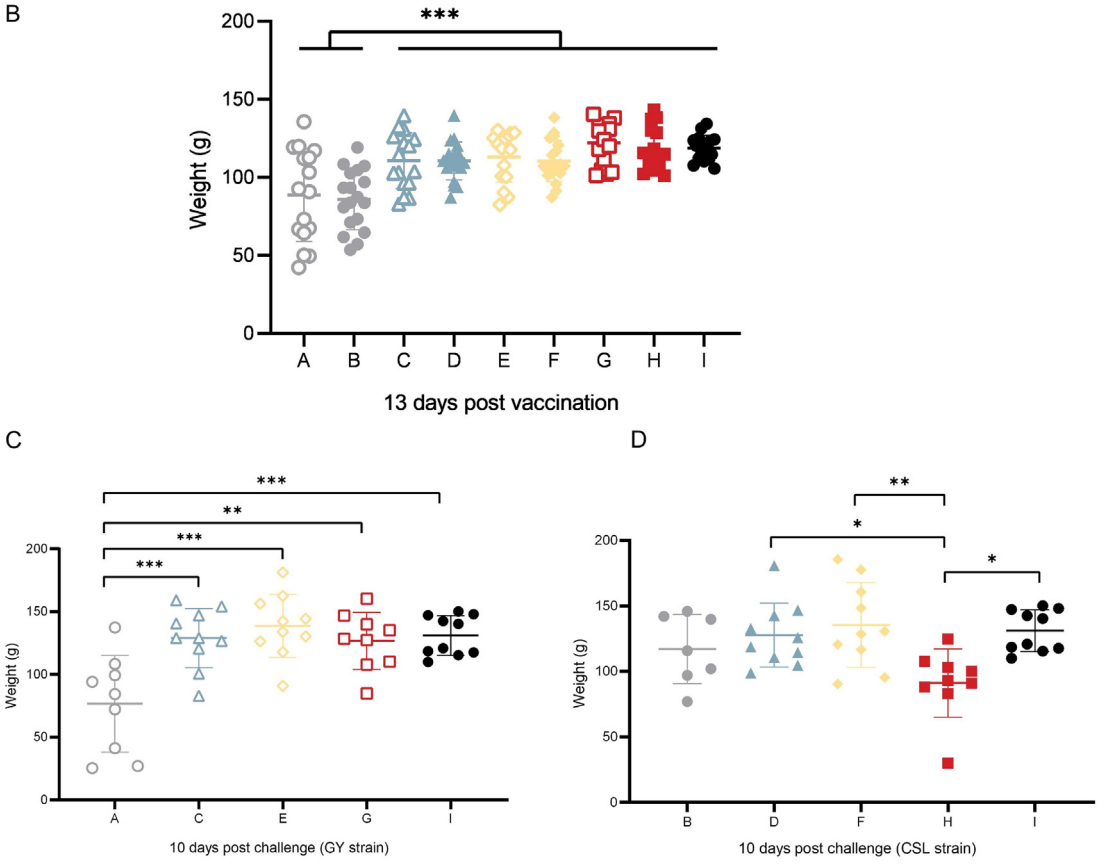


Fig. 4. Determination of body weight gain in chickens after vaccine immunization and virus challenge. One-day-old chickens in the immunization groups (Groups A-F) were intranasally vaccinated, subsequently challenged via the same route at 14 days of age (Groups A–H), and euthanized for necropsy 10 dpc. **A** Immunization, challenge procedures and grouping information of vaccine evaluation test. Combined ND and IB commercial vaccine are combined Newcastle disease (La Sota strain) and infectious bronchitis (H120 strain) live attenuated commercial vaccine. **B** Weight gain of chickens in each group at the day 13 post-vaccination. **C, D** Weight gain of chickens in each group at day 10 post-challenge with GY and CSL strains. One-way ANOVA was used to identify the differences in the weight impact and vaccine colonization amount in the tissues. * P <0.05, ** P <0.01, *** P <0.001.

Tracheal ciliostasis test score and lesion score of different tissues

To measure the effect of a virus on the tracheal mucosa, the ciliostasis test was conducted at 5 dpc. We found that all six vaccinated-challenged groups had tracheal ciliostasis scores below 1 point, indicating the absence of disease onset. In contrast, the two challenge control groups showed tracheal ciliostasis scores exceeding 2 points, indicative of disease onset. Importantly, there were a highly significant difference (P < 0.001) in ciliostasis scores between the vaccinated-challenged groups and the blank control group (Fig. 6A).

At 7 dpv, the IBV antibody positivity rates in the three vaccinated-challenged groups were all 0%. However, at 13 dpv (prior to the virus challenged), the positive rate of the combined ND and IB commercial vaccine group was 19%, while the H120-FQH QX3 and H120-CSL groups exhibited positive rates of 13% and 0%, respectively (Table 5).

At 10 dpc, all chickens were euthanized and dissected to record gross pathological characters (Table 6). Lesions of varying degrees were observed in the air sacs, kidneys, glandular stomachs, and muscular stomachs, and were scored accordingly (Fig, 6B and E). Mild symptoms of myogastritis were observed in all experimental groups (Groups A to H).

