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Development and efficacy of a novel live-attenuated QX-like nephropathogenic infectious bronchitis virus vaccine in China

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ABSTRACT

In this study, we attenuated a Chinese QX-like nephropathogenic infectious bronchitis virus (IBV) strain, YX10, by passaging through fertilized chicken eggs. The 90th passage strain (YX10p90) was selected as the live-attenuated vaccine candidate strain. YX10p90 was found to be safe in 7-day-old specific pathogen free chickens without induction of morbidity or mortality. YX10p90 provided nearly complete protection against QX-like (CH I genotype) strains and partial protection against other two major Chinese genotype strains. YX10p90 also showed no reversion to virulence after five back passages in chickens. An IBV polyvalent vaccine containing YX10p90 was developed and showed that it could provide better protection against major Chinese IBV virulent strains than commercial polyvalent vaccines. In addition, the complete genome sequence of YX10p90 was sequenced. Multiple-sequence alignments identified 38 nucleotide substitutions in the whole genome which resulted in 26 amino acid substitutions and a 110-bp deletion in the 3′ untranslated region. In conclusion, the attenuated YX10p90 strain exhibited a fine balance between attenuation and immunogenicity, and should be considered as a candidate vaccine to prevent infection of Chinese QX-like nephropathogenic IBV.

1. Introduction

Avian infectious bronchitis (IB) is an acute and highly contagious disease caused by the avian infectious bronchitis virus (IBV). It has a major impact on the poultry industry worldwide and causes severe economic losses [1–3]. This disease, which affects chickens of all ages, leads to not only respiratory illness but also nephritic syndromes, and such complications as proventriculitis and decreased egg production and quality [4].

IBV belongs to the genus *Gammacoronavirus* of the family Coronaviridae in the order Nidovirales. The IBV genome consists of a linear, non-segmented, positive-sense, single-stranded RNA of approximately 27.6 kilobases (kb) in length [5]. Due to the inaccuracy of the coronavirus RNA-dependent RNA polymerase (RDRP) and high frequency of homologous RNA recombination, emergence of variant strains, genotypes and serotypes of IBV is constantly reported [6–9], and at least 30 serotypes have been identified worldwide. Some studies have indicated that IBV immunity is serotype-specific, and low degrees of cross-protection were observed among IBV genotypes [9].

Since the QX IBV strain was detected in Shandong province, China in the 1990s, QX-like genotype IBV strains mainly causing nephritis have spread widely in Asia and Europe, where flocks are commonly vaccinated with Massachusetts (Mass) and 4/91 vaccines [2]. Previous studies have shown that at least six genotypes (CH I–CH VI) are prevalent in China, and the QX-like (CH I) genotype, accounting for more than 60% of newly isolated IBV strains in the past 10 years, have become predominant [10–14]. Current vaccine strains used in China (H120, H52, W93 and Ma5) belong to the Mass genotype and are evolutionarily distant from QX-like genotype strains [15,16]. Although various live-attenuated and inactivated vaccines derived from Mass genotype strains have been widely used in chicken farms, their efficacies are poor, and IB disease

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Table 1
Viruses used in this study.

Strain	Abbreviation	Geographic origin	Usage	Genotype	Accession number
H120		Holland	Vaccine strain	Mass	M21970
28/86		Italy	Vaccine strain	Mass	AY846750
4/91		UK	Vaccine strain	CH II (793B)	AF093794
LDT3-A		China	Vaccine strain	CH III	AY702975
YX10p5		Zhejiang China	Challenge strain	CH I (QX-like)	JX84041
CK/CH/TJ/NH10	NH10	Tianjin China	Challenge strain	CH II (793B)	HQ018917
CK/CH/GD/LZ12	LZ12	Guangdong China	Challenge strain	CH I (QX-like)	KJ524638
CK/CH/GX/GL12	GL12	Guangxi China	Challenge strain	CH I (QX-like)	KJ524616
CK/CH/GX/YL12	YL12	Guangxi China	Challenge strain	CH III	KC692263
CK/CH/GX/ZS12	ZS12	Guangxi China	Challenge strain	CH V	KC692259

outbreaks occur frequently in vaccinated chicken flocks. Therefore, developing a novel live-attenuated vaccine from a Chinese QX-like nephropathogenic IBV strain is necessary.

In the present study, a live-attenuated IBV vaccine strain based on the Chinese QX-like IBV strain YX10 was developed, and its attenuation, efficacy and safety profile were analyzed. We prepared a polyvalent vaccine containing YX10 and evaluated its protective efficacy. Differences in the complete genome between attenuated YX10p90, YX10p102 and virulent YX10p5 were studied. The results demonstrated that the newly developed vaccine strain achieved a desired level of immunogenicity and attenuation of virulence. Most of the tests described here were performed in accordance with the requirements of the Veterinary Pharmacopoeia of the People's Republic of China (CVP) 2010 Edition.

2. Materials and methods

2.1. Viruses

IBV strain YX10, used for vaccine development, was isolated in Zhejiang province, China in 2010. Phylogenetic analysis revealed that this recombinant nephropathogenic strain belongs to the QX-like (CH I) genotype. The complete genome of the IBV strain YX10 was sequenced and deposited in GenBank under accession number JX84041 in our previous study [17].

Four vaccine strains and six challenge strains were used to evaluate the efficacy and cross-protective effects of the live-attenuated vaccine in this study. The vaccine strains were H120 (Dahuanong Animal Health Products Co., Ltd., Guangdong, China), LDT3-A (Weike Biotechnology Development Co., Ltd., Harbin, China), 4/91 (Intervet International B.V., Boxmeer, Holland) and 28/86 (China Institute of Veterinary Drug Control, IVDC). The six challenge strains (YX10p5, CK/CH/GD/LZ12, CK/CH/GX/GL12, CK/CH/TJ/NH10, CK/CH/GX/YL12 and CK/CH/GX/ZS12) were all nephropathogenic strains and isolated in diseased chicken flocks in China during 2010–2012. The geographic origin, genotypes and accession numbers of all the strains were listed in Table 1.

