



Altered pathogenicity, immunogenicity, tissue tropism and 3'-7 kb region sequence of an avian infectious bronchitis coronavirus strain after serial passage in embryos

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ABSTRACT

In this study, we attenuated a Chinese LX4-type nephropathogenic infectious bronchitis virus (IBV) strain, CK/CH/LHLJ/04V, by serial passage in embryonated chicken eggs. Based on sequence analysis of the 3'-7 kb region, the CK/CH/LHLJ/04V virus population contained subpopulations with a mixture of genetic mutants. The titers of the virus increased gradually during serial passage, but the replication capacity decreased in chickens. The virus was partially attenuated at passage 40 (P40) and P70, and was fully attenuated at P110. It lost immunogenicity and kidney tropism at P110 and P70, respectively. Amino acid substitutions were found in the 3'-7 kb region, primarily in the spike (S) protein. Substitutions in the S1 subunit occurred between P3 and P40 and all subpopulations in a virus passage showed the same substitutions. Other substitutions that occurred between P70 and P110, however, were found only in some subpopulations of the virus passages. A 109-bp deletion in the 3'-UTR was observed in most subpopulations of P70 and P110, and might be related to virus replication, transcription and pathogenicity. The changes described in the 3'-7 kb region of the virus are possibly responsible for virus attenuation, immunogenicity decrease and tissue tropism changes; however, we cannot exclude the possibility that other parts of the genome may also be involved in those changes.

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1. Introduction

Coronaviruses belong to the family *Coronaviridae* and the order *Nidovirales*, and are classified into three groups based on the absence of genetic and antigenic relationships between the species of the different groups [1,2]. They are known to cause upper and lower respiratory diseases, gastroenteritis, and central nervous system infection in a number of avian and mammalian hosts, including humans [3]. The infectious bronchitis virus (IBV) belongs to the group 3 coronaviruses. It primarily causes respiratory disease in domestic fowl, although it also replicates on epithelial surfaces of the alimentary tract, oviduct, and kidney, and is one of the most economically important pathogens in the poultry industry [4]. IBV has four essential structural proteins: the phosphorylated nucleocapsid (N) protein, and the three membrane proteins spike (S), integral membrane (M), and small envelope (E). Although the S1 subunit of the S protein carries virus-neutralizing and serotype-specific deter-

minants, the S2 subunit may also induce neutralizing antibodies, and IBV strains can be grouped by the sequence of S2 [5]. The N gene and N-terminal region of the IBV M protein also vary between strains [6]. Furthermore, mutations and recombination events have been observed in multiple structural genes of IBV recovered from naturally occurring infections. Interspersed among the structural protein genes are genes 3 and 5, for two small accessory proteins [7]. These vary in number and sequence among the IBVs [2,6,8,9]. Gene 3 is functionally tricistronic [10], with three ORFs, 3a, 3b, and 3c. ORFs 3a and 3b encode two small accessory proteins of unknown function, and the structural protein E is encoded by ORF 3c [10–12]. Neither the RNA nor the proteins of ORFs 3a and 3b are essential for replication [13,14].

Live-attenuated infectious bronchitis (IB) vaccines have been used worldwide since the 1950s. Because of the many IBV serotypes, different multiple live-attenuated IB vaccines are in use around the world. IBV vaccines are attenuated by multiple serial passages, generally 52 or more, in embryonated eggs [15–18]. During this process, selective pressure results in the adaptation of the virus population to its new host, the embryo, reflected by more efficient replication and higher lethality to the embryo. In addition, during passages in

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embryonated eggs, recombination between virus subpopulations, and accumulation of mutations in the S1 region can lead to the formation of attenuated viruses [19].

The importance of S1 in determining cell and tissue tropism has been demonstrated for several coronaviruses, such as murine hepatitis virus [20–28], porcine transmissible gastroenteritis virus [29,30], and severe acute respiratory syndrome coronavirus [31]. In the case of IBV, the S1 subunit of the S protein determines the serotype of IBV and is responsible for viral attachment to cells. Furthermore, it has been shown that S1 is a major determinant of cell tropism in culture [32], and the majority of changes accumulated during the adaptation of IBV to Vero cells are in the S gene [13]. However, differences in one or more other genes are responsible for the highly attenuated phenotype of the Beaudette IBV laboratory strain [13], and the roles of these gene products in the attenuation process have yet to be determined.

In spite of the extensive use of vaccines, nephrotropic IBV outbreaks are frequent in China [33–36]. LX4-type has been the predominant IBV type in China in recent years [34–36], and appears to have become widespread in several countries in Europe, causing severe losses to both the layer and broiler industries [6]. In addition, this type of IBV has increased in recent years in both China and European countries; thus, the development of an efficacious live-attenuated vaccine against LX4-type IBV is important. We are developing an IB vaccine by serial passage of the IBV strain CK/CH/LHLJ/04V, which represents the LX4-type, in embryonated eggs. Evaluating the attenuation, the growth of viruses in embryos, the efficacy in poultry populations, and the changes in molecular characteristics after serial passage were the primary focus and objectives of the present study. In a future study, practical considerations regarding the development of such a vaccine will be examined.

2. Materials and methods

2.1. Viruses

We used a virulent IBV strain, CH/CK/LHLJ/04V, which was previously isolated during an outbreak of IB in 2004 at a broiler farm in the Heilongjiang province in China. Clinical signs and lesions observed during the outbreak included nephritis and mortality. The virus was isolated from the kidney of a dead broiler using 9-day-old embryonated specific pathogen-free (SPF) chicken eggs. The IBV strain was identified by means electron microscope examination, reverse transcriptase-polymerase chain reaction (RT-PCR) and sequencing of the entire S1 protein gene, as described previously [34]. An analysis of the molecular characteristics showed that this virus exhibited limited homology (not more than 83% of amino acids) to genotypes representing the vaccine strains H120 and W93 [34]. Phylogenetic analysis showed that this virus was an LX4-type [34]. The virus stock for this study was produced by inoculating the virus into embryonated SPF chicken eggs via the allantoic cavity and collecting the infectious allantoic fluid 72 h post-inoculation. The allantoic fluid was clarified by centrifugation at $3000 \times g$ for 10 min and filtered with a Teflon membrane. In addition, three IBV strains, CK/CH/LDL/04II, CK/CH/LXJ/02I and CK/CH/LSHH/03I, were used as references for the pathogenicity study; their backgrounds and types were reported previously [34].

2.2. Eggs and chicks

Fertile White Leghorn SPF chicken eggs, and White Leghorn SPF chicks were obtained from the Laboratory Animal Center, Harbin Veterinary Research Institute, the Chinese Academy of Agricultural Sciences, China. The birds were maintained in isolators with negative pressure, and food and water were provided *ad libitum*.