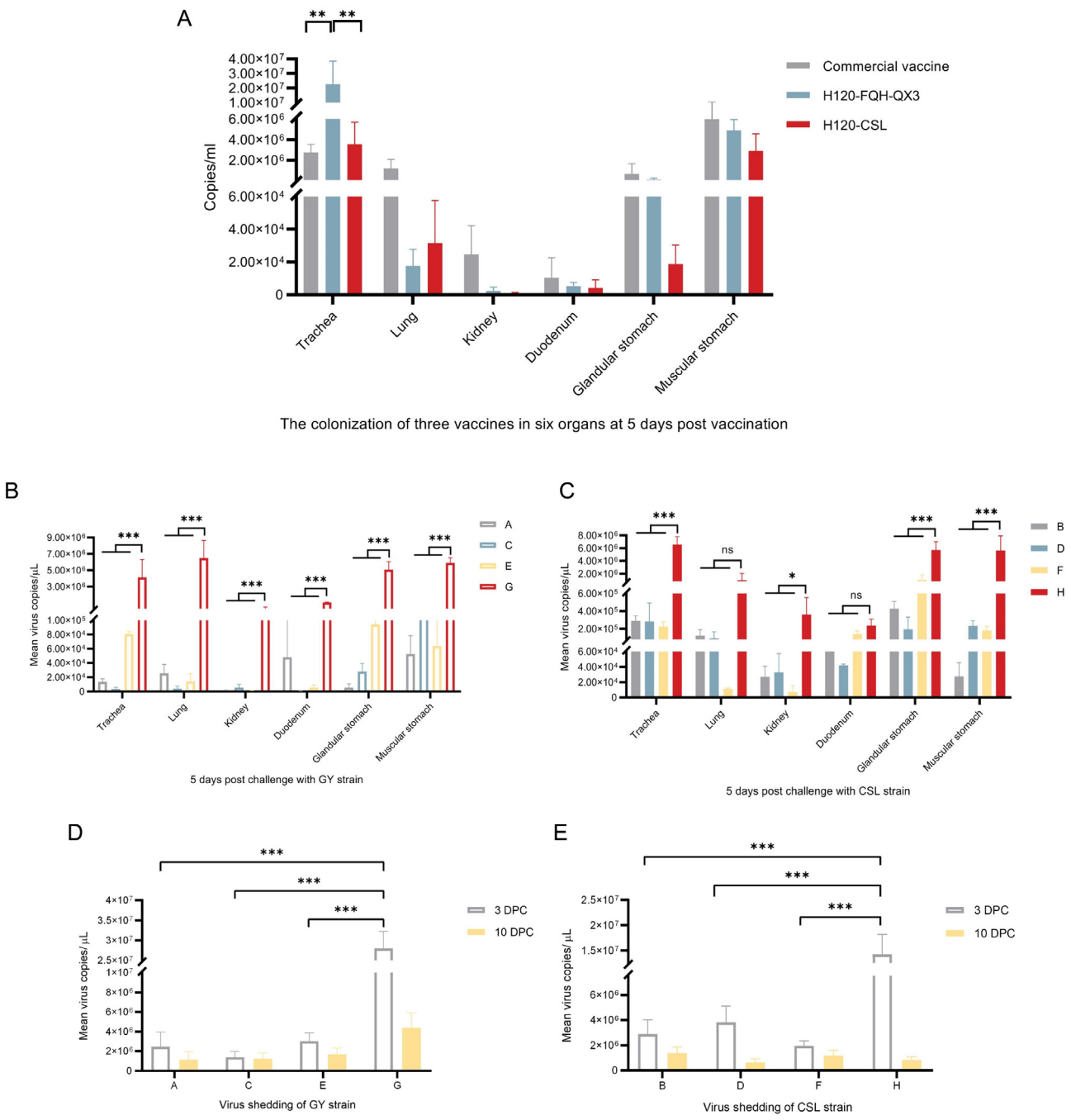


Fig. 5. Virus shedding and tissue viral load detection. **A** Viral loads of three vaccines colonized in tissues at 5 days post vaccination. **B** Tissue viral load of each group at 5 days post challenge with GY strain. **C** Tissue viral load of each group at 5 days post challenge with CSL strain. **D** Detection of virus shedding in throat and cloaca swabs at 3 and 10 dpc with GY and CSL strains. One-way ANOVA was used to identify the differences in virus shedding and tissue viral load levels. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$, ns, not sifnificant.

Table 5
Results of positive rates of IBV antibodies in different immune groups.

Group	Serum positivity rate at different stages after immunization	
	7dpv	13dpv
Combined ND and IB commercial vaccine	0% (0/16)	19% (3/16)
H120-FQH QX3	0% (0/16)	13% (2/16)
H120-CSL	0% (0/16)	0% (0/16)

Except for the blank control group (Group I), Group A and Group E, all other groups exhibited symptoms of renal pallor during autopsy. The scores for air sac and kidney lesions showed no significant difference between the six vaccinated-challenged groups and the blank group. However, the scores for air sac and kidney lesions in the six vaccinated-challenged groups were significantly lower than those in the GY and CSL positive challenge groups ($P < 0.001$). Furthermore, the scores for gastric mucosal lesions in the six vaccinated-challenged groups were significantly lower than those in the CSL positive challenge group ($P < 0.05$),

Table 6
Analysis results of the vaccine efficacy test.

Group	Immune vaccine	Challenge strain	Anatomical number	Anatomical result	Incidence rate	Relative protection rate
A	Combined ND and IB commercial vaccine	GY	10	One died (mottled kidney biopsy) and three had mild myogastritis	10%	90%
B	Combined ND and IB commercial vaccine	CSL	7	1 kidney bilateral white + mild myogastritis; 3 mild myogastritis	14.3%	85.7%
C	H120-FQH QX3	GY	10	1 kidney bilateral white + moderate glandular gastritis + moderate myogastritis; 3 kidneys slightly white; 2 mild myogastritis	40%	60%
D	H120-FQH QX3	CSL	10	1 kidney bilateral swelling + mild myogastritis; two kidneys were slightly white on both sides; 1 case of mild myogastritis	20%	80%
E	H120-CSL	GY	10	2 mild myogastritis; 1 patient with moderate myogastritis	0%	100%
F	H120-CSL	CSL	10	1 kidney was slightly white on both sides; 2 mild myogastritis	10%	90%
G	/	GY	10	One died (mottled kidney); 4 patients with balloon inflammation + kidney pallor and swelling; 1 balloon inflammation; three of the kidneys were pale; 1 mild and 1 mild myogastritis	90%	/
H	/	CSL	10	One died (mottled kidney and urate deposition); 4 patients with balloon inflammation + kidney pallor and swelling; five kidneys were pale and swollen; there were 5 mild muscles and 1 severe muscle gastritis	100%	/
I	Normal saline	Normal saline	10	Ten chickens were asymptomatic	/	/

Note: Relative protection rate = (total − number of cases)/total × 100 %.

with no significant differences observed among the groups for gastric mucosal lesion scores.

In the GY strain challenge group, the H120-CSL recombinant vaccine candidate strain achieved a relative protection rate of 100% against the GY strain, while the H120-FQH QX3 recombinant vaccine candidate strain had a relative protection rate of 60%, showing the lowest level of protection. In the CSL strain challenge group, the relative protection rates of combined ND and IB commercial vaccine, H120-FQH QX3, and H120-CSL recombinant vaccine candidate strains were 87.5%, 80%, and 90%, respectively.

Pathological section results

At 10 dpc, pathological sections of the trachea, lungs, and kidneys of each chicken were examined (Fig. 7). No significant abnormalities were observed in the trachea, lungs, and kidneys of the blank control group and Group F. In the vaccinated-challenged group, except for Group F, the tracheal tissue mucosal layer in the other groups showed exfoliation, inflammatory cell infiltration, and submucosal edema. However, no obvious lesions were found in the lungs and kidneys. In the control group, mucosal layer detachment and inflammatory cell infiltration were observed in the tracheal tissue mucosa layer. Inflammatory cell infiltration was noted in the lungs, and symptoms such as hemorrhage and nephritis were observed in the kidneys. These results indicate that all three vaccines were effective in protecting the visceral organs of chickens from damage caused by GY and CSL strains.