2.2. Chicken embryos and chickens

Fertilized white leghorn specific pathogen free (SPF) eggs and chickens were obtained from the Guangdong DHN poultry and egg products Co., Ltd., China. The birds were maintained in isolators with negative pressure, and food and water were provided *ad libitum*. For production of virus and titrations, eggs were incubated at 37 °C in a relative humidity of 55%. All study procedures and animal care activities were conducted in accordance with the national and institutional guidelines for the care and use of laboratory animals.

2.3. Attenuation

IBV strain YX10 was passaged 102 times by inoculating 9-day-old fertilized SPF eggs by the allantoic sac route, each with a volume of 0.1 ml. Inoculated eggs were then incubated at 37 °C for 36–48 h, and the allantoic fluid was harvested. RT-PCR was performed to detect the presence of virus as previously described [17] every five passages. The passaged viruses were titrated by inoculating 10-fold serial dilutions in phosphate-buffered saline (PBS) of the virus stocks into the allantoic sac of 10-day-old fertilized SPF eggs, and the titer was calculated by the Reed and Muench method [18]. The passaged viruses were designated as YX10p*N*, with the *N* denoting the passage number. For example, virus at the 50th passage was recorded as YX10p50.

2.4. Evaluation of vaccine safety

Safety testing was conducted according to guidelines of the CVP 2010 Edition, Volume III, which states that if none of 10 vaccinated 7-day-old SPF chickens show respiratory signs, nervous system signs and/or death, the vaccine is considered to be safe. In this study, at least 80 SPF 7-day-old chickens were divided into 8 groups with at least 10 chickens in each group. The birds in the experimental groups were inoculated intranasally with 10 doses of the IBV strains (YX10p5, YX10p25, YX10p58, YX10p75, YX10p90, YX10p102 and H120) at $10^{4.5}$ embryo infectious dose [EID_{50}]/0.1 ml, while those in the control group were inoculated with phosphate buffered saline (PBS). All experimental groups and the control group were observed twice daily for clinical signs for 21 days.

2.5. Evaluation of vaccine efficacy

2.5.1. Testing efficacy against challenge with YX10p5

Sixty-two-week-old SPF chickens were divided into 5 groups of 12 chickens each. Chickens in experimental groups were each vaccinated intranasally with one dose of YX10p75, YX10p90, YX10p102 or H120 at $10^{3.5}$ EID_{50}/0.1 ml. The control group was inoculated with PBS. Fourteen days post-vaccination, sera were collected for analysis using an enzyme-linked immunosorbent assay (ELISA) (IDEXX, USA), and then all birds were challenged intranasally with YX10p5 at $10^{5.5}$ EID_{50}/0.1 ml. The chickens were observed twice daily for clinical signs for 21 days. Fourteen days post-challenge, sera were also collected for ELISA.

2.5.2. Testing efficacy against challenge with strains representing major China IBV genotypes

One hundred forty-four 1-day-old SPF chickens were divided into 3 groups of 48 chickens. The birds were given feed and water *ad libitum*. At 5 and 10 days of age, chickens in immune groups vaccinated intranasally with one dose of YX10p90 or H120 at $10^{3.5}$ EID_{50}/0.1 ml. Control group birds were inoculated with PBS. At 17 days of age, each group was divided into 4 challenge groups of 12

chickens each and challenged with Chinese isolated strains LZ12, GL12, YL12 or ZS12 at $10^{5.5}$ EID_{50}/0.1 ml. All birds were observed twice daily for clinical signs for 21 days.

2.5.3. Monitoring reversion of virulence

In order to examine YX10p90 for potential reversion of virulence, 7-day-old SPF chickens were divided into two groups of six chickens per group. The inoculated group was inoculated intranasally with 10 doses of YX10p90 at $10^{4.5}$ EID_{50}/0.1 ml. Birds in the control group were inoculated with PBS. At 5 days post-inoculation, the kidneys and tracheas were collected from three birds of each group, and the tissue homogenates were prepared in PBS with antibiotics for virus detection by RT-PCR. Subsequently, the tissue homogenates were inoculated into the next group of chickens and five fertilized SPF eggs. The other three chickens of each group were observed twice daily for clinical signs for 21 days, and then birds were sacrificed for pathological examinations of the tracheas and kidneys. This experiment was repeated five times.

2.6. Testing efficacy of polyvalent vaccines

To expand the cross-protective effects of YX10p90 strain and research the combined effects with other IBV vaccine strains, polyvalent vaccine A was prepared. One dose contained $10^{3.5}$ EID_{50} of YX10p90, 4/91 and H120, respectively. Two other commercial polyvalent vaccines were also obtained for comparison. Polyvalent vaccine B contained H120, 28/86 and 4/91 strain, and polyvalent vaccine C contained H120, LDT3-A and 4/91 strain. One dose polyvalent vaccine contained $10^{3.5}$ EID_{50} of each strain.

Four hundred and twenty 1-day-old SPF chickens were divided into 5 groups: 3 immune groups of 100 chickens in each group, positive control group of 100 and negative control group of 20. The birds were given feed and water *ad libitum*. At 5 and 10 days of age, chickens in experimental groups were vaccinated intranasally with one dose of polyvalent vaccine A, B or C. Positive and negative control groups were inoculated with PBS. At 17 days of age, each immune group and positive control group was divided into 5 challenge groups of 20 chickens in each group and challenged intranasally with Chinese isolated strains LZ12, GL12, NH10, YL12 or ZS12 at $10^{5.5}$ EID_{50}/0.1 ml. The negative control group was inoculated with PBS. Five days post-challenge, 10 chickens of each challenge group were killed humanely and all organs, especially the kidney, were examined. Nephritis, when observed, was characterized by enlarged, pale and marbled kidneys, with urate deposits in the ureters and cloaca. Renal pathology was used to calculate the degree of renal protection. The remainder of the birds in each group was checked twice a day for clinical signs for a period of 21 days.