2.3. Pathogenicity study

Fifty-one-day-old White Leghorn SPF chickens were used to assess the pathogenicity of the CK/CH/LHLJ/04V strain. Five groups of 10 chickens were kept in isolators with negative pressure. At the age of 15 days, groups 1–4 were inoculated intranasally with 0.1 ml per chick containing $10^{4.7}$ – $10^{4.8}$ median embryo infectious doses (EID₅₀) at passage level 3 of strains CH/CK/LHLJ/04V, CK/CH/LDL/04II, CK/CH/LXJ/02I and CK/CH/LSHH/03I. Group 5 was mock-inoculated with sterile allantoic fluid and served as a control (Table 1). The chicks were examined daily for signs of infection for 30 days after inoculation. Tracheal swabs and blood samples were collected from all 10 birds in each treatment groups at 4, 8, 12, 16 and 20 days post-inoculation. Serum was stored at -70°C until ELISA testing was performed. The tracheal swabs were used for virus recovery attempts in embryonated chicken eggs.

2.4. Attenuation

The CH/CK/LHLJ/04V strain was serially passaged 110 times by inoculating 9-day-old SPF chicken eggs by the allantoic cavity route as described previously [19]. Inoculated eggs were incubated for 48–72 h at 37°C in an egg incubator (Heraeus, Germany). The chorioallantoic fluids were harvested and stored at -70°C or used directly for subsequent passage. At every 15th passage starting with passage 30, the virus was examined for viability by inoculation of two to three additional eggs for 7 days and observation of the embryos for clinical signs consistent with IBV infection. In addition, these selected passages were examined by negative contrast electron microscopy (JEM-1200, EX) for the presence of coronavirus, and by reverse transcriptase-polymerase chain reaction (RT-PCR) and sequencing [34] to verify the virus type. Passage 3 (P3) for the pathogenic strain, and P40, P70 and P110 were examined in more detail. The viruses of these four passages were propagated once in 10-day-old embryonated SPF chicken eggs as described for field isolates [34], to obtain titers of 10^6 – 10^8 EID₅₀ per 0.1 ml. Before use, viruses from the allantoic fluids of inoculated eggs were confirmed by negative contrast electron microscopy and by RT-PCR and sequencing.

2.5. Experimental design

2.5.1. Experiment 1

One hundred fifteen, 1-day-old SPF White Leghorn chicks were housed in different isolators and divided into five groups. Groups 1–4 had 25 birds each, and group 5 included 15 birds. Chickens in groups 1–4 were inoculated with P3, P40, P70 and P110, respectively, by ocular application at 15 days of age with a dose of $\log_{10}^{4.7}$ – $\log_{10}^{4.8}$ EID₅₀ per chick (Table 2). Birds in group 5 were mock-inoculated with sterile allantoic fluid and served as the control. Five birds from each group were killed humanely 5 days post-inoculation. The trachea and kidney were collected for virus titration. Blood samples from 10 birds in each group were collected at 4, 8, 12, 16 and 20 days post-inoculation. The serum was stored at -70°C for ELISA testing. The chicks were examined daily for signs of infection for 30 days after inoculation.

2.5.2. Experiment 2

Ninety, 1-day-old SPF White Leghorn chicks were housed in different isolators and divided into five groups. Groups 1–3, and the positive control group, had 20 birds each, and the negative control group included 10 birds. Chickens in groups 1–3 were inoculated with P40, P70 and P110, respectively, by ocular application at 15 days of age with a dose of $\log_{10}^{4.7}$ – $\log_{10}^{4.8}$ EID₅₀ per chick (Table 3). Birds in the positive and negative control groups were mock-inoculated with sterile allantoic fluid. At 20 days post-

Table 1
Results of the pathogenicity study using four IBV strains.

Group ^a	Dose, median embryo infectious doses (log 10) ^b	Morbidity (%)	Mortality (%)	Virus recovery ^c					Antibody (%) ^d				
				4 ^e	8	12	16	20	4 ^e	8	12	16	20
CK/CH/LHLJ/04V ^f	4.8	100	40	+	+	+	–	–	0/9(0)	6/7(86)	6/6(100)	6/6(100)	6/6(100)
CK/CH/LDL/04II	4.7	100	30	+	+	+	–	–	0/9(0)	7/7(100)	7/7(100)	7/7(100)	7/7(100)
CK/CH/LXJ/02I	4.8	100	20	+	+	–	–	–	3/10(30)	8/8(100)	8/8(100)	8/8(100)	8/8(100)
CK/CH/LSHH/03I	4.8	100	20	+	–	–	–	–	0/10(0)	8/8(100)	8/8(100)	8/8(100)	8/8(100)
Control	–	0	0	–	–	–	–	–	0/10(0)	0/10(0)	0/10(0)	0/10(0)	0/10(0)

^a Ten chicks per group.^b Dose per chick, 100 µL.^c Two procedures were used for virus recovery after challenge. First, lesions in embryos that had been inoculated with pooled samples (tracheal swabs) were observed. Second, RT-PCR using oligonucleotide primers N(+) and N(–) on RNA recovered from allantoic fluid of the same eggs was conducted. The results from the two procedures were identical.^d Number seroconverted/number inoculated.^e Days after challenge.^f CK/CH/LHLJ/04V passage 3 (P3) was used for the virulence study.**Table 2**
Pathogenicities of CK/CH/LHLJ/04V P3, P40, P70 and P110 to SPF chickens.

Passage ^a	Dose, median embryo infectious doses (log 10) ^b	Morbidity (%)	Mortality (%)	Antibody (%) ^c			
				4 ^{d,e}	8	12	16
P3	4.8	20/20(100)	8/20(40)	0/10(0)	8/10(80)	10/10(100)	10/10(100)
P40	4.8	20/20(100)	2/20(10)	0/10(0)	3/10(30)	10/10(100)	10/10(100)
P70	4.7	10/20(50)	0/20(0)	0/10(0)	4/10(40)	7/10(70)	9/10(90)
P110	4.7	0/20(0)	0/20(0)	0/10(0)	0/10(0)	4/10(40)	4/10(50)
Control	–	0/10(0)	0/10(0)	0/10(0)	0/10(0)	0/10(0)	0/10(0)

^a Twenty-five chicks in groups 1–4 and fifteen chicks in group 5.^b Dose per chick, 100 µL.^c Number seroconverted/number inoculated.^e Days after challenge.

inoculation, birds in groups 1–3 and in the positive control group were challenged by ocular/nasal application with 10^{4.8} EID₅₀/0.1 ml of pathogenic CH/CK/LHLJ/04V virus, while the birds in the negative control group were mock-inoculated again with sterile allantoic fluid. Ten birds each from groups 1–3 and the positive control group, and 5 birds from the negative control group were killed humanely 5 days post-challenge. The trachea and kidney were collected for virus recovery. Blood samples from 10 birds in each group were collected at 4, 8, 12, 16 and 20 days after challenge. The serum was stored at –70 °C for ELISA testing. The chicks were examined daily for signs of infection for 30 days after inoculation.