DISCUSSION

Vaccines are an effective means of control IBV in the poultry industry. However, gene mutations and high-intensity immunization pressure lead to the emergence of IBV variants, which eventually lead to immune failure. The cross-protection between different IBV serotypes is limited, and there is little correlation between IBV genotypes and serotypes (Ariyoshi et al., 2010; Fan et al., 2019), which also aggravates the difficulty and complexity of IBV prevention and control. Therefore, timely understanding of the genetic evolution of IBV and the antigenic correlation between vaccine and epidemic strains play a crucial role in the selection and use of vaccine in the poultry industry.

Neutralization testing of antisera and viruses is important to help select vaccine candidates. In the initial stage of vaccine development, antisera-neutralizing virus assays can help to select viral strains capable of triggering a strong immune response, so that the most promising vaccine candidates can be selected for further development. In this study, the average neutralizing titer of FQH QX3 antiserum was the highest, 93.3, with a broad neutralizing spectrum, which is expected to become an inactivated vaccine candidate strain for further study. In contrast, the CSL strain was difficult to be neutralized by serum, indicating the need to strengthen the prevention and control of this strain. In addition, sero-neutralization tests can monitor the adaptability of mutant strains: as the virus evolves, prevalent strains may escape the surveillance of the immune system. Regular antiserum neutralization tests can help us to evaluate the ability of existing vaccines to neutralize epidemic strains, and thus guide vaccine updates and improvements. In this study, the antisera to the existing transmission vaccine strains LDT3, 4/91 and H120 had low neutralization potency against infections with QH QX3 and CSL strains. This suggests that the existing vaccine strains may not provide effective protection against infection with these two prevalent strains, resulting in persistent prevalence of the strains in vaccinated chickens.

Two recombinant H120 vaccine candidates, H120-FQH QX3 and H120-CSL, were constructed in our laboratory against the prevalent strains FQH QX3 and CSL, expressing the S1 and N proteins of FQH QX3 and CSL strains, respectively. The safety and immunoprotective efficacy of the two recombinant vaccine candidates were evaluated in this study. In research studies evaluating the immunoprotective effect of IBV vaccine, virulent strains prevalent in poultry production are commonly used to challenge immunized chickens. The protective effect of the IBV vaccine is assessed based on clinical signs, histopathology scores, tracheal ciliostasis score, changes in viral load in tissues, and reduced virus shedding from the trachea in chickens (Johnson et al., 2010). After immunization, good propagation and colonization of the IB vaccine in tissues such as the trachea is a key component in stimulating subsequent immune protection. In this study, viral loads were measured in six tissues, namely the trachea, lung, kidney, duodenum, glandular stomach and muscular stomach, after immunization and challenge. The results revealed that five days after immunization, the viral load was highest in the trachea and muscular stomach for all three vaccines, followed by the glandular stomach and lung tissue, with the lowest load found in the kidneys and duodenum. The chicken's trachea comprises cartilage tissue, smooth muscle, and connective tissue, while the mucosal surface of the muscular stomach is covered with a keratinous membrane called the cuticula gastrica. This membrane, formed by secretions from mucosal myogastric glands and shed epithelial cells in an acidic environment, acts as a protective barrier. The high viral load of the vaccine virus in the trachea and muscular stomach indicates a strong affinity of the vaccine virus for these tissues. IBV is a respiratory disease that primarily infects the trachea before spreading within the organ. The observation of IBV colonization in the trachea is consistent with previous research.

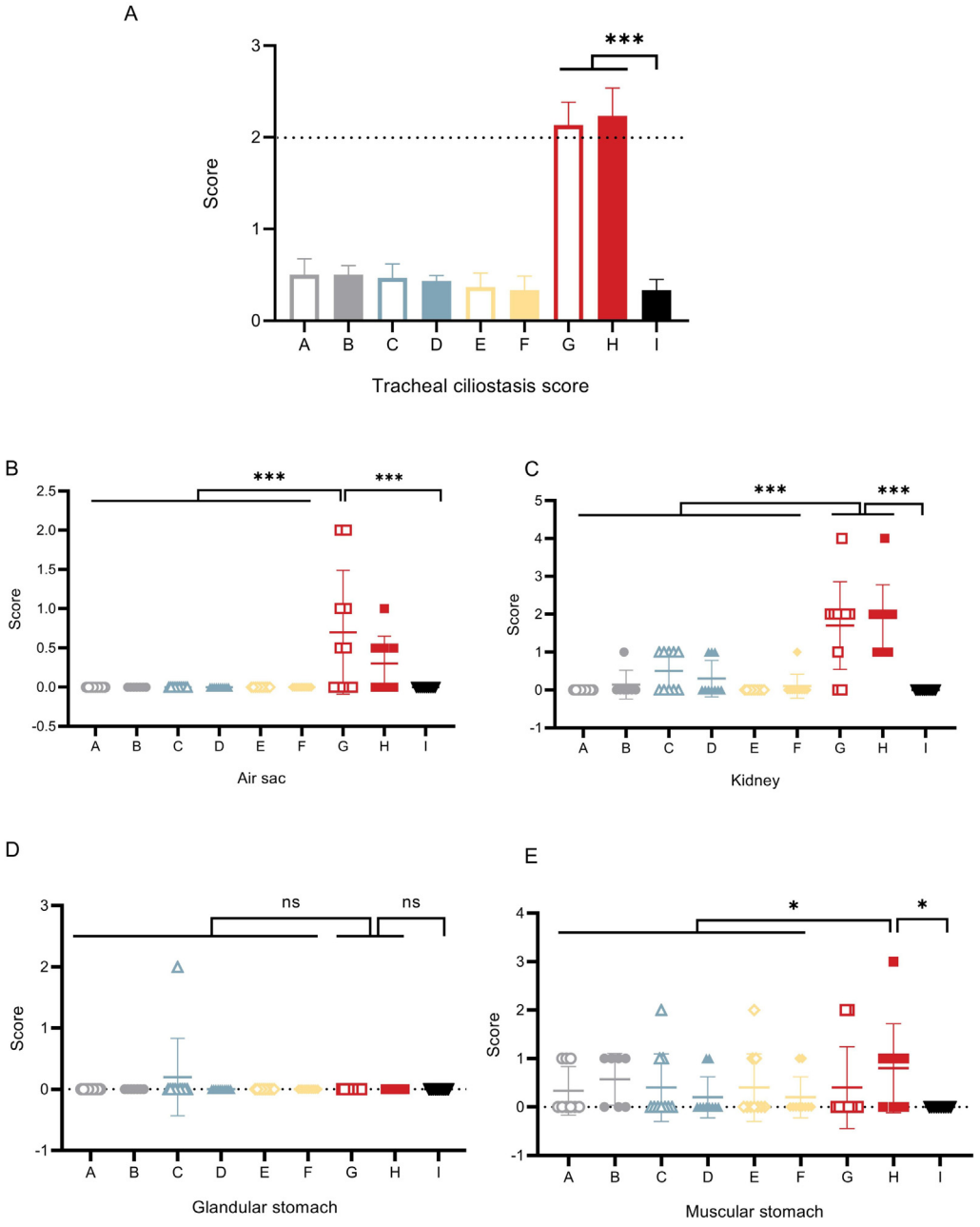


Fig. 6. Tracheal ciliostasis score and tissue lesion score. Ciliostasis score was conducted on the trachea of three chickens in each group at 5 dpc. All chickens were euthanized for observation of pathological changes in the air sac, kidney, glandular stomach, and muscular stomach at 10 dpc, and the degree of tissue damage was scored for these organs. **A** Tracheal ciliostasis score. If the average score of ciliostasis exceeds 2 points, it is considered as diseased, and if it is less than 2 points, it is considered as not diseased. **B** Air sac lesion score. **C** Kidney lesion score. **D** Glandular stomach lesion score. **E** Muscular stomach lesion score. One-way ANOVA was used to identify the differences in groups. * $P < 0.05$, *** $P < 0.001$, ns, not significant.