2.7. Sequence analysis of complete genome of YX10p90 and YX10p102

As described in our previous study [17], 24 pairs of primers used for amplifying the complete genome sequence of strain YX10 were designed and synthesized by AuGCT DNA-SYN Biotechnology Co., Ltd. (Beijing, China). Viral RNA extracted from the allantoic fluid of YX10p90 and p102 were amplified with the 24 primer pairs by reverse transcription-polymerase chain reaction (RT-PCR). Products of each RT-PCR were ligated to the TA cloning vector pMD19-T (Takara, Japan) and transformed into competent cells. Positive clones were screened by PCR and then sequenced by Shanghai Sang-gong Biological Engineering Technology & Services Co., Ltd. (Shanghai, China). The 24 nucleotide sequences of the YX10p90 and p102 strain were assembled into a complete genome sequence and compared with that of the YX10p5 strain using DNAstar software.

Table 2
Safety testing of IBV YX10 at different passages.

Group	Passage level/ vaccine/control	Dosage[a] (EID_{50})	Morbidity	Mortality
1	p5	$10^{4.5}$	12/12	3/12
2	p25	$10^{4.5}$	12/12	2/12
3	p58	$10^{4.5}$	9/12	1/12
4	p75	$10^{4.5}$	0/12	0/12
5	p90	$10^{4.5}$	0/12	0/12
6	p102	$10^{4.5}$	0/12	0/12
Vaccine	H120	$10^{4.5}$	0/12	0/12
Control	PBS		0/12	0/12

[a] Seven-day-old SPF chickens were inoculated with 10 doses of IBV (passaged virus $10^{5.5}$ EID_{50}/bird or vaccine strain H120 $10^{4.5}$ EID_{50}/bird) via the intranasal route.

3. Results

3.1. Virus passaging

As the IBV strain YX10 was passaged, the virus increasingly adapted to SPF chicken embryos. Consequently, lesions of SPF chicken embryos became more and more apparent, and times to death gradually shortened. In order to obtain the appropriate virus titer in the allantoic fluid, the incubation times were adjusted accordingly. Before the 50th passage, the incubation time was 42–48 h. After the 50th passage, the optimum incubation time was 34–40 h. Notably after the 70th passage, if the incubation time was more than 42 h, the allantoic fluid would become turbid with a low virus titer due to the deposition of urate crystals. Titers of the allantoic fluids ranged between $10^{6.0}$ and $10^{7.5}$ EID_{50}/0.1 ml.

3.2. Safety

With continuous passage, the virus became more pathogenic to SPF chicken embryos, while pathogenicity to SPF chickens declined (Table 2). When administered intranasally, YX10p5, YX10p25 and YX10p58 respectively induced 100% (12/12), 100% (12/12) and 75% (9/12) of morbidity, and 25% (3/12), 16.7% (2/12) and 8.3% (1/12) of mortality. YX10p75, YX10p90, YX10p102, H120 and the control did not cause any clinical sign or death.

3.3. Efficacy

3.3.1. Efficacy of different virus passages against IBV YX10p5 challenge

As shown in Table 3, the vaccination challenge test showed that the attenuated strains YX10p75 and YX10p90 could confer protection against challenge with the homologous virulent virus, pathogenic strain YX10p5, with no morbidity (0/12) or mortality (0/12). The chickens inoculated with the YX10p102 strain showed morbidity and mortality of 41.6% (5/12) and 8.3% (1/12), respectively. The chickens immunized with vaccine strain H120 showed 75% morbidity (9/12) and 75% mortality (9/12) post-challenge. The positive control chickens showed 100% (12/12) morbidity and 50% (6/12) mortality, and the negative control chickens showed no morbidity or mortality. Nephritis was observed in dead chickens and was characterized by enlarged, pale and marbled kidneys, frequently with urate deposits in the ureters and cloaca.

Serum samples were collected for ELISA antibody tests from 4-week-old (14 day post-vaccination) and 6-week-old (14 day post-challenge) chickens. Only 8.3–25% of the birds inoculated with YX10p75, YX10p90, YX10p102 or H120 showed a positive serum antibody response at 14 days post-vaccination, by contrast, 50–100% at 14 days post-challenge. Only 33.3% (2/6) of chickens in the positive control group showed a positive serum antibody response at 14 days post-challenge.

Table 3
Protective effects in chickens immunized with YX10p75, YX10p90, YX10p102 or H120 against challenge with virulent YX10p5.

Group	Vaccination at day 14	Dosage[a] (EID_{50})	Challenge at day 28[b]	Morbidity	Mortality	Protected	Positive rate of ELISA antibody[c]	
							14 d post-vaccination	14 d post-challenge
1	p75	$10^{3.5}$	Yes	0/12	0/12	12/12	3/12	9/12
2	p90	$10^{3.5}$	Yes	0/12	0/12	12/12	2/12	6/12
3	p102	$10^{3.5}$	Yes	5/12	1/12	7/12	1/12	6/11
Vaccine	H120	$10^{3.5}$	Yes	9/12	3/12	3/12	1/12	9/9
Positive control	PBS	NA	Yes	12/12	6/12	0/12	0/12	2/6
Negative control	PBS	NA	No	0/12	0/12	12/12	0/12	0/12

[a] Fourteen-day-old SPF chickens were inoculated with one dose of IBV (passaged virus $10^{3.5}$ EID_{50}/bird or vaccine strain H120 $10^{3.5}$ EID_{50}/bird) via the intraocular route.
[b] Two weeks after vaccination, chickens were challenged with IBV YX10p5 at $10^{5.5}$ EID_{50}/bird via the intranasal route.
[c] Number seroconverted/number inoculated.

Table 4
Protective effects in SPF chickens immunized with YX10p90 or commercial vaccines against challenge with IBV isolates representing the three major Chinese genotypes.