2.6. Virus titration, recovery and detection

The virus stocks used for the pathogenicity study, and the tissue samples of tracheas and kidneys collected 5 days post-inoculation from Experiment 1, were used for RT-PCR amplification and virus

titration. Tissue samples were homogenized individually and RT-PCR was conducted using primers N(+) and N(–) as described previously [9]. Virus titrations were performed in 9-day-old embryonated chicken SPF eggs via the allantoic cavity route of inoculation, and titers were expressed as 50% (median) embryo infectious doses (EID₅₀) [9,37]. Serial 10-fold dilutions were used for titrations. At each dilution, five embryos received 0.1 ml inoculum. The embryos were candled daily and examined for one week; those showing characteristic IBV lesions, such as dwarfing, stunting, or curling of embryos, were recorded as infected by IBV.

The 10 swab samples taken in the pathogenicity study from each group at each time point were pooled, and the tissue samples of tracheas and kidneys collected 5 days post-challenge from Experimental 2 were homogenized individually for virus isolation. For virological examination, the pooled samples were clarified by centrifugation at 300 × g for 5 min and filtered with a Teflon membrane. For virus isolation from the trachea and kidney, individual samples

Table 3
Results of vaccination-challenge test by CK/CH/LHLJ/04V P40, P70 and P110.

Group ^a	Morbidity (%) ^b	Mortality (%) ^b	Antibody response ^c				Virus recovery ^d	
			4 ^e	8	12	16	Trachea	Kidney
P40	0/10(0)	0/10(0)	10/10(100)	10/10(100)	10/10(100)	10/10(100)	0/10(0)	0/10(0)
P70	0/10(0)	0/10(0)	10/10(100)	10/10(100)	10/10(100)	10/10(100)	1/10(10)	0/10(0)
P110	3/10(30)	2/10(20)	8/10(80)	10/10(100)	10/10(100)	10/10(100)	6/10(60)	5/10(50)
Positive control	10/10(100)	4/10(40)	0/10(0)	5/7(70)	6/6(100)	6/6(100)	10/10(100)	10/10(100)
Negative control	0/5(0)	0/5(0)	0/10(0)	0/5(0)	0/5(0)	0/5(0)	0/5(0)	0/5(0)

^a Twenty chicks in groups P40, P70, P110 and the positive control group, and 10 chicks in the negative control group.^b The morbidity and mortality were those of P40, P70 and P110 vaccinated chickens after challenge.^c Number seroconverted/number inoculated.^d Two procedures were used for virus recovery after challenge. First, lesions in embryos that had been inoculated with individual tissue samples (trachea or kidney) were observed. Second, RT-PCR using oligonucleotide primers N(+) and N(–) on RNA recovered from allantoic fluid of the same eggs was conducted. The results from the two procedures were identical.^e Days after challenge.

Table 4

Sequence and position of the oligonucleotides used in RT-PCR.

Oligonucleotide	Sense ^a	Sequence (5'–3')	Gene	Position in genome ^b
IBV-257	+	TATTGATTAGAGATGTGG	S1	20356–20373
S1Oligo3'	–	CATACTAACATAAGGGCAA	S1	22002–22021
IBV-167	+	GCTTCTGAGAA(T/C)CAGTTT	S2, gene 3 and partial M (5')	21921–21941
IBV-281	–	GCCACTGACC(C/A)TCACAATAAAG	S2, gene 3 and partial M (5')	24955–24976
IBV-280	+	CCC(C/A)GAATCTAATGCCGTAGG	Partial M (3'), gene 5 and partial N (5')	24846–24866
IBV-171	–	AACCAAGATGCATTCCAGA	Partial M (3'), gene 5 and partial N (5')	25960–25979
N(+)	+	GACGCCCCAGCGCCAGTCATTA	N and partial 3'-UTR	25903–25926
N(–)	–	ACGCGGAGTACGATCGAGGTACA	N and partial 3'-UTR	27484–27507

^a Negative-sense (–) or positive-sense (+) primer.^b The nucleotide positions correspond to the sequence of the IBV Beaudette genome, GenBank accession number M95169.

were homogenized, diluted 1:10 with PBS, clarified by centrifugation at $300 \times g$ for 5 min and filtered with a Teflon membrane. The filtered samples were inoculated into at least four SPF embryonated eggs via the allantoic cavity (0.2 ml per egg). The eggs were candled daily to record embryo mortality, and allantoic fluid from two of the inoculated embryos was collected 72 h post-inoculation for RT-PCR amplification. After 7 days, the remaining embryos were chilled at 4 °C and examined for characteristic IBV lesions such as the dwarfing, stunting, or curling of embryos. Embryo mortality recorded in the first 24 h post-inoculation was considered non-specific. Samples were considered negative if the embryos did not show lesions after three blind passages of 7-day duration. A positive sample was recorded if the specific lesions were observed and the RT-PCR amplification was positive.

2.7. Serum antibody detection

Serum samples were assayed using a commercial total antibody ELISA (IDEXX Corporation, Westbrook, Maine, USA) according to the manufacturer's instructions. Each sample was usually tested in triplicate. Serum-to-positive ratios (S/P ratios) were calculated as described previously [38,39]. Individual serum titers were calculated from these S/P ratios, evaluated as positive or negative, and expressed as OD_{650 nm} values according to the manufacturer's instructions.

2.8. Cloning and sequencing of the 3'-7 kb region of CK/CH/LHLJ/04V strains

The strategy for cloning the 3'-7 kb region of CK/CH/LHLJ/04V strains was described previously [9]. Briefly, four fragments spanning the 3'-7.8 kb region of the IBV genome were obtained by RT-PCR from each of the four virus passage levels. The sequences and locations of the primers used in this study are in Table 4.

Briefly, viral RNA was extracted from 200 µl of allantoic fluid from P5, P40, P70 and P110 virus stocks using TRIzol reagent (Invitrogen, Grand Island, USA) according to the manufacturer's instructions. RNA was air-dried for 2–10 min, re-dissolved in 30 µl RNase-free water and stored at –70 °C until use. Reverse transcription (RT) was performed with M-MLV Reverse Transcriptase (Invitrogen, Grand Island, USA) using the reverse primer N(–). RT procedures were performed using 20 µl of RNA in a 40 µl reaction volume as previously described [40]. Each cDNA fragment was amplified from RT products by PCR as previously described [9]. PCR products were purified from agarose gels using a DNA extraction kit (Boehringer Mannheim, Germany) and sequenced directly or cloned into the pMD-18T (TaKaRa, Dalian, China) vector following the manufacturer's instructions.

RNA extraction, cDNA generation, PCR amplification and gene fragment cloning and sequencing were independently conducted 4 times for each of the four passages, P3, P40, P70 and P110. The viral stocks used were from independently inoculated embryos. In total,

20 clones of each gene fragment were selected and sequenced for each of the CK/CH/LHLJ/04V passages. Five clones were selected for sequencing each time.

2.9. ORF determination and sequence analysis

Sequences were compiled and ORFs were determined using the Gene Runner program, version 3.00 (<http://www.generunner.com>). Nucleotide and amino acid sequences for the 3'-7.8 kb fragments were assembled and aligned using the MEGALIGN program (DNAS-tar).