In the current tissue tropism studies of IBV, the main focus has been on respiratory organs, kidney and glandular stomach lesions, and lesions of the myogastric stomach have been less studied (Hoerr, 2021). In the safety evaluation and immunoprotection test of the candidate vaccines in this study, varying degrees of damage to the cuticula gastrica of the stomach was observed in both the immunized and challenged groups, and symptoms of myogastritis were also observed in the pathogenicity test of 6 selected strains (data not shown). In addition, the viral load of IBV in the cuticle of gizzard was higher than that of the glandular stomach in this study, although there was no direct positive correlation between viral load and pathogenicity, which suggested that we should pay more attention and focus on muscular stomach in the study of IBV.

The respiratory tract is the most important organ for shedding IBV virus, and reduction of virus excretion in the trachea is a necessary indicator of the protective efficacy of the vaccine. In addition, fecal excretion from cloacal can also cause virus spread; therefore, tracheal and cloacal swabs were used as qRT-PCR test samples in this study. Three days after challenge with GY and CSL strains, viral loads in the trachea and cloaca of the three immunization groups (A, C and E) were significantly lower than those in the challenge control group (G group) ($P < 0.0001$). The protective effect of the vaccine can be visually evaluated by comparing the weight gain of the immunized animals through clinical and necropsy observations. According to the clinical characters observed in this study, there was no symptoms of transmission after immunization

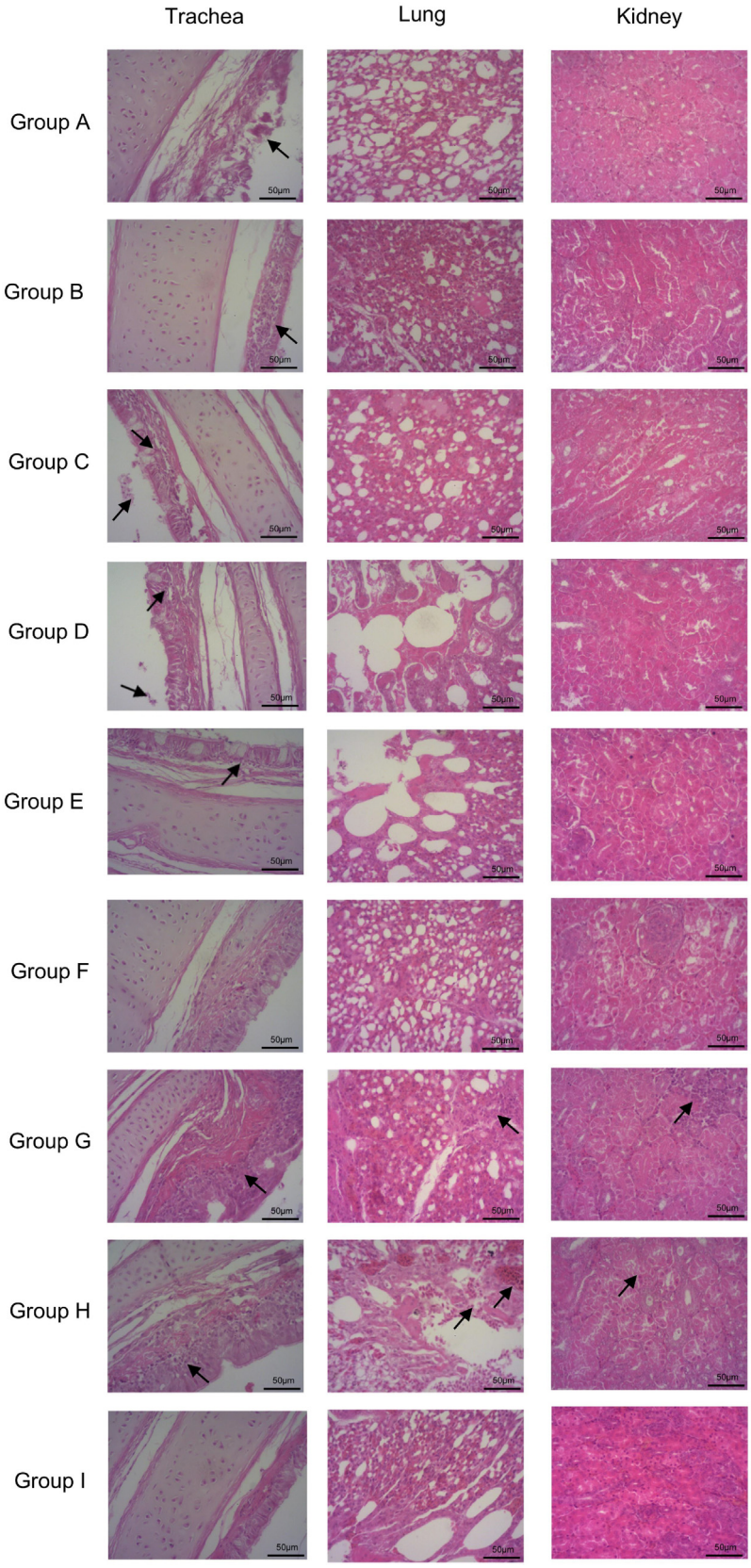


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Fig. 7. Histopathological sections of tissues after virus challenge. Trachea, lungs, and kidneys from three randomly selected chickens per group were examined for pathology at 10 dpc. All tissue sections were observed under a 200 × microscope (200 ×). All tissues with lesions are marked with black arrows. Group A: Exfoliation of tracheal mucosal epithelial cells. Group B: Inflammatory cell infiltration was observed in the tracheal mucosa layer. Group C, D: Exfoliated epithelial cells and inflammatory cell infiltration were observed in the mucosal layer. Group E: Inflammatory cell infiltration was observed in the mucosal layer. Group G: The mucosal layer of the trachea shows swelling and shedding of epithelial cells; pulmonary inflammatory cell infiltration, renal interstitial nephritis. Group H: Tracheitis cell infiltration; pulmonary inflammatory cell infiltration and congestion; renal tubular epithelial shown sign of coagulative necrosis, hypereosinophilia of the cytoplasm in the epithelia lining the renal tubules. Group F, I: No lesions were observed in the pathological sections of the trachea, lungs and kidneys.

with the two recombinant vaccine candidate strains, but dead and weak chickens were found in the commercial vaccine group (3 chickens died in group B). The average weight gain of the chickens in the commercial vaccine group was lower than that in the two recombinant vaccine candidate strains and the blank control group at 13 dpv, which indicated that this commercial vaccine needs to be further optimized and improved in terms of safety.