Immune group (vaccine strain)[a]	Challenge group	Challenge strain (genotype)[b]	Morbidity	Mortality
A (YX10p90)	A1	LZ12 (CH I)	1/12	1/12
	A2	GL12 (CH I)	0/12	0/12
	A3	YX10p5 (CH I)	0/12	0/12
	A4	YL12 (CH III)	4/12	2/12
	A5	ZS12 (CH V)	5/12	2/12
B (H120)	B1	LZ12 (CH I)	9/12	7/12
	B2	GL12 (CH I)	4/12	1/12
	B3	YX10p5 (CH I)	8/12	4/12
	B4	YL12 (CH III)	3/12	2/12
	B5	ZS12 (CH V)	5/12	1/12
C (control)	C1	LZ12 (CH I)	8/12	4/12
	C2	GL12 (CH I)	10/12	4/12
	C3	YX10p5 (CH I)	12/12	6/12
	C4	YL12 (CH III)	8/12	5/12
	C5	ZS12 (CH V)	7/12	5/12

[a] SPF chickens were inoculated with one dose of IBV (passage virus $10^{3.5}$ EID_{50}/bird or vaccine H120 $10^{3.5}$ EID_{50}/bird) via the intranasal route at 5 and 10 days of age.
[b] At 17 days old, chickens were challenged with five isolated IBV strains each at $10^{5.5}$ EID_{50}/bird via the intranasal route.

3.3.2. *Efficacy against challenge with strains representing major China IBV genotypes*

As shown in Table 4, chickens immunized with YX10p90 and commercial vaccine H120 were challenged with three isolated QX-like (CH I) genotype strains and two strains belonging to CH III and CH V genotype. Chickens inoculated with YX10p90 acquired protection against CH I genotype strains (LZ12, GL12 and YX10p5), showing morbidity and mortality rate of 0–8.3%. However, YX10p90 only provided partial protection to vaccinated chickens against challenge with CH III and CH V genotype strains (YL12 and ZS12), which resulted in morbidity rate of 33.3–41.7% and mortality rate of 16.6%. Sufficient protection was not provided by the commercial vaccine H120 against challenge with the five isolated strains, and the morbidity ranged from 25 to 75% and mortality rate of 8.3–58.3%. By comparison, the unvaccinated chickens (control) showed 58.3–100% morbidity and 33–50% mortality.

3.4. *Reversion to virulence*

IBV was detected in tissue homogenates of inoculated groups at every passage by RT-PCR, and chicken embryos inoculated with these tissue homogenates of every passage showed characteristic lesion of IBV: stunted and curled embryos with feather dystrophy (clubbing) and urate deposits in the mesonephros. These phenomena were not observed in control groups. During the observation period, no clinical signs, pathological change or deaths were observed in all chickens inoculated with tissue homogenates and the control chickens over five passages. These results indicate that no reversion to virulence of YX10p90 occurred in the chickens.

3.5. *Analysis of complete genome of YX10p90 and YX10p102*

In order to find correlations between specific genomic sequences and IBV pathogenicity, the complete genome of the YX10p90 strain and YX10p102 strain was sequenced, and then compared with that of virulent YX10p5. The complete genome of YX10p90 is 27,564 bp in length. The YX10p90 and YX10p5 exhibit nucleotide similarity as high as 99.9%. Comparing with YX10p5, thirty-eight nucleotide changes resulted in twenty-six amino acid substitutions (Table 5) and a 110-bp deletion located ten nucleotides downstream of the stop codon of the N gene in the 3′-untranslated region (3′-UTR) (Fig. 1) were observed in YX10p90. Among these twenty-six amino acid substitutions, twenty took place in the RNA polymerase protein (non-structure protein, NSP) encoded by gene 1, two in 3a protein (NSP), one in E protein (structure protein, SP) and three in S protein (SP). Three amino acid substitutions in S protein include two in S1 subunit and one in S2 subunit. The complete genome of YX10p102 is 27,564 bp, with the same length of YX10p90. When p102 genome sequence is compared with that of p90 four nucleotide substitutions are observed (Table 5). These nucleotide changes resulted in 3 amino acid substitutions: two in RNA polymerase protein and one in S1 subunit.

3.6. *Efficacy of polyvalent vaccines against challenge with strains representing major China IBV genotypes*

The chickens immunized with polyvalent vaccines were challenged with five isolated nephropathogenic IBV strains which represented major China IBV genotypes. As shown in Table 6, the renal protective effects of the polyvalent vaccine A against all individual challenge strains were obviously higher than those

Table 5
Nucleotide and deduced amino acid substitutions in complete genome of IBV YX10p5 and embryo-passaged derivatives.