2.10. Accession number

The genomic sequences of the 3'-7 kb region of IBV CK/CH/LHLJ/04V P5, P40, P70 and P110 have been submitted to the GenBank database and have been assigned the accession number FJ641062 and FJ821732 to FJ821773.

3. Results

3.1. IBV CK/CH/LHLJ/04V is a nephropathogenic strain with high mortality in SPF chickens

In the pathogenicity study, all four IBV strains produced typical IB-induced disease. All chicks exhibited respiratory clinical signs at about 4–15 days post-challenge with all four IBV strains. Clinical signs included tracheal rales, watery eyes, nasal mucus, and sneezing, similar to those caused by other IBV strains with affinity for the respiratory tract [39]. All IBV strains caused death 3–12

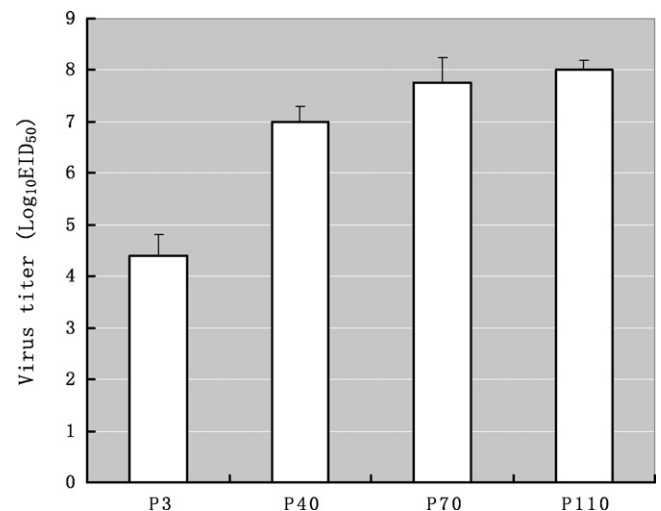


Fig. 1. Comparison of titers of IBV CK/CH/LHLJ/04V passages (P3, P40, P70 and P110) in 9-day-old SPF chicken embryos, evaluated by EID₅₀.

days post-challenge; however, strain CK/CH/LHLJ/04V caused the highest mortality. Gross lesions of the dead chickens were mainly confined to the kidneys. The kidney parenchyma of the affected birds was pale, swollen and mottled; tubules and urethras were distended with uric acid crystals. Hemorrhagic lesions of the cecal tonsil and respiratory tract were also observed in some of the affected chickens. The clinical signs of the inoculated birds tended to disappear gradually after 20 days of challenge. No clinical signs and gross lesions were observed in the negative-control group (data not shown).

All of the challenge IBV strains could be detected in the trachea at 4 days post-challenge by the recovery of the virus using 9-day-old embryos and subsequent RT-PCR; however, strains CK/CH/LHLJ/04V and CK/CH/LDL/04II could be detected in the trachea of the birds at day 12 post-challenge. The virus was not detected in the trachea of the unchallenged negative-control birds. As summarized in Table 1, most of the chicks challenged with the four IBV strains showed no seroconversion at 4 days post-challenge, but antibodies were detected by ELISA in most of the birds after 8 days post-challenge.

3.2. Titer of CK/CH/LHLJ/04V increased with serial passage in SPF embryonated chicken eggs

Nine-day-old embryonated eggs were used to determine the growth ability of P3, P40, P70 and P110 in vitro. Equal doses (10^2 EID₅₀) of each virus at each passage level were used to inoculate three 9-day-old embryos. The inoculated embryos were incubated at 37 °C, and allantoic fluid was harvested at 72 h for virus titration. Based on movement and the extent of bleeding, curling, and dwarfing, all of the inoculated embryos were determined to be infected but alive after 72 h. EID₅₀ was determined for each sample. The titers of CK/CH/LHLJ/04V increased gradually from P3 to P110,

indicating an increase during serial passage in SPF embryonated chicken eggs (Fig. 1).

3.3. The IBV CK/CH/LHLJ/04V strain was attenuated by serial passage

Clinical signs, mortality and gross lesions were used to assess the attenuation of IBV CK/CH/LHLJ/04V P3, P40, P70 and P110 using SPF chickens. As summarized in Table 2, all birds given P3 and P40, and 50% of birds given P70 by oculonasal application showed overt disease, as did the birds challenged with virulent CK/CH/LHLJ/04V in the pathogenicity study, in comparison with P110-inoculated and negative control chicks. Clinical signs were observed from day 3 or 4, to day 15 post-inoculation. Eight chicks inoculated with P3 and two chicks inoculated with P40 died during the experiment. Gross lesions of dead chicks were mainly confined to the kidneys, and were similar to those in the birds in the pathogenicity study. In addition, mucous exudate was observed at 5 days post-challenge in the tracheas of all 5 birds inoculated with P3 and P40, while only one of the chickens in the P40-inoculated group exhibited a respiratory lesion. Chickens in both the P110-inoculated and negative control groups showed no clinical signs, death or gross lesions. Based on the clinical response, mortality and gross lesions, we can conclude that IBV CK/CH/LHLJ/04V P40 and P70 were partly attenuated, but P110 was fully attenuated by serial passages in embryos.

3.4. Immunogenicity of CK/CH/LHLJ/04V decreased with serial passage

Two criteria were used for evaluating altered immunogenicity of selected IBV CK/CH/LHLJ/04V passages. First, serum IgG antibodies specific for IBV CK/CH/LHLJ/04V were measured with an indirect ELISA test. As shown in Table 2 and Fig. 2, none of