The primary goal of vaccines is to provide protection by eliciting an immune response. Antibody level is one of the important indicators of a vaccine's effectiveness. High level of antibody was usually associated with stronger immune protection, which can effectively prevent infection or alleviate disease symptoms. However, the relationship between antibody levels and immune protection is not simply linear. In some cases, higher antibody levels do not always mean better protection. For example, certain vaccines can provide protection by activating cellular immune mechanisms, and viral infections confer immune protection even at low antibody levels (Xia et al., 2018).

In the ELISA assay, the antibody positive rates of commercial vaccine, H120-FQH QX3 and H120-CSL recombinant vaccine candidate strains were 19%, 13% and 0%, respectively, at 13dpv. Although the H120-CSL recombinant vaccine candidate strain did not produce antibodies before challenge, the vaccine provided the best protection against challenged with GY and CSL strains. This may be the commercial vaccine is a live attenuated vaccine, which mainly stimulated the body to produce humoral immune response in the early stage, so the antibody positive rate of the commercial vaccine will be higher, while the H120-CSL recombinant vaccine may mainly stimulated the cellular level of immunity. According to the principle that the higher the antigenic correlation between the homotypic serum strains, the better the vaccine immunoprotection (Gelb et al., 1991), our experimental result was unexpected. Compared with the CSL challenged group, the H120-FQH QX3 recombinant vaccine provided lower protection against GY. Similarly, the H120-CSL vaccine provided lower protection against CSL than the GY strain. The relationship between the serotype of a vaccine and the rate of protection is a complex issue, and heterologous serotype vaccines may in some cases elicit a broader antigenic response, thereby improving immunogenicity. In some cases, heterologous serotype vaccines may provide better cross-protection, that is, protection against pathogens of multiple serotypes, not just those of the same serotype as the vaccine (Fan et al., 2018). According to the results of viral load in tissues, among the three immunized groups, the lowest viral loads were found in the trachea, lung and kidney tissues of the H120-CSL recombinant vaccine immunized group, especially challenged with CSL strain at 5 dpc. In addition, the viral load in kidney tissue of mice immunized with the recombinant H120-CSL vaccine was similar to that of the commercial vaccine challenged with GY strain at 5 dpc. This result implied that the H120-CSL recombinant vaccine could be a potential vaccine candidate for the control of renal-type pathogenic strains.

In conclusion, among the six epidemic strains, the FQH QX3 strain was found to have the best monospecific antisera neutralizing antigen spectrum by serum cross-neutralization test, which could be used as a potential inactivated vaccine candidate. However, the serum of the existing vaccine strain is difficult to neutralize the CSL strain, so it is necessary to strengthen the prevention and control of the disease. The H120-CSL recombinant vaccine candidate strain had the best protective effect and could be used as a recombinant vaccine candidate strain for further research and evaluation. The *S1* and *N* genes of the CSL strain had good immunogenicity and could be used as a candidate strain for screening antigen genes. Because the *N* gene is highly conserved, and the variation in T cell epitopes is relatively minor; it is important to study the effect of replacement antigens on T cell epitopes. In the follow-up study, we will strengthen the comparative analysis of IBV *S1* gene in different T cell epitopes of different serotypes, including the possible immune escape mechanism and its influence on immune response.

CONCLUSIONS

In this study, we selected six IBV strains from previous research, and through serum cross-neutralization experiments, it was found that the existing vaccine strains are poorly neutralized by the serum against the CSL strain, indicating a need for enhanced prevention and control measures. Among these, the FQH QX3 strain exhibited the best broad-spectrum neutralization ability as a monospecific serum neutralizing antigen. Therefore, we chose the FQH QX3 and CSL strains as the antigen gene donor strains. Subsequently, using reverse genetics technology, we successfully constructed and rescued two recombinant vaccine candidates: H120-FQH QX3 and H120-CSL, which express the S1 and N proteins of the FQH QX3 and CSL strains, respectively. Immunoprotective efficacy assays demonstrated that the H120-CSL strain exhibited excellent protective capability. The *S1* and *N* genes of the CSL strain showed good immunogenicity, making them potential candidate antigen genes for future vaccine development. The H120-CSL strain has the potential for further research and evaluation as a recombinant vaccine.

MATERIALS AND METHODS

Viruses and vaccine

Six candidate strains (Supplementary Table S1) were preserved based on previous work and have been maintained in our laboratory. Additionally, a combined Newcastle disease and infectious bronchitis vaccine, an attenuated live vaccine (LaSota strain + H120 strain) was obtained from a commercial manufacturer. The H120-FQH QX3 recombinant live vaccine and H120-CSL recombinant live vaccine were constructed and prepared in this experiment.

The titer of screened strains (EID_{50})

The six purified screened strains were subjected to a 10-fold dilution using normal saline. Subsequently, 0.1 mL of each diluted strain was inoculated into 10-day-old SPF chicken embryos at four different dilution levels (10^{-5}, 10^{-6}, 10^{-7}, 10^{-8}) via the allantoic cavity. Five eggs were included for each dilution group, with a negative control. Following incubation at 37 °C, any deceased chicken embryos within 24 h were discarded, and daily observations of embryo lesions were conducted. After a 144-h incubation period, the viral titers of screened strains were determined as the 50% embryo infectious dose (EID_{50}) using the Reed-Muench calculation method.

Preparation of inactivated vaccine

Thirteen strains of IBV, including six candidate strains (PYG QX1, ZQF QX2, FQH QX3, LYZ QX4, XXX QX5, CSL) and seven genotypic strains such as QX (D90 vaccine strain), 4/91 (4/91 vaccine strain), LDT3 (LDT3 vaccine strain), HN08 (XWF strain), TW (GZ14 strain), TC07-2 (MXF strain), and Mass (H120 vaccine strain), were purified, passaged, and

expanded. The information about seven genotypic strains can be found in Supplementary Table S2. Subsequent analysis of nucleotide and amino acid sequence similarities between the six candidate strains and these seven genotypic strains was summarized in Supplementary Fig. S1. The allantoic fluid of these strains was diluted, inactivated with formaldehyde, and tested for sterility and inactivation following Chinese Veterinary Pharmacopoeia guidelines (2015 edition). Afterward, the allantoic fluid of the thirteen strains was meticulously diluted and titrated with physiological saline to achieve a concentration of $10^{6.0}$ EID_{50}/0.1 mL. Each allantoic fluid was then inactivated by the addition of formaldehyde solution (final concentration of 0.2%), thoroughly mixed, and incubated on a shaker table at 37 °C with a rotation speed of 70 rpm for 36–48 h. Sterility tests were conducted in accordance with the guidelines outlined in the Veterinary Pharmacopoeia of China (2015 Edition). Specifically, 0.2 mL of the inactivated allantoic fluid was introduced into the allantoic cavity of chicken embryos, which were then incubated for a duration of 36–48 h. Following three generations of blind transmission, IBV was not detected.