Gene		Position (nt)[a]	Nucleotide substitution		Amino acid substitution	
			p5 → p90	p90 → p102	p5 → p90	p90 → p102
Gene 1	1a	371	C → T	None (C)	T → N	None (N)
		671	C → T	None (C)	S → L	None (L)
		939	T → C	None (T)	Silent (N)	None (N)
		947	C → T	None (C)	T → I	None (I)
		949	C → T	None (C)	Silent (L)	None (L)
		1710	T → C	None (T)	Silent (C)	None (C)
		2079	T → C	None (T)	Silent (V)	None (V)
		3840	T → C	None (T)	Silent (A)	None (A)
		4162	T → G	None (T)	C → R	None (R)
		4658	A → G	None (A)	K → R	None (R)
		5696	None (T)	T → G	None (M)	M → T
		6367	A → → T	None (A)	T → S	None (S)
		6872	C → T	None (C)	T → I	None (I)
		6902	A → G	None (A)	E → G	None (G)
		7356	T → A	None (T)	N → K	None (K)
		9995	C → A	None (A)	T → K	None (K)
		10797	A → G	None (A)	Silent (T)	None (T)
	1b	647	A → G	None (A)	Y → C	None (C)
		802	G → A	None (G)	V → I	None (I)
		1417	G → A	None (G)	Silent (R)	None (R)
		2535	G → T	None (G)	Silent (S)	None (S)
		3009	C → T	None (C)	S → F	None (F)
		3225	A → C	None (A)	S → R	None (R)
		3269	G → A	None (G)	G → S	None (S)
		4061	A → G	None (A)	N → S	None (S)
		4077	C → T	None (C)	Silent (R)	None (R)
		4083	C → T	None (C)	Silent (C)	None (C)
		4093	G → A	None (G)	V → I	None (I)
		5460	T → C	None (T)	Silent (D)	None (D)
		5852	A → T	None (A)	N → Y	None (Y)
		6155	T → C	None (T)	L → S	None (S)
		6630	C → T	None (C)	P → S	None (S)
		7405	G → A	None (G)	Silent (E)	None (E)
		7534	None (G)	G → A	None (V)	V → I
S gene	S1	202	G → A	None (G)	V → I	None (I)
		1125	None (C)	C → A	None (A)	Silent (A)
		1456	C → T	None (C)	L → F	None (F)
		1523	None (T)	T → G	None (I)	I → R
	S2	2654	C → T	None (C)	S → F	None (F)
Gene3	3a	95	A → T	None (A)	Q → L	None (L)
		99	T → A	None (T)	H → Q	None (Q)
	E	209	G → T	None (G)	R → I	None (I)

[a] Position of nucleotides from the AUG start codon in ORF of each gene.

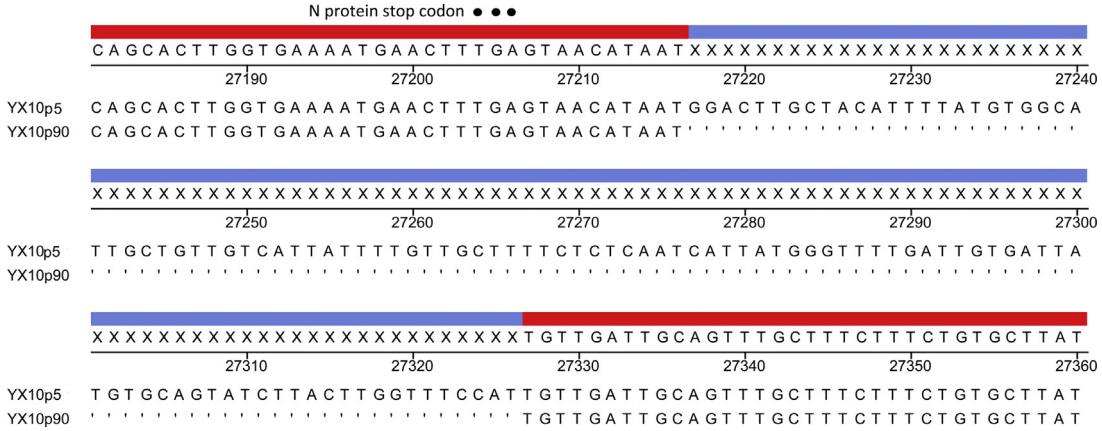

Fig. 1. Sequences of 3′-UTR downstream of the N protein gene of strain YX10p5 and YX10p90 were compared. A 110-bp deletion located 10 nucleotides downstream of the stop codon of the YX10N gene in the 3′-UTR during serial passage. The stop codon (●●●) of the N protein gene is indicated. Insertions and deletions within the sequences are shown with crosses (×××).

of the commercial polyvalent vaccines B and C. Polyvalent vaccine A could provide protection against nephropathogenic IBV strains. The morbidity and mortality rates of immune group A were lower than those of immune group B and C, and the protection provided by polyvalent vaccine A against the genotype CH I and CH II strains reached 100%. The protection against genotype CH III and CH V strains was improved compared with the univalent vaccine YX10p90. The total clinical protective rate of polyvalent vaccine A

Table 6
Protective effects in 2-week-old SPF chickens immunized with prepared and commercial polyvalent vaccines against challenge with IBV isolates representing the major Chinese genotypes.

Immune group (vaccine)[a]	Challenge group	Challenge strain (genotype)[b]	Renal protection[c]	Total renal protection[d]	Morbidity	Mortality	Total clinical protection[e]
Polyvalent vaccine A (YX10p90 + H120 + 4/91)	A1	LZ12 (CH I)	8/10	76%	0/10	0/10	90%
	A2	GL12 (CH I)	8/10		0/10	0/10	
	A3	NH10 (CH II)	7/10		0/10	0/10	
	A4	YL12 (CH III)	7/10		3/10	1/10	
	A5	ZS12 (CH V)	8/10		2/10	1/10	
Polyvalent vaccine B (H120 + 28/86 + 4/91)	B1	LZ12 (CH I)	5/10	52%	4/10	1/10	60%
	B2	GL12 (CH I)	5/10		3/10	1/10	
	B3	NH10 (CH II)	5/10		4/10	1/10	
	B4	YL12 (CH III)	6/10		4/10	3/10	
	B5	ZS12 (CH V)	5/10		5/10	1/10	
Polyvalent vaccine C (H120 + LDT3-A + 4/91)	C1	LZ12 (CH I)	6/10	48%	3/10	1/10	62%
	C2	GL12 (CH I)	6/10		3/10	1/10	
	C3	NH10 (CH II)	6/10		4/10	1/10	
	C4	YL12 (CH III)	3/10		5/10	2/10	
	C5	ZS12 (CH V)	3/10		4/10	2/10	
D (positive control)	D1	LZ12 (CH I)	0/10	0	10/10	3/10	0
	D2	GL12 (CH I)	0/10		10/10	3/10	
	D3	NH10 (CH II)	0/10		10/10	5/10	
	D4	YL12 (CH III)	0/10		10/10	4/10	
	D5	ZS12 (CH V)	0/10		10/10	4/10	
E (negative control)		None	10/10	100%	0/10	0/10	100%

[a] SPF chickens were inoculated with one dose of polyvalent vaccine via the intraocular route at 5 and 10 days of age.
[b] At 17 days old, chickens were challenged with five isolated IBV strain each at $10^{5.5}$ EID_{50}/bird via the intranasal route.
[c] Number renal lesions/number inoculated.
[d] Average renal protection of each immune group.
[e] Number without clinical symptom/number inoculated of each immune group.

was 90% compared with 60% for polyvalent vaccine B and 62% for polyvalent vaccine C.