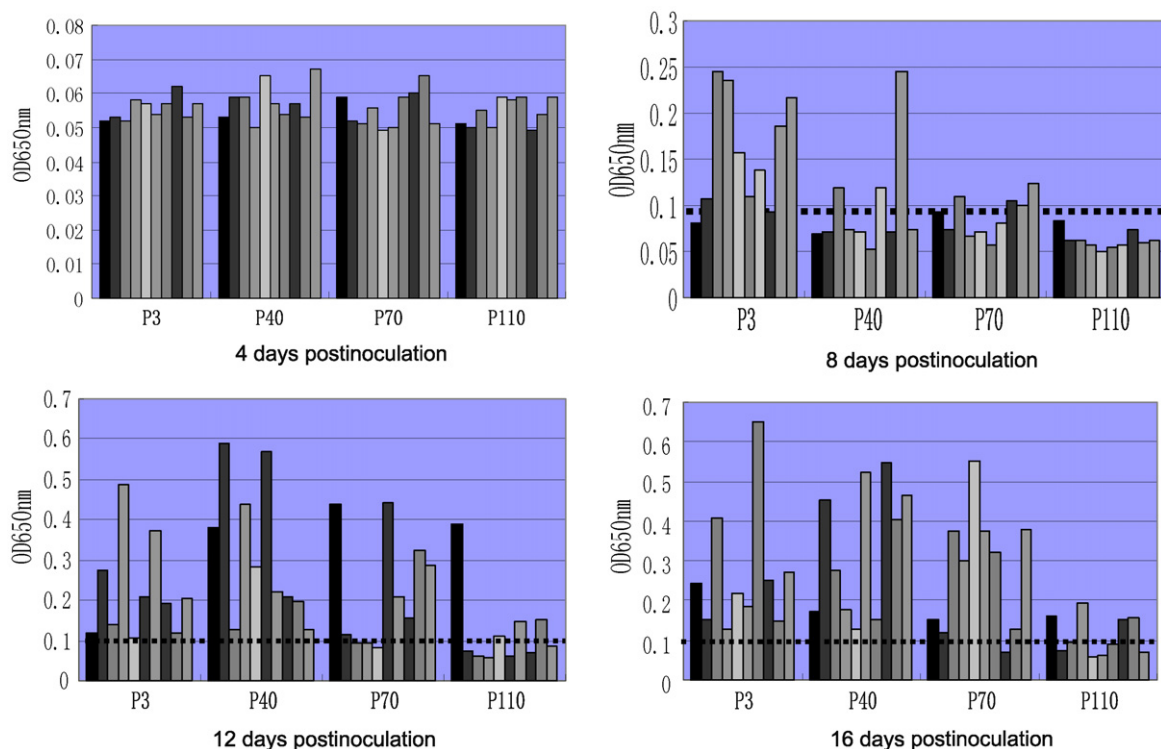


Fig. 2. Humoral immune responses in SPF chickens inoculated with IBV CK/CH/LHLJ/04V passages evaluated by indirect ELISA. Ten chickens were tested in each inoculated group at 4, 8, 12 and 16 days after inoculation. Dashes show the S/P ratios, calculated as described in Section 2. Serum samples with S/P ratios equal or above the dashes were considered positive, and those below were considered negative. The serum sample S/P ratios of chickens in the negative control group were all below the dashes and are not indicated in figures.

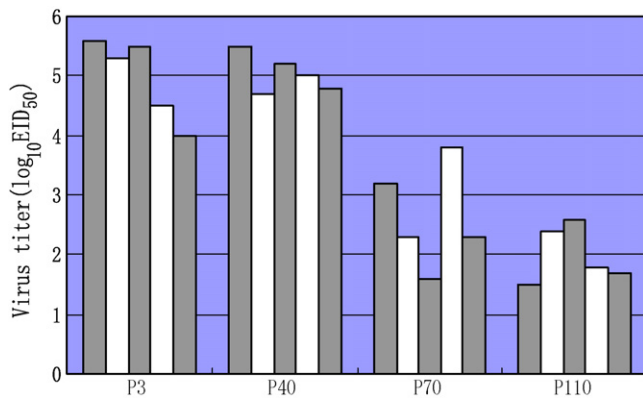


Fig. 3. Virus titers evaluated by EID₅₀ in the respiratory tracts of chickens inoculated with IBV CK/CH/LHLJ/04V passages. Five chickens were tested in each group at 5 days after inoculation. Virus was not detected in the negative control group.

the chickens inoculated with the four selected CK/CH/LHLJ/04V passages showed seroconversion at 4 days post-inoculation. At 8 days post-inoculation, 80%, 30% and 40% of the P3-, P40- and P70-inoculated chickens showed seroconversion, respectively. However, none of the P110-inoculated chickens showed seroconversion at that time. All chickens inoculated with P3 and P40 showed seroconversion from 12 days on, and only 70% and 40% of the P70- and P110-inoculated chickens, respectively, showed seroconversion at 12 days post-inoculation. Fewer than 40% of the P110-inoculated chickens showed seroconversion at 16 days post-inoculation. However, almost all chickens inoculated with P3, P40 and P70 showed seroconversion at that time point. Second, the vaccination-challenge test was used to evaluate the immunogenicity of IBV CK/CH/LHLJ/04V after serial passage in SPF embryonated eggs. As summarized in Table 3, none of chickens inoculated with P40 or P70 showed clinical signs or death after challenge with the virulent P3 level strain, indicating good clinical protection provided by vaccination with P40 or P70. However, 30% of the P110-vaccinated chickens showed clinical signs, and 2 of 10 vaccinated chickens died after P3 challenge. Parallel to the clinical protection results, vaccination with P40 and P70 also offered good trachea and kidney protection against virulent P3 challenge, although one of the P70-vaccinated chickens was positive for virus recovery in the trachea after P30 challenge. In contrast, more than 50% of the P110-vaccinated chickens were positive for virus recovery from both tracheas and kidneys after challenge with the virulent P3 strain, indicating poor trachea and kidney protection after P110 vaccination. Based on these results, the partly attenuated P40 and P70 viruses were considered capable of stimulating systemic immunity in chicks; however, immunogenicity against the fully attenuated P110 decreased as expected.

3.5. CK/CH/LHLJ/04V lost kidney tropism during serial passage

Viruses were identified by RT-PCR at 5 days post-inoculation in both the tracheas and kidneys of the P3-, P40-, P70- and P110-inoculated chickens. All trachea samples, and kidney samples from P3- and P40-inoculated chickens, were positive by PCR; however, only one kidney sample from P70- and P110-inoculated chickens was positive, respectively. All trachea and kidney samples were titrated at that point, and as shown in Fig. 3, viral titers steadily decreased with passage. P40 had almost the same titer in the trachea as P3; however, the titers of P70 and P110 in the trachea were lower than those of P3 and P40, as expected. It is puzzling that viral titers in the kidneys rapidly decreased and were eventually lost when the corresponding virus adapted to embryonated eggs. Compared to the virulent P3 virus, P40 showed a lower titer in the

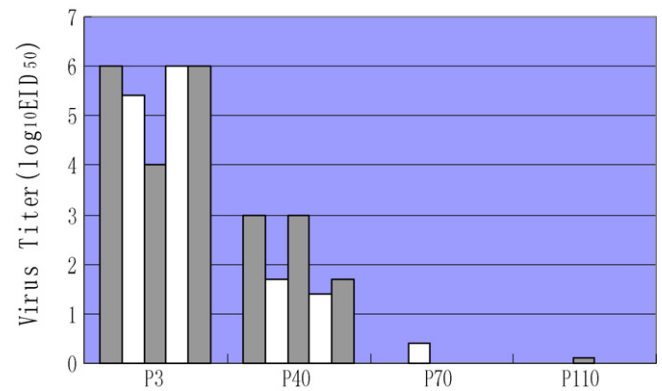


Fig. 4. Virus titers evaluated by EID₅₀ in kidneys of chickens inoculated with IBV CK/CH/LHLJ/04V passages. Five chickens were tested in each group at 5 days after inoculation. Virus was not detected in the negative control group.

kidneys, while P70 and P110 had already lost kidney tropism after serial passages in SPF embryonated chicken eggs (Fig. 4).

