



## Serotype shift of a 793/B genotype infectious bronchitis coronavirus by natural recombination



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### ABSTRACT

An infectious bronchitis coronavirus, designated as ck/CH/LHLJ/140906, was isolated from an infectious bronchitis virus (IBV) strain H120-vaccinated chicken flock, which presented with a suspected infectious bronchitis virus (IBV) infection. A phylogenetic analysis based on the S1 gene clustered ck/CH/LHLJ/140906 with the 793/B group; however, a pairwise comparison showed that the 5' terminal of the S1 gene (containing hypervariable regions I and II) had high sequence identity with the H120 strain, while the 3' terminal sequence was very similar to that of IBV 4/91 strain. A SimPlot analysis of the complete genomic sequence, which was confirmed by a phylogenetic analysis and nucleotide similarities using the corresponding gene fragments, suggested that isolate ck/CH/LHLJ/140906 emerged from multiple recombination events between parental IBV strains 4/91 and H120. Although the isolate ck/CH/LHLJ/140906 had slightly higher S1 amino acid sequence identity to strain 4/91 (88.2%) than to strain H120 (86%), the serotype of the virus was more closely related to that of the H120 strain (32% antigenic relatedness) than to the 4/91 strain (15% antigenic relatedness). Whereas, vaccination of specific pathogen-free chickens with the 4/91 vaccine provided better protection against challenge with ck/CH/LHLJ/140906 than did vaccination with the H120 strain according to the result of virus re-isolation. As the spike protein, especially in the hypervariable regions of the S1 domain, of IBVs contains viral neutralizing epitopes, the results of this study showed that recombination of the S1 domain resulted in the emergence of a new serotype.

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### 1. Introduction

Infectious bronchitis virus (IBV), the prototype of the subfamily *Coronavirinae*, which is in the family *Coronaviridae* of the order *Nidovirales*, is an important pathogen in chickens, and it infects the respiratory tract, kidneys and oviduct, causing reduced performance, reduced egg quality and quantity, increased susceptibility to infection with other pathogens and increased mortality (Cavanagh, 2003). The genome of IBV is a single-stranded, positive-sense RNA of approximately 27.6 kb, one-third of which encodes four structural proteins, including the spike glycoprotein (S), the membrane glycoprotein (M), the phosphorylated nucleocapsid protein (N) and the small membrane protein (E).

Interspersed among the structural protein genes are two small accessory protein genes (genes 3 and 5) that vary in number and sequence among IBVs. Two-thirds of the 5' region in the IBV genome encodes the 1a and 1ab polyproteins, which are proteolytically cleaved by two virus-encoded replicase proteins (the papain-like and 3C-like proteinases) into 15 nonstructural proteins (nsp2–nsp16) (Ziebuhr et al., 2001). The 5' and 3' untranslated regions (UTRs) usually harbor important structural elements that are involved in replication and/or translation (Masters, 2006).

The S1 subunit of the S protein of IBV carries virus-neutralizing and serotype-specific determinants. IBVs exist as dozens of serotypes, which are defined by the virus neutralization test. In keeping with the large number of IBV serotypes, the S1 protein is very variable; the amino acid sequences of serotypes commonly differ by 20% to 25%, and can differ by up to 50%, (Cavanagh et al., 2005; Wickramasinghe et al., 2014). Massachusetts and 793/B are among the most important and prevalent serotypes of IBV worldwide (Jackwood, 2012). Until the mid-1950s, the Massachusetts serotype viruses were believed to be the only serotype found in the

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USA and other regions of the world, until a second IBV serotype, Connecticut, was reported. The use of commercially produced, Massachusetts-type modified, live virus vaccines began in the 1950s and has continued to the present. These types of vaccines, such as the Massachusetts (M41) and H120 strains, are the most commonly used around the world because such vaccines have been proven to confer protection against a wide range of IBV strains, including homologous and non-homologous IBV genotype/serotypes (Cavanagh and Gelb, 2008). The first known strain of the 793/B serotype, also known as 4/91 (Parsons et al., 1992) and CR88, was isolated in France in 1985 (Picault et al., 1995). This serotype may have entered the UK in the winter of 1990/91, when it was sometimes associated with deep pectoral muscle myopathy, in addition to the more usual manifestations of infectious bronchitis (Gough et al., 1992; Parsons et al., 1992). Subsequently, it was discovered that this serotype had been present in most European countries (de Wit et al., 2011). The first record of the 793/B serotype in Asia was in China in 2003; the virus isolate was named Taian-03, and the sequence of the S1 gene has been deposited in the GenBank database with the accession number AY837465. Currently, 793/B type viruses have been detected in several other Asian countries, including Japan (Mase et al., 2008), India (Sumi et al., 2012) and Iran (Bijanzed et al., 2013).