4. Discussion

The broad variety of circulating serotypes and genotypes may contribute to the low cross-protection afforded by current IBV vaccine strains; hence, the best protective effect that can be achieved would be only against strains of the same serotype or genotype [19,20]. To date, most countries, which have carried out domestic epidemiological investigations of IBV, have chosen the dominant strain to develop live-attenuated vaccines [21,22]. A previous study showed that IBV strains of at least six genotypes were found in China, and the epidemic IBV strains are evolutionarily distant from the commercial vaccine strains of the Mass genotype. In recent years, the CH I (QX-like) genotype had become dominant and accounted for 60% of isolated IBV strains in China [23]. Therefore, we aimed to develop a novel live-attenuated vaccine from IBV YX10, which is a Chinese QX-like nephropathogenic strain.

In this study, we attenuated the virulent YX10 strain by passaging in fertilized SPF eggs. Related research has shown that the virulence and pathogenicity of IBV strains are closely related to their passage level. The viruses at initial passages seldom cause the death of chicken embryos. Over multiple passages, the virulence to chicken embryos and virus titer are known to be obviously enhanced, while the virulence to chickens is weakened. In the present study, as the virulent YX10 strain was passaged, the virus caused increasing mortality of embryos and significant pathological changes such as curled and dwarfed embryos, while the virus titer ranged from $10^{6.0}$ to $10^{7.5}$ EID_{50}/0.1 ml.

In order to evaluate the safety of the passaged viruses, 15-day-old SPF chickens were inoculated intranasally with 10 doses of virus from different passages, and their clinical signs were observed. Passage strains YX10p5, YX10p25 and YX10p58 caused varying degrees of morbidity and mortality, while none of the clinical signs or death was observed in chickens inoculated with YX10p75, YX10p90 or YX10p102. On the whole, the morbidity and mortality declined as the passage level increased, suggesting enhanced safety.

Based on their safety profiles, the passage strains YX10p75, YX10p90 and YX10p102 were selected to carry out efficacy testing. Birds inoculated with these three strains separately were challenged with the virulent YX10p5 strain. Chickens inoculated with YX10p75 or YX10p90 were 100% protected without morbidity and mortality, indicating that these two passage strains preserved immunogenicity and could provide protection against challenge with a homologous virulent virus. Excessive passaging has been reported to decrease immunogenicity and lead to poor protective effects. These outcomes were confirmed in the current study, as the chickens inoculated with YX10p102 showed morbidity and mortality rates of 41.6% (5/12) and 8.3% (1/12), respectively. The high morbidity and mortality of the H120 vaccinated group and the positive control group also illustrated the poor protective effects of Mass-type vaccines against challenge with QX-like isolated strains. In the evaluation of efficacy, the ELISA antibody positive rates of all experimental group including p75 and p90 group were low post-vaccination, but the protection provided by p75 and p90 strain was effective. This finding was predictable and typical for chickens vaccinated against IBV. Humoral immunity is not a determining factor for protection against IBV infection, and circulating antibody titers may not positively correlate with anti-IBV effects [24]. Local antibody responses and cell-mediated immunity both play significant roles in protection against IBV infection [25]. However, surveillance of specific IgG antibodies remains an important method for evaluating immune responses to an IBV vaccine [26].

In subsequent efficacy tests, in order to study the cross-protective effects of YX10p90 against other genotype strains, YX10p5 and other four isolated strains belonging to three major Chinese genotypes (CH I, CH III and CH V) were selected as challenge viruses. YX10p90 provided varying degrees of cross-protection

against the major Chinese genotype strains. The YX10p90 immunized chickens showed effective protection (91.7–100%) against CH I genotype strains (YX10p5, LZ12 and GL12), but partial protection (58.3–66.7%) against CH III and CH V genotype strains (YL12 and ZS12). Thus, developing polyvalent vaccines is a means of improving the cross-protection of IBV vaccine strains and controlling the complex IBV epidemic in China. The polyvalent vaccine A prepared in this study contained the combination of strains YX10p90, H120 and 4/91. Compared with the two commercial polyvalent vaccines B and C, polyvalent vaccine A provided higher levels of renal protection and clinical protection. Importantly, the polyvalent vaccine formulation expanded the level of cross-protection over that individual vaccine strains achieved. However, it was reported that the use of polyvalent live vaccine may contribute to the variation and recombination of IBV, and bring about more complex IBV epidemic [15,27,28], therefore, the biological safety of polyvalent live vaccine need to be further verified.

Testing for reversion to virulence is important for evaluating an attenuated virus due to a potential for the formation of variant viruses by recombination with field strains. In this study no reversion to a virulent IBV strain was observed after five passages in chickens of the attenuated YX10p90 strain.