3.6. Changes in the 3'-7 kb region of IBV CK/CH/LHLJ/04V after serial passage

The IBV CK/CH/LHLJ/04V strain was propagated in embryonated eggs and passaged 110 times. To investigate whether nucleotide and/or amino acid sequences changed during passage, P3, P40, P70 and P110 were chosen for RT-PCR amplification and sequence analysis of the 3'-7 kb region. All RT-PCR products were analyzed, and a single band of expected size was visible after ethidium bromide staining of the products on a 1.0% agarose gel. Twenty independent clones of each fragment from four independent RT-PCRs were selected and sequenced, so the sequence profiles represented all genetic diversity within the populations of the viral RNAs from a given passage.

As summarized in Table 5, no sequence changes were observed in ORF 3a or gene 5 (ORF5a and ORF5b) after 110 passages. Only one to two nucleotide mutation(s) occurred in the ORF 3b, 3c (E), M and N genes after 110 passages, and all amino acid substitutions in those regions occurred between P70 and P110. However, most nucleotide mutations were found in the S region between P3, P40, P70 and P110 and all of the nucleotide mutations resulted in amino acid substitutions. Significantly, a majority of the sequence changes in the S1 region of the S protein occurred between P3 and P40. Most of the sequence changes in the S2 region were observed between P70 and P110, however. In addition, a single base mutation (G → T) was observed in 10 out of 20 clones of P3, and this mutation was located at the 3'-terminal region of the S gene, at nucleotide 3469 from the AUG start codon of the S1 gene. This caused a frameshift that would result in a C-terminally truncated product, if synthesized. The truncated product would contain 1157 residues instead of the 1166 residues of the normal S protein (Fig. 5). Furthermore, the S proteins of P40, P70 and P110 all contained the mutation for truncated S.

To investigate whether serial passage of IBV CK/CH/LHLJ/04V in embryonated eggs can cause sequence changes in the 3'-untranslation region (3'-UTR), the 3'-UTR sequences of P3, P40, P70 and P110 were amplified and sequenced. Surprisingly, a 109-bp deletion, located 8 nucleotides downstream of the stop codon of the N protein gene, was found in the 3'-UTR of 70% and 85% clones of P70 and P110, respectively, as shown in Fig. 6. Interestingly, both the deleted and non-deleted 3'-UTR sequences of the secreted offspring viruses were detected in the respiratory tracts of the chickens inoculated with IBV CK/CH/LHLJ/04V P110 at 5 days post-inoculation (data not shown).

Table 5

Nucleotide and amino acid changes in the 3' region of IBV CK/CH/LHLJ/04V and embryo-passaged derivatives.

Gene	Position (nt) ^a	Nucleotide change			Codon change			Amino acid change		
		P3 → P40	P40 → P70	P70 → P110	P3 → P40	P40 → P70	P70 → P110	P3 → P40	P40 → P70	P70 → P110
S gene	65–73	ATTCTGATA (10/20) ^b	None	None				SDN	None (SDN)	None (SDN)
	172	None (A)	A → G (13/20)	A → G (14/20)	AGT → AAT	AAT → GAT	AAT → GAT	Ser → Asn	Asn → Asp	Asn → Asp
	173	G → A	None (A)	None (A)	AGT → AAT	AAT → GAT	AAT → GAT	Ser → Asn	Asn → Asp	Asn → Asp
	194	A → G	None (G)	None (G)	GAG → GGG	None (GGG)	None (GGG)	Glu → Gly	None (Gly)	None (Gly)
	397	C → T	None (T)	None (T)	CAT → TAT	None (TAT)	None (TAT)	His → Tyr	None (Tyr)	None (Tyr)
	914	A → G	None (G)	None (G)	TAT → TGT	None (TGT)	None (TGT)	Tyr → Cys	None (Cys)	None (Cys)
	1741	None (C)	None (C)	C → T (12/20)	None (CTT)	None (CTT)	CTT → TTT	None (Leu)	None (Leu)	Leu → Phe
	2476	None (G)	None (G)	G → T (15/20)	None (GAT)	None (GAT)	GAT → TAT	None (Asp)	None (Asp)	Asp → Tyr
	2503	None (C)	None (C)	C → T (15/20)	None (CTT)	None (CTT)	CTT → TTT	None (Leu)	None (Leu)	Leu → Phe
	3088	None (T)	None (T)	T → C (15/20)	None (TTT)	None (TTT)	TTT → CTT	None (Phe)	None (Phe)	Phe → Leu
	3469	G → T (10/20)	G → T	None (T)	GAA → TAA	GAA → TAA	None (TAA)	Glu → # ^c	Glu → #	None (#)
Gene 3	ORF 3a				None ^d					
	ORF 3b	153	None (G)	None (G)	None (GAG)	None (GAG)	GAG → GAT	None (Glu)	None (Glu)	Glu → Asp
	ORF 3c (E)	264	C → T	None (T)	AAC → AAT	None (AAT)	None (AAT)	None (Asn)	None (Asn)	None (Asn)
M gene		638	None (A)	None (A)	None (GAG)	None (GAG)	GAG → GCG	None (Glu)	None (Glu)	Glu → Ala
Noncoding		241 ^e	None (C)	None (C)	NA ^f					
Gene 5	ORF 5a				None ^d					
	ORF 5b				None ^d					
N gene	283–284	None (TA)	None (TA)	TA → AC (13/20)	None (TAC)	None (TAC)	TAC → ACC	None (Tyr)	None (Tyr)	Tyr → The

^a The position of nucleotides from the AUG start codon of each gene ORF.^b A 9-bp sequence, ATTCTGATA, was deleted in 10 out of 20 clones of CK/CH/LHLJ/04V P3, resulting in three deletions from amino acids 23–25 of S. The viruses P40, P70 and P110 do not have this deletion. Shown is the number of clones showing nucleotide mutations/number of clones sequenced.^c #, stop codon.^d No nucleotide mutations or amino acid substitutions were found in those ORFs between CK/CH/LHLJ/04V virus passages.^e 241 bp downstream of stop codon of M gene.^f NA: not applicable.

(a)

LX4	ATTAGTAAGTGTGGTAAGAAATCTTCTTATTACACTACTTTTGAT
CK/CH/LHLJ/04V P3 (50%)	ATGAGTAAGTGTGGTAAGAAATCCTCTTATTACACGACATTTGAT
CK/CH/LHLJ/04V P3 (50%)	ATGAGTAAGTGTGGTAAGAAATCCTCTTATTACACGACATTTGAT
	stop codon ***
LX4	AATGATGTGGTAAC <u>GAA</u> CAATACAGACCTAAAAAGTCTGTTTAA
CK/CH/LHLJ/04V P3 (50%)	AATGATGTGGTAAC <u>GAA</u> CAATACAGACCTAAGAAGTCTGTTTAA
CK/CH/LHLJ/04V P3 (50%)	AATGATGTGGTAAC <u>TAA</u> CAATACAGACCTAAGAAGTCTGTTTAA

(b)

LX4	ISKCGKKSSYYTTFDNDVVTEQYRPKKS#		GAA (Glu) → TAA
CK/CH/LHLJ/04V P3 (50%)	MSKCGKKSSYYTTFDNDVVTEQYRPKKS#	P3 50%	50%
CK/CH/LHLJ/04V P3 (50%)	MSKCGKKSSYYTTFDNDVVTEQYRPKKS#	P40 0%	100%
CK/CH/LHLJ/04V P3 (50%)	MSKCGKKSSYYTTFDNDVVTEQYRPKKS#	P70 0%	100%
CK/CH/LHLJ/04V P3 (50%)	MSKCGKKSSYYTTFDNDVVTEQYRPKKS#	P110 0%	100%

Fig. 5. Comparison of the nucleotide (a) and amino acids (b) sequences of the 3-terminal region of the S protein gene of CK/CH/LHLJ/04V passages. IBV LX4 was the comparison reference strain (GenBank accession number: AY189157). The mutation (GAA → TAA) that changed a Glu codon to a stop codon is underlined. The stop codon (*** of the normal S gene is indicated. The percentage of deleted and non-deleted sequences in each of the virus passage was estimated and is indicated on the right.