Add Tween 80 (antigen volume: Tween 80 vol = 50:2) into 13 portions of inactivated allantoic fluid that have passed the test, vortex for 10 min, mix the allantoic fluid and Tween 80 completely. Then 13 aliquots of allantoic fluid and Tween 80 mixture were mixed with vaccine compound adjuvant (antigen volume: white oil and span compound adjuvant = 2: 3) When using a high shear dispersion emulsifier for emulsification, first add the compound adjuvant to the beaker, followed by the antigen. Set the shear speed to 3000 rpm–6000 rpm, starting with a slow shear. As the speed gradually increase, maintain the whole emulsification process with the beaker placed in an ice bath to reduce the heat generated by the high shear and stirring process, thereby preventing excessive temperatures. The completion of emulsification is characterized by the presence of emulsified liquid droplets floating in water that exhibits condensed morphology rather than a dispersed state. To verify emulsification success, a portion of the prepared oil emulsion was centrifuged at 8000 rpm for 5 minutes. The absence of oil-water stratification after centrifugation confirms success. The inactivated oil emulsion was placed in the refrigerator at 4 °C for storage.

Preparation of monospecific antisera

Following Chen Tong's methodology (Tong, 2019), monospecific antisera against the above 13 IBV inactivated vaccines were prepared. SPF chickens were divided into 13 groups, each containing 5 chickens, and housed in isolators with food and water. At 40 days of age, the 13 groups were immunized with the inactivated oil emulsion vaccines via subcutaneous injection in the neck every two weeks, for a total of three immunizations, with 1 mL per animal. On the 10th day after the third immunization, venous blood samples were collected from each chicken to isolate serum.

The antibody levels of the vaccinated chickens were assessed using an IBV antibody ELISA detection kit (IDEXX, Beijing, China). Chickens with positive antibodies and titers ≥2000 had blood collected using a combination of cardiac and carotid artery methods. The blood was left at room temperature for 4 h, then centrifuged to isolate the high-immunity monospecific antiserum. Penicillin-Streptomycin Solution was added to each serum at a 2% volume, packed separately, and stored at −80 °C for cross-neutralization tests.

Serum-neutralization test

Multiple cross serum neutralization tests were conducted by reacting individual antigens with homologous and heterologous antisera using the constant-virus varying-serum method (β method). Antibody titers of prepared virus samples were determined with serial dilutions of antiserum (1:8, 1:16, 1:32, 1:64, 1:128). Each antiserum dilution (0.5 mL) was mixed with an equal volume of virus sample (100 EID_{50}/0.1 mL) and incubated at 37 °C for 45 min. Subsequently, 0.2 mL of the serum and virus mixture was inoculated into four 10-day-old 818 broiler chicken embryos via the allantoic cavity pathway.

Positive control (0.1 mL of 100 EID_{50} antigen per embryo) and blank control (0.1 mL of 0.9% physiological saline per embryo) were established simultaneously. The inoculated chicken embryos were placed in a constant-temperature incubator at 37 °C and observed for 144 h, with their development monitored. Characteristic lesions indicative of IBV infection in the chicken embryos were noted, and the number of lesions in each embryo body gradient in each group was recorded (excluding embryos that died within 24 h). Finally, the half-protection dose (PD_{50}) was calculated using the Reed-Muench method.

Antigenic relationships between IBV strains

The antigenic relationship of IBV strains was evaluated through cross serum neutralization tests using hyperimmune sera. The antigenic correlation value (R) was calculated using the formula: $R = \sqrt{r1 \times r2} \times 100\%$, where r1 and r2 represent the titers of heterologous sera divided by the titers of homologous sera. An R value greater than 80% indicated strains of the same subtype, while values between 25% and 80% indicated different subtypes within the same serotype. An R value below 25% indicated different serotypes (Lee et al., 2003).

Evaluation for safety of recombinant vaccine candidates

In order to assess the safety of two recombinant vaccine candidates, H120-FQH QX3 and H120-CSL , in chickens, a total of 45 one-day-old SPF chickens were randomly divided into two immunization groups and one control group, with 15 chickens in each group. The chickens were housed in isolators and allowed free access to food and water. On the first day of life, chickens in the immunization groups were nasally administered 10 times the dose of the recombinant vaccine ($10^{5.5}$ EID_{50}/0.1 mL/chicken), while chickens in the control group received normal saline. Detailed group information can be found in Supplementary Table S3. The safety of the vaccine candidates was evaluated by monitoring clinical characteristics, tracheal cilia motility, pathological changes, and weight gain in the experimental chickens post-immunization.

Establishing models for challenging QX and CSL IBV strains

Given that the FQH QX3 strain typically exhibits insufficient virulence to challenge 14-day-old SPF chickens, rendering it unsuitable for challenge model establishment, we opted to employ the GY virulent strain, which shares the same serotype as the FQH QX3 strain, to establish the challenge model for IBV QX type. In parallel, the challenge model for IBV CSL type was established using the CSL strain. The serum cross-neutralization titer, antigen correlation, and *S1* gene similarity of the GY, FQH QX3 and CSL strains were shown in Supplementary Table S4, Table S5 and Supplementary Fig. S2, respectively.

Sixty 14-day-old SPF chickens were randomly assigned to five challenge groups, along with a blank control group, with each group comprising 10 chickens. These 6 groups of chickens were individually housed in 6 isolators and provided with ad libitum access to food and water. At 14 days of age, the chickens were exposed to the virus challenge. Details of the specific groupings and virus challenge procedures can be found in Supplementary Table S6. Following the challenge, clinical signs of the chickens were monitored and recorded daily. Subsequently, all chickens were necropsied ten days after the virus challenge (at 24 days old), and symptoms were observed during the autopsy.