Correlating pathogenicity with specific viral gene sequences is a major focus of IBV research. The S1 glycoprotein plays an important role in tissue tropism, attachment to the host cell and induction of neutralizing antibodies. Therefore, many studies on IBV pathogenicity have been limited to the S1 gene [1,29,30]. Several amino acid changes at the N-terminus of the S protein have been shown to result in the change of tissue tropism and virulence of a coronavirus [19,31,32]. With the development of reverse genetic manipulations, the S1 glycoprotein was found to not affect the virulence of IBV [33,34]. In previous pathogenicity experiment of YX10p5 (date not shown), samples (kidney, trachea, lung, thymus, bursa and proventriculus) were collected from diseased and dead chickens for virus recovery. The kidney and trachea were the main tissues and organs for virus recovery rate and the recovery rate (100%) was much higher than other tissues. In this study, virus was always recovered from samples of kidney and trachea in virulence reversion test of YX10p90. It suggested that 26 amino acids substitutions, including 3 in S protein, and 110-bp deletion in 3′-UTR do not alter main tissue tropism but attenuate the pathogenicity and virulence of IBV. Cavanagh et al. [35,36] reported that gene 1 (replicase gene) of IBV is a determinant of pathogenicity. Huang and Wang [37] found a 49-bp deletion in the 3′-UTR immediately downstream from the N protein at passage 76 of strain TW2296/95 that is not present in the pathogenic parent. Liu et al. [38] found a 109-bp deletion located 8 nucleotides downstream of the stop codon of the N gene in the 3′-UTR during attenuation of QX-like IBV strain CK/CH/LHLJ/04V by passaging in fertilized SPF eggs. Notably, the length and location of the 110-bp deletion in the 3′-UTR of YX10p90 was very similar to the 109-bp deletion during attenuation of QX-like IBV strain CK/CH/LHLJ/04V reported by Liu et al. [38]. It was noticed that YX10p102 strain was less capable to induce a protective immune response compared to p90 in efficacy test. Complete genome sequence analysis showed that there were 4 nucleotide differences between p90 and p102, and these differences could bring about 3 amino acid substitutions. It is speculated that these 3 amino acid substitutions may contribute to the further decrease of virulence and immunogenicity of YX10p102 strain. However, it is not easy to conclude that certain gene related to the virulence or immunogenicity of IBV, or certain nucleotide mutation could bring about attenuation. Identifying specific changes involved in the attenuation, virulence and immunogenicity of IBV will require further investigation by reverse genetics and animal studies to verify the exact function of the amino acid substitutions, insertion and deletion in the IBV genome.

The CVP (2010) defines the safety and efficacy of IBV vaccines. The guideline for safety tests states that if none of 10 vaccinated 7-day-old SPF chickens show respiratory signs, nervous system signs and/or death, the vaccine is considered to be safe. The efficacy test states that at least 80% of immunized chickens be free of clinical signs, the vaccine is considered to be effective. Based on our evaluations of the safety, efficacy and potential for reversion to virulence, the IBV strain of YX10p90 conforms to these guidelines of the CVP (2010), exhibiting a fine balance between attenuation and immunogenicity. Thus, YX10p90 may be considered as a vaccine candidate to prevent infection by the Chinese QX-like nephropathogenic IBV.

Authors' contributions

Keyu Feng and Yu Xue carried out most of the experiments and wrote the manuscript, and contributed equally to this work, should be considered as first authors. Jinglan Wang and Weiguo Chen helped with the experiment. Feng Chen, Yingzuo Bi and Qingmei Xie critically revised the manuscript and designed the experiment. All of the authors read and approved the final version of the manuscript.

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References

[1] Cavanagh D. Coronavirus avian infectious bronchitis virus. Vet Res 2007;38(2):281–97.
[2] Cook JKA, Jackwood M, Jones RC. The long view: 40 years of infectious bronchitis research. Avian Pathol 2012;41(3):239–50.
[3] Jackwood MW. Review of infectious bronchitis virus around the world. Avian Dis 2012;56(4):634–41.
[4] Yu L, Jiang Y, Low S, Wang Z, Nam SJ, Liu W, et al. Characterization of three infectious bronchitis virus isolates from China associated with proventriculus in vaccinated chickens. Avian Dis 2001;45(2):416–24, 2001-04-01.
[5] Cavanagh D, Davis PJ, Cook JKA, Li D, Kant A, Koch G. Location of the amino acid differences in the S1 spike glycoprotein subunit of closely related serotypes of infectious bronchitis virus. Avian Pathol 1992;21(1):33–43, 1992-01-19.
[6] Stern DF, Sefton BM. Coronavirus proteins: biogenesis of avian infectious bronchitis virus virion proteins. J Virol 1982;44(3):794–803, 1982-12-01.
[7] Cavanagh D, Davis PJ, Darbyshire JH, Peters RW. Coronavirus IBV: virus retaining spike glycopolypeptide S2 but not S1 is unable to induce virus-neutralizing or haemagglutination-inhibiting antibody, or induce chicken tracheal protection. J Gen Virol 1986;67(Pt 7):1435–42, 1986-07-01.
[8] Duffy S, Shackelton LA, Holmes EC. Rates of evolutionary change in viruses: patterns and determinants. Nat Rev Genet 2008;9(4):267–76, 2008-04-01.
[9] Cowen BS, Hitchner SB. Serotyping of avian infectious bronchitis viruses by the virus-neutralization test. Avian Dis 1975;19(3):583–95, 1975-07-01.
[10] Li L, Xue C, Chen F, Qin J, Xie Q, Bi Y, et al. Isolation and genetic analysis revealed no predominant new strains of avian infectious bronchitis virus circulating in South China during 2004–2008. Vet Microbiol 2010;143(2–4):145–54, 2010-07-14.
[11] Ji J, Xie J, Chen F, Shu D, Zuo K, Xue C, et al. Phylogenetic distribution and predominant genotype of the avian infectious bronchitis virus in China during 2008–2009. Virol J 2011;8(1):184, 2011-01-20.
[12] Luo H, Qin J, Chen F, Xie Q, Bi Y, Cao Y, et al. Phylogenetic analysis of the S1 glycoprotein gene of infectious bronchitis viruses isolated in China during 2009–2010. Virus Genes 2012;44(1):19–23, 2012-02-01.
[13] Xie Q, Ji J, Xie J, Chen F, Cai M, Sun B, et al. Epidemiology and immunoprotection of nephropathogenic avian infectious bronchitis virus in southern China. Virol J 2011;8(1):484, 2011-01-20.
[14] Feng K, Xue Y, Wang F, Chen F, Shu D, Xie Q. Analysis of S1 gene of avian infectious bronchitis virus isolated in southern China during 2011–2012. Virus Genes 2014;49(2):292–303, 2014-10-01.
[15] Liu SW, Zhang QX, Chen JD, Han ZX, Liu X, Feng L, et al. Genetic diversity of avian infectious bronchitis coronavirus strains isolated in China between 1995 and 2004. Arch Virol 2006;151(6):1133–48, 2006-06-01.
[16] Liu S, Zhang X, Wang Y, Li C, Han Z, Shao Y, et al. Molecular characterization and pathogenicity of infectious bronchitis coronaviruses: complicated evolution and epidemiology in China caused by cocirculation of multiple types of infectious bronchitis coronaviruses. Intervirology 2009;52(4):223–34, 2009-01-20.