New serotypes and genotypes of IBV emerge frequently in different parts of the world (Al Tarcha et al., 1990; Cook, 1983; Gelb et al., 1991; Gough et al., 1992; Jia et al., 1995; Liu and Kong, 2004; Zanella et al., 2003). A number of factors, among which mutation and recombination are two major forces, account for the emergence and evolution of new genetic variants, and they play an important role in increasing the number of variants (Cavanagh, 1992; Estevez et al., 2003; Jia et al., 1995; Kusters et al., 1990; Wang et al., 1993). Although mutations are very common in IBV genomes, recombination between strains has also been widely reported (Davidson and Silva, 2008; Hewson et al., 2014; Jia et al., 1995; Kusters et al., 1990). It has been suggested that turkey coronavirus, another group of the gamma-corona viruses, emerged from a host infected with a known IBV strain and an uncharacterized coronavirus that resulted in recombination in the S1 gene and a subsequent host shift from chickens to turkeys (Jackwood et al., 2010).

Recently, a couple of “novel” IBVs have been isolated in China, and they were shown to have emerged from a recombination event between distant strains (Feng et al., 2014; Liu et al., 2013, 2014; Zhao et al., 2013; Zhou et al., 2014) by molecular characterization using sequencing and phylogenetic analysis. However, the biological characteristics, such as antigenicity and pathogenicity, of most of these IBVs have not been fully evaluated (Feng et al., 2014; Zhao et al., 2013; Zhou et al., 2014). In this study, a new variant of IBV, designated as ck/CH/LHLJ/140906, which was isolated in Heilongjiang province in China in 2014, was comprehensively analyzed by comparing its genome with those of two other IBV strains, 4/91 and H120, which were suspected of being the parental viruses of ck/CH/LHLJ/140906. In addition, the antigenicity of the isolate was further analyzed using cross virus-neutralization and vaccination-challenge tests.

## 2. Materials and methods

### 2.1. Viral isolation

An IBV strain, designated as ck/CH/LHLJ/140906, was isolated from swollen kidneys from diseased chickens in a chicken flock suspected of having an IBV infection during the course of our conventional surveillance activities for IBV in China in 2014. Three different ages of broilers, at 10-day intervals, were reared in the same house. All 1-day-old chickens were vaccinated against IBV

with the commercial live attenuated H120 vaccine and then boosted when 14 days old. The broilers in two of the flocks showed early signs of respiratory disease when they were 15 and 25 days old, and the chickens in the third flock, which were 35 days old, showed no clinical signs. Gross examinations showed mild to severe tracheitis, nephritis and proventriculitis. The morbidities were 50% and 10%, respectively, and the mortalities were 20% and 5%, respectively.

The kidney samples of the diseased chickens were collected, pooled and used for virus isolation as previously described (Liu and Kong, 2004). Three blind passages were performed when the characteristic embryo changes appeared, such as dwarfing, stunting, or curling of the embryos, which were observed from 26 to 72 h. In addition, the IBV vaccine strains 4/91 and H120 were used in the cross virus-neutralization and vaccination-challenge tests in this study. Embryo-propagated viral stocks of ck/CH/LHLJ/140906, 4/91 and H120 were produced by inoculating the virus into embryonated specific pathogen-free (SPF) chicken eggs via the allantoic cavity, and the infectious allantoic fluid was collected 48 h post-inoculation as previously described (Liu and Kong, 2004). The titers of the three viruses were determined by inoculation of 10-fold dilutions into groups of five 10-day-old embryonated chicken eggs. The median embryo infectious dose (EID<sub>50</sub>) was calculated using the method of Reed and Muench (1938).

### 2.2. Eggs and chicks

White Leghorn SPF chickens and fertile SPF chicken eggs were obtained from the Harbin Veterinary Research Institute. The birds were maintained in isolators with negative pressure, and food and water were provided ad libitum. All experimental procedures were approved by the Ethical and Animal Welfare Committee of Heilongjiang province, China.

### 2.3. RNA extraction, amplification and sequencing

Genomic RNA was extracted from virus-infected allantoic fluid with the TRIzol reagent (Invitrogen, Carlsbad, CA, USA) following the manufacturer's instructions. The extracted RNA was used as template in a reverse transcription PCR (RT-PCR). Overlapping primers were designed in a manner such that each pair of primers covered approximately 2 kb of the genome based on the conserved sequences among the LX4-, 793/B- and Massachusetts-type IBVs (