Quantitative real-time RT-PCR

The total viral RNA was isolated from tissues using the Pure Viral RNA/DNA Extraction Kit (TianLong, Xi'an, China) according to the suppliers' instructions. DNA was synthesized using PrimeScript One Step RT-PCR Kit Ver (TaKaRa, Dalian, China). Real-time PCR amplification was done with the Evo M-MLV One Step RT-qPCR Kit

III(Probe)(Accurate Biology, Hunan, China) with a forward primer (5'-CCTCTAAGGGCTTTTGAG-3'), a reverse primer (5'-GTCACTGTC-TATTGTATGTC-3'), and the IBV Probe (5'-FAM CACCACCA-GAACCTGTCACCTC BHQ1-3') (Wu et al., 2022). The reverse transcription PCR (RT-PCR) reaction was performed in a 25 μL reaction volume containing 12.5 μL of 2 × 1 Step Buffer (Dye Plus), 1 μL each of the two primers, 2 μL template,1 μL of PrimeScript One Step Enzyme Mix and 7.5 μL of ddH2O. The optimum conditions for RT-PCR were as follows: 50 °C for 30 min; 94 °C for 4 min, followed by 32 cycles of 94 °C for 30 seconds, 53 °C for 35 seconds, and 72 °C for 12 seconds, with a final extension at 72 °C for 10 minutes. The RT-PCR products were examined using agarose gel electrophoresis, and after ethidium bromidestaining, the predicted size of the target gene was isolated from agarose gels and purified. Recombinant plasmid was constructed, and its concentration was determined as decribed in a previous report (Wei et al., 2015). The recombinant plasmid was subjected to a 10-fold serial dilution (10^0, 10^{-1}, $10^{-2,}$ 10^{-3}, 10^{-4}, 10^{-5}, 10^{-6}), and each dilution was used as a template for quantitative real-time RT-PCR. Three replicates were performed for each dilution, ultimately establishing a standard curve. The reaction conditions were as follows: 42 °C for 5 min, 95 °C for 30 s, and 40 cycles of 95 °C for 5 s and 60 °C for 34 s on the Applied Biosystems Gentier 96E/96R (TianLong, Xi'an, China).

Evaluation of immune efficacy of recombinant vaccine candidates

A total of 165 one-day-old SPF male and female chickens were randomly assigned to nine groups, which comprised six different immune challenge groups (Groups A to F), two challenge control groups (Groups G and H), and one blank control group (Group I), as detailed in Fig. 4A. At one day of age, the six immune groups received immunization, while at 14 days of age, the six immune groups and two challenged control groups underwent vaccination. The blank control group received no treatment. Throughout the rearing period, each isolator was equipped with insulation measures for the chicks, enabling them to freely access food and water. Data on chicken weight was collected at three time points: the total weight of all chickens in each group was measured prior to vaccination on Day 1 (pre-immunization), and the average weight was calculated; individual chicken weights were then recorded 13 days after vaccination (pre-challenge, at 14 days of age) and again at 10 days post-challenge (at 24 days of age). The average weight for each time point was determined, and the difference value between each chicken's weight and the group's average weight from the previous time point was calculated. This difference value served as a basis for comparing the weight gain among chickens following different vaccination and challenge scenarios.

Following the 1-day immunization and 14-day challenge, daily clinical observations were conducted for each chicken group. Antibody levels were assessed by collecting blood samples from ten randomly selected chickens in each group via the wing vein at 7 and 13 days post-immunization. Ciliostasis was evaluated by dissecting and collecting trachea samples from three randomly chosen chickens in each group, 5 days after the virus challenge. Pathological sections were examined and scored 10 days post-challenge using samples from three randomly selected chickens in each group. Trachea, lungs, and kidneys were preserved in 4% formaldehyde for section preparation.

Five days post-vaccination, three chickens from each of three immune groups (only one group for the same vaccine type) and the control group were randomly selected. Samples were collected from six tissues: Trachea, lungs, kidneys, duodenum, glandular stomach, and muscle stomach, for the detection of vaccine virus colonization.

At 3 and 10 days post-challenge, eight chickens from each of the nine groups were randomly selected for virus shedding detection. Throat and cloaca swabs were collected from each chicken and pooled in a single centrifuge tube for analysis. This was done to assess the virus shedding profile of the immune-challenged groups. Five days post-challenge, three chickens from each group were randomly selected for tissue collection. Samples were taken from six tissues: trachea, lung, kidney, duodenum, glandular stomach, and muscle stomach. These samples were used for virus load analysis to evaluate the replication and colonization of challenged strain in the immune chickens.

Ten days post-challenge, remaining chickens underwent dissection, and symptoms were observed. Pathological lesions were scored to evaluate the efficacy of the two recombinant vaccines (refer to Supplementary Table S7 for scoring criteria).

Statistical analysis

The results such as viral load, body weight change, cilia swing score, and lesion score were examined by one-way analysis of variance (ANOVA) in GraphPad software 8.0. The significance levels were indicated as follows: ns (no significant difference between groups, $P > 0.05$), * (significantly different between groups, $P < 0.05$), ** (significantly different between groups, $P < 0.01$), *** (significantly different between groups, $P < 0.001$). The sequence of IBV was initially analyzed by Mega software, and then the homology (similarity) of nucleotide and amino acid sequences of IBV *S1* and *N* genes was compared by Clustal W method in MegAlign software.

DATA AVAILABILITY

The original data presented in the study are included in the article and supplementary material. In addition, the *S1* and *N* gene sequences of the 6 screened strains involved in this study have not been published for the time being. If necessary, you can contact the corresponding authors for more information.

ETHICS STATEMENT

All procedures involving animals adhered strictly to the guidelines for the use of animals in experimental research reviewed by the Committee of the Ethics on Animal Care and Experiments at South China Agricultural University (approval no. SYXK-2019-0136).

AUTHOR CONTRIBUTIONS

Benli Huang: methodology, formal analysis and investigation. Shen Chen and Xinheng Zhang: conceptualization, writing-original draft. Zhanxin Wang and Keyu Feng: supervision and project administration. Yutao Teng, Ruoying Li, Guanming Shao and Jiaqian Rao: investigation. Qingmei Xie: funding acquisition and writing-review & editing.

CONFLICT OF INTEREST

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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APPENDIX A. SUPPLEMENTARY DATA

Supplementary data to this article can be found online at https://doi.org/10.1016/j.virs.2025.03.008.

REFERENCES

Ariyoshi, R., Kawai, T., Honda, T., Tokiyoshi, S., 2010. Classification of IBV S1 genotypes by direct reverse transcriptase-polymerase chain reaction (RT-PCR) and relationship between serotypes and genotypes of strains isolated between 1998 and 2008 in Japan. J. Vet. Med. Sci. 72, 687–692.

Bande, F., Arshad, S.S., Omar, A.R., Hair-Bejo, M., Mahmuda, A., Nair, V., 2017. Global distributions and strain diversity of avian infectious bronchitis virus: a review. Anim. Health Res. Rev. 18, 70–83.

Cavanagh, D., 2007. Coronavirus avian infectious bronchitis virus. Vet. Res. 38, 281–297.