4. Discussion

IBV CK/CH/LHLJ/04V is a nephropathogenic IBV strain of the LX4-type that is highly pathogenic to SPF chickens, with 100% morbidity and approximately 40% mortality. LX4-type IBV has been one of the major types of IBV circulating both in China and European countries in recent years [34,41–44]. However, both experimental infections and field results have shown that available commercial vaccines provide poor protection against the LX4-type IBV [39]. Hence, we selected the LX4-type IBV CK/CH/LHLJ/04V strain for attenuation by serial passage in SPF chicken embryonated eggs. Coronaviruses, including IBV, have been shown to exist as a mixture of genetic mutants within an isolate [45–47]. This is also the case for the IBV isolate CK/CH/LHLJ/04V. Based on sequence data from cloned RT-PCR products, 50% of the sequences in the low-level virus populations (P3) showed a 9-bp deletion upstream of hypervariable

region 1 (HVR1) of the S1 gene compared to the other sequences in the same population. However, this deletion was not observed in all of the S1 sequences in the high-level virus populations (P40, P70 and P110). We cannot determine if this reversion of the S1 gene sequence was due to a recombination event or the selection of a subpopulation in the process of serial passage in chicken eggs. In addition, a stop codon was observed in 50% of the S protein sequences of P3 at residue 1157 due to a mutation changing a Glu codon to a stop codon, resulting in an S protein with 9 amino acids missing at its carboxy-terminal end. All sequences in P40, P70 and P110 showed this mutation. This indicates that this region is not necessary for virus formation. The above mentioned S gene sequence heterogeneity indicated the presence of different proportions of subpopulations in CK/CH/LHLJ/04V. Most genetic changes occur in the S1 gene during adaption to the host [19,47,48]. However, until now, it was not clear if mutations or the selection of a

	N protein***			
Tw2296/95 path.	AATGAACTTTGAGTAACATAATGGACTTGCTGCATTTTGTGCGCATTTCTTTGTGA			
Tw2296/95 att.	AATGAACTTTGAGTAACATA-----			
CK/CH/LHLJ04 VP3	AATGAACTTTGATTAAACATAATGGACTTGCTGCATTTTATGTGCGCATTTGCTGTTGTT			109-bp → deletion
CK/CH/LHLJ04VP110 (85%)	AATGAACTTTGATTAAACATA-----	P3	100%	0%
Tw2296/95 path.	CTTATTTTGTAGATTCTCACAATTATTACTGGAGTTGATTGTGATTATGTGCAAT	P40	100%	0%
Tw2296/95 att.	-----GATTTCTCACAATTATTACTGGAGTTGATTGTGATTATGTGCAAT	P70	30%	70%
CK/CH/LHLJ04 VP3	ACTATTCTCTCTGCTTTCTCTCAATTATTGTGGGCTTTGATTGTGATTATGTGCACT	P110	15%	85%
CK/CH/LHLJ04VP110 (85%)	-----			
Tw2296/95 path.	TTCTTAGTTGGTTTAATATGCTTGTTGCAGTTTGCTTTCTTTCTGTGCTTATTGTTT			
Tw2296/95 att.	TTCTTAGTTGGTTTAATATGCTTGTTGCAGTTTGCTTTCTTTCTGTGCTTATTGTTT			
CK/CH/LHLJ04 VP3	ACCTTAGTTGGTTTCCCTTGTTGTTTGCAGTTTGTGTTCTTTCTGTGCTTATTGTTT			
CK/CH/LHLJ04VP110 (85%)	-----CCTTGTTGTTTGCAGTTTGTGTTCTTTCTGTGCTTATTGTTT			

Fig. 6. A 109-bp deletion, located 8 nucleotides downstream of the stop codon of the CK/CH/LHLJ/04V N gene during serial passage in 9–11-day-old embryonated SPF eggs. The pathogenic and attenuated TW2296/95 (TW2296/95 path and TW2296/95 att.) were used as comparison reference strains. The stop codon (*** of the N protein gene is indicated. Deletions within the sequences are shown with dashes (---). The percentage of deleted and non-deleted sequences in each of the virus passage was estimated and is indicated on the right.

fit subpopulation was responsible for the changes observed when coronaviruses were attenuated or adapted to a particular host system [48].

Routinely, IB attenuated vaccines are developed by multiple passages (generally 52 or more) of a field isolate, in embryonated domestic fowl eggs, until the desired blend of non-pathogenicity and immunizing capacity has been achieved. The mutations that cause the attenuation of pathogenicity are not known. We found 13 amino acid substitutions between pathogenic CK/CH/LHLJ/04V P3 and its embryo-passaged, attenuated derivative P110. Of these substitutions, 10 were in the S protein (5 substitutions each in the S1 and S2 regions). Importantly, a common substitution at residue 132 in the S1 protein (His to Tyr), found in the D207 and TW2296/95 IBV strain and related to antibody attachment [49], was also found in the S1 protein of CK/CH/LHLJ/04V after 40 passages. This substitution was maintained for P70 and P110, indicating that this residue was likely to be important for virus pathogenicity. However, other amino acid substitutions in the S1 protein were not observed between the M41 vaccine strain and M41 challenge strain [50], the 4/91 pathogenic and 4/91 attenuated strains [9,51], the TW1171/92 pathogenic and TW1171/92 attenuated strains, the TW2296/95 pathogenic and TW2296/95 attenuated strains, the TW2575/98 pathogenic or TW2575/98 attenuated strains [48], or the ArkDPI strain [52]. Investigations with other coronaviruses have shown that the S protein is a determinant of pathogenicity; however, the replacement of the S protein gene of the apathogenic Beaudette strain with that of the pathogenic M41 strain resulted in a recombinant virus that was still not pathogenic [53]. Thus proteins other than, or in addition to, the S protein must affect pathogenicity. Our findings were consistent with this result. We found that nearly all of the amino acid substitutions in the S1 subunit were between P3 and P40, and other substitutions were between P70 and P110. The virus was still pathogenic to chickens after 40 passages and it was fully attenuated after 110 passages in embryonated eggs. The structural E gene in SARS-CoV is a virulence factor, and a SARS-CoV that lacks the E gene is attenuated in vitro and in vivo [54]. In this study, a synonymous mutation in the E gene was found between P3 and P40, and three substitutions in the 3a, M and N proteins were also observed between P70 and P110. Nonetheless, the relationship between those substitutions and virus pathogenicity is unknown. A single mutation, Tyr639His, in mouse hepatitis virus MHV-A59 nsp14 resulted in attenuated virus pathogenesis in mice [55]. Sequence changes in the 5'-two-thirds of the genome, which contains two overlapping replicase genes, were not investigated in this study and should be further studied.