[17] Xue Y, Xie Q, Yan Z, Ji J, Chen F, Qin J, et al. Complete genome sequence of a recombinant nephropathogenic infectious bronchitis virus strain in China. J Virol 2012;86(24):13812–3, 2012-12-15.
[18] Reed LJ, Muench H. A simple method of estimating fifty per cent endpoints. Am J Epidemiol 1938;27(3):493–7.
[19] Hofstad MS. Cross-immunity in chickens using seven isolates of avian infectious bronchitis virus. Avian Dis 1981;25(3):650–4, 1981-07-01.
[20] Cook JK, Orbell SJ, Woods MA, Huggins MB. A survey of the presence of a new infectious bronchitis virus designated 4/91 (793B). Vet Rec 1996;138(8):178–80, 1996-02-24.
[21] Bijlenga G, Cook JKA, Gelb JJ, Wit JJD. Development and use of the H strain of avian infectious bronchitis virus from the Netherlands as a vaccine: a review. Avian Pathol 2004;33(6):550–7, 2004-12-01.
[22] Huang Y, Wang C. Development of attenuated vaccines from Taiwanese infectious bronchitis virus strains. Vaccine 2006;24(6):785–91, 2006-02-06.
[23] Han Z, Sun C, Yan B, Zhang X, Wang Y, Li C, et al. A 15-year analysis of molecular epidemiology of avian infectious bronchitis coronavirus in China. Infect Genet Evol 2011;11(1):190–200, 2011-01-01.
[24] de Wit JJ, Davelaar FG, Braunius WW. Comparison of the enzyme linked immunosorbent assay, the haemagglutination inhibition test and the agar gel precipitation test for the detection of antibodies against infectious bronchitis and Newcastle disease in commercial broilers. Avian Pathol 1992;21(4):651–8, 1992-12-01.
[25] Seo SH, Collisson EW. Specific cytotoxic T lymphocytes are involved in in vivo clearance of infectious bronchitis virus. J Virol 1997;71(7):5173–7, 1997-07-01.
[26] Toro H, Espinoza C, Ponce V, Rojas V, Morales MA, Kaleta EF. Infectious bronchitis: effect of viral doses and routes on specific lacrimal and serum antibody responses in chickens. Avian Dis 1997;41(2):379–87.
[27] Sjaak De Wit JJ, Cook JKA, van der Heijden HMJF. Infectious bronchitis virus variants: a review of the history, current situation and control measures. Avian Pathol 2011;40(3):223–35.
[28] Cavanagh D. Coronaviruses in poultry and other birds. Avian Pathol 2005;34(6):439–48, 2005-12-01.
[29] Kusters JG, Jager EJ, Niesters HG, van der Zeijst BA. Sequence evidence for RNA recombination in field isolates of avian coronavirus infectious bronchitis virus. Vaccine 1990;8(6):605–8, 1990-12-01.
[30] Ignjatovic J, Galli U. Immune responses to structural proteins of avian infectious bronchitis virus. Avian Pathol 1995;24(2):313–32, 1995-06-01.
[31] Ballesteros ML, Sánchez CM, Enjuanes L. Two amino acid changes at the N-terminus of transmissible gastroenteritis coronavirus spike protein result in the loss of enteric tropism. Virology 1997;227(2):378–88, 1997-01-20.
[32] Sanchez CM, Izeta A, Sanchez-Morgado JM, Alonso S, Sola I, Balasch M, et al. Targeted recombination demonstrates that the spike gene of transmissible gastroenteritis coronavirus is a determinant of its enteric tropism and virulence. J Virol 1999;73(9):7607–18, 1999-09-01.
[33] Casais R, Dove B, Cavanagh D, Britton P. Recombinant avian infectious bronchitis virus expressing a heterologous spike gene demonstrates that the spike protein is a determinant of cell tropism. J Virol 2003;77(16):9084–9, 2003-08-15.
[34] Britton P, Armesto M, Cavanagh D, Keep S. Modification of the avian coronavirus infectious bronchitis virus for vaccine development. Bioengineered 2014;3(2):114–9, 2012-03-01.
[35] Cavanagh D, Casais R, Armesto M, Hodgson T, Izadkhasti S, Davies M, et al. Manipulation of the infectious bronchitis coronavirus genome for vaccine development and analysis of the accessory proteins. Vaccine 2007;25(30):5558–62.
[36] Armesto M, Cavanagh D, Britton P. The replicase gene of avian coronavirus infectious bronchitis virus is a determinant of pathogenicity. PLoS ONE 2009;4(10):e7384, 2009-10-09.
[37] Huang Y, Wang C. Sequence changes of infectious bronchitis virus isolates in the 3′ 7.3 kb of the genome after attenuating passage in embryonated eggs. Avian Pathol 2007;36(1):59–67, 2007-02-01.
[38] Liu S, Zhang X, Gong L, Yan B, Li C, Han Z, et al. Altered pathogenicity, immunogenicity, tissue tropism and 3′–7 kb region sequence of an avian infectious bronchitis coronavirus strain after serial passage in embryos. Vaccine 2009;27(34):4630–40.