Cavanagh, D., Picault, J.P., Gough, R., Hess, M., Mawditt, K., Britton, P., 2005. Variation in the spike protein of the 793/B type of infectious bronchitis virus, in the field and during alternate passage in chickens and embryonated eggs. Avian Pathol. 34, 20–25.

Cook, J.K.A., Jackwood, M., Jones, R.C., 2012. The long view: 40 years of infectious bronchitis research. Avian Pathol. 41, 239–250.

Fan, W., Tang, N., Dong, Z., Chen, J., Zhang, W., Zhao, C., He, Y., Li, M., Wu, C., Wei, T., Huang, T., Mo, M., Wei, P., 2019. Genetic analysis of avian coronavirus infectious bronchitis virus in yellow chickens in Southern China over the past decade: revealing the changes of genetic diversity, dominant genotypes, and selection pressure. Viruses 11, 898.

Fan, W.S., Li, H.M., He, Y.N., Tang, N., Zhang, L.H., Wang, H.Y., Zhong, L., Chen, J.C., Wei, T.C., Huang, T., Mo, M.L., Wei, P., 2018. Immune protection conferred by three commonly used commercial live attenuated vaccines against the prevalent local strains of avian infectious bronchitis virus in southern China. J. Vet. Med. Sci. 80, 1438–1444.

Franzo, G., Massi, P., Tucciarone, C.M., Barbieri, I., Tosi, G., Fiorentini, L., Ciccozzi, M., Lavazza, A., Cecchinato, M., Moreno, A., 2017. Think globally, act locally: phylodynamic reconstruction of infectious bronchitis virus (IBV) QX genotype (GI-19 lineage) reveals different population dynamics and spreading patterns when evaluated on different epidemiological scales. PLoS One 12, e0184401.

Gelb, J.J., Wolff, J.B., Moran, C.A., 1991. Variant serotypes of infectious bronchitis virus isolated from commercial layer and broiler chickens. Avian Dis. 35, 82–87.

Han, Z., Sun, C., Yan, B., Zhang, X., Wang, Y., Li, C., Zhang, Q., Ma, Y., Shao, Y., Liu, Q., 2011. A 15-year analysis of molecular epidemiology of avian infectious bronchitis coronavirus in China. Infect. Genet. Evol. 11, 190–200.

Hoerr, F.J., 2021. The pathology of infectious bronchitis. Avian Dis. 65, 600–611.

Jackwood, M.W., 2012. Review of infectious bronchitis virus around the world. Avian Dis. 56, 634–641.

Jackwood, M.W., Hall, D., Handel, A., 2012. Molecular evolution and emergence of avian gammacoronaviruses. Infect. Genet. Evol. 12, 1305–1311.

Jackwood, M.W., Hilt, D.A., Callison, S.A., Lee, C.W., Plaza, H., Wade, E., 2001. Spike glycoprotein cleavage recognition site analysis of infectious bronchitis virus. Avian Dis. 45, 366–372.

Johnson, D.I., Vagnozzi, A., Dorea, F., Riblet, S.M., Mundt, A., Zavala, G., Garcia, M., 2010. Protection against infectious laryngotracheitis by in ovo vaccination with commercially available viral vector recombinant vaccines. Avian Dis. 54, 1251–1259.

Kant, A., Koch, G., van Roozelaar, D.J., Kusters, J.G., Poelwijk, F.A., van der Zeijst, B.A., 1992. Location of antigenic sites defined by neutralizing monoclonal antibodies on the S1 avian infectious bronchitis virus glycopolypeptide. J. Gen. Virol. 73, 591–596.

Lee, C.W., Hilt, D.A., Jackwood, M.W., 2003. Typing of field isolates of infectious bronchitis virus based on the sequence of the hypervariable region in the S1 gene. J. Vet. Diagn. Invest. 15, 344–348.

Legnardi, M., Tucciarone, C.M., Franzo, G., Cecchinato, M., 2020. Infectious bronchitis virus evolution, diagnosis and control. Vet. Sci. 7, 79.

Lian, J., Wang, Z., Xu, Z., Chen, T., Shao, G., Zhang, X., Qin, J., Xie, Q., Lin, W., 2021. Distribution and molecular characterization of avian infectious bronchitis virus in southern China. Poult. Sci. 100, 101169.

Masters, P.S., 2006. The molecular biology of coronaviruses. Adv. Virus Res. 66, 193–292.

Promkuntod, N., Thongmee, S., Yoidam, S., 2015. Analysis of the S1 gene of the avian infectious bronchitis virus (IBV) reveals changes in the IBV genetic groups circulating in southern Thailand. Res. Vet. Sci. 100, 299–302.

Tang, M., Wang, H., Zhou, S., Tian, G., 2008. Enhancement of the immunogenicity of an infectious bronchitis virus DNA vaccine by a bicistronic plasmid encoding nucleocapsid protein and interleukin-2. J. Virol. Methods 149, 42–48.

Tong, C., 2019. Research on IBV Isolates Molecular Epidemiology in 2016-2018 and TW Type IBV Attenuated Vaccine. Master, South China Agricultural University, p. 92 (In Chinese).

Wei, Z.J., Wang, F.Y., Guo, M.P., Duan, Z.Z., Zou, N.L., Liu, P., Yan, Q.G., Wen, X.T., Cao, S.J., Huang, Y., 2015. Dynamic changes of virus load in supernatant of primary CEK cell culture infected with different generations of avian infectious bronchitis virus strains Sczy3 as revealed by real-time reverse transcription-polymerase chain reaction. Genet. Mol. Res. 14, 6340–6349.

Wu, Z., Fang, H., Xu, Z., Lian, J., Xie, Z., Wang, Z., Qin, J., Huang, B., Feng, K., Zhang, X., Lin, W., Li, H., Chen, W., Xie, Q., 2022. Molecular characterization analysis of prevalent infectious bronchitis virus and pathogenicity assessment of recombination strain in China. Front. Vet. Sci. 9, 842179.

Xia, J., He, X., Du, L.J., Liu, Y.Y., You, G.J., Li, S.Y., Liu, P., Cao, S.J., Han, X.F., Huang, Y., 2018. Preparation and protective efficacy of a chicken embryo kidney cell-attenuation GI-19/QX-like avian infectious bronchitis virus vaccine. Vaccine 36, 4087–4094.

Yu, D., Han, Z., Xu, J., Shao, Y., Li, H., Kong, X., Liu, S., 2010. A novel B-cell epitope of avian infectious bronchitis virus N protein. Viral Immunol. 23, 189–199.

Zhao, W., Gao, M., Xu, Q., Xu, Y., Zhao, Y., Chen, Y., Zhang, T., Wang, Q., Han, Z., Li, H., Chen, L., Liang, S., Shao, Y., Liu, S., 2017. Origin and evolution of LX4 genotype infectious bronchitis coronavirus in China. Vet. Microbiol. 198, 9–16.