An additional ORF, detected within the 3'-UTR of several IBV isolates, and with the potential to encode hydrophobic proteins is referred to as ORF7 [48]. It was also observed in the IBV CK/CH/LHLJ/04V strain. In other coronaviruses, such as porcine transmissible gastroenteritis virus (TGEV) and FCoV, a relationship between gene 7 and virus virulence has been observed [49], although it is difficult to compare the hydrophobic proteins of these coronaviruses directly with that of IBV. Until now, the corresponding protein and its IBV function have not been clear. However, it is hypothesized that the sequences in the IBV 3'-UTR are involved in regulating viral RNA replication and transcription [56]. In addition, Sapats et al. [57] reported that shorter forms of the 3'-UTR in the Australian N1/88, Q3/88, and V18/91 strains are associated with a decrease in virulence. Huang and Wang [48] 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. In this study, we observed a longer (109-bp) deletion at the same position in the 3'-UTR in most subpopulations of P70 and P110 (70% and 85%, respectively), indicating that not only this deletion, but also the size of the deletion may be correlated with IBV attenuation. In addition, both the deleted and non-deleted

sequences in the 3'-UTR of the offspring viruses were detected in the respiratory tracts of chickens inoculated with P110 at 5 days post-inoculation, implicating that this deleted region is not necessary for viral replication in the chicken respiratory tract.

Although multiple passage of a field isolate in embryonated domestic fowl eggs is the usual method for development of IB attenuated vaccines, not all IBV strains stimulate the immunity after serial passages. The Beaudette strain is apathogenic in chickens after many serial passages in embryonated chicken eggs [58]. In addition, this embryo-passaged virus is considered to be poorly immunogenic [58], and consequently, has never been used as a vaccine strain. This is also the case with the IBV CK/CH/LHLJ/04V strain. The immunogenicity of the virus has been gradually decreased by serial passage in embryonated eggs. The CK/CH/LHLJ/04V P110 did not confer immunity to SPF chickens when compared to P3, P40, P70 and the negative control. The reduced immunogenicity of the attenuated virus may correlate with its reduced replication efficiency and infectivity in chickens. The S glycoprotein induces protection against virulent challenge, and several epitopes that induce virus-neutralizing antibodies have been mapped within the S protein [59–64]. These epitopes showed the importance of inducing the CTL response in primary infections and neutralizing the antibody response against secondary exposure to the same virus [65,66]. In this study, 3 amino acid substitutions were observed in the above-mentioned epitopes of S1 protein, and no substitutions were found in the epitopes residues of the S2 protein. In addition, N is another important protein that induces protection in IBV. In this study, a substitution (Tyr → The) was observed in the N protein between P70 and P110 viruses. This substitution was located in an identified epitope in the IBV N protein that induces a T-cell response and protection [67,68]. The amino acid substitution at residue 188 (Thr to Ile/Ala) in the M protein, which was observed to be related to antigenicity and/or virulence of IBV strains H52/H120, TW2296/95 and Arkansas [48,69], was not observed in our CK/CH/LHLJ/04V strain.

The IBV strains, as a group, infect a large range of epithelial surfaces, literally from the top to the bottom of the chicken. Isolates differ in their extent of replication in non-respiratory tissues, and some produce clinical disease in non-respiratory tissues, most notably the kidney and proventriculus. CK/CH/LHLJ/04V is a nephropathogenic strain; however, it lost kidney tropism after 70 serial passages in embryonated eggs. Using a reverse genetics system, the S glycoproteins for a group 2 coronavirus (MHV), a group 1 coronavirus (TGEV) and a group 3 coronavirus (IBV) were demonstrated to be involved in the tropism of these coronaviruses [26–30,68,70]. For the TGEV, several amino acid changes at the N-terminus of the S protein resulted in the loss of enteric tropism [29,30]. In this study, an amino acid substitution was found at residue 581 (Leu → Phe) between P70 and P110; however, loss of kidney tropism of CK/CH/LHLJ/04V occurred between P40 and P70. The only amino acid difference between P40 and P70 was at residue 58; however, this residue is not likely to be a determinant of tissue tropism of CK/CH/LHLJ/04V, because some viral subpopulations in P70 and P110 showed this change. Several other substitutions were found in S and other proteins in this study. Further investigation by reverse genetics and animal studies is needed to verify the exact function of substitutions.

In this study, the titers of the embryo-adapted IBV CK/CH/LHLJ/04V strain increased gradually with the serial passage in embryonated eggs, indicating that the virus had a high replication capacity in vitro; however, its capacity for in vivo replication decreased dramatically. The N, M and E proteins of IBV play a role in viral replication and assembly [2]. It is difficult to conclude that the decreased replication of the virus in vitro in this study was due to substitutions in M and N proteins. A point mutation in the coronavirus HCoV-229E and the arterivirus EAV NendoU (nsp15) resulted in a lack of viral genome replication and transcription, indi-

cating that this RNase mostly affected viral production [71–73]. It is unclear whether this is the case for IBV CK/CH/LHLJ/04V.

The embryo-adapted IBV strains appeared to contain a mixture of genetic variants, and selection and mutations occurred in the viral populations during the passages in the embryos. In the process of serial passage, almost all of the amino acid substitutions in S1 proteins occurred between P3 and P40, and all the subpopulations in the virus passages showed those substitutions; however, other substitutions were found between P70 and P110 and only parts of the subpopulations in the virus passages showed those substitutions. The exact roles of different subpopulations in changes in virus replication, pathogenicity, antigenicity, immunogenicity and tissue tropism are unknown; we have not succeeded in isolating the different subpopulations from the virus population by limited dilution (data not shown).

Understanding the molecular mechanism of IBV attenuation, tissue tropism and immunogenicity changes is important, because not only is this virus of economical importance to the poultry industry, but it also shows antigenic and biological similarities and differences to other coronaviruses. Although it is reasonable to conclude that some of the few sequence changes described in this study in the 3'–7 kb region are responsible for virus attenuation, decrease in immunogenicity and tissue tropism changes, we cannot conclude that they are the only predictors for these changes. We also cannot completely exclude the possibility that other parts of the genome are responsible for the observed changes, because IB coronavirus has a large genome (27.6 kb). In addition, similar to other reports [19,47,48,59], we found that none of the sequence changes were shared by all pathogenic IBV strains and their attenuated derivatives, indicating that there may be many factors and pathways that affect virus replication, pathogenicity, antigenicity, immunogenicity and tissue tropism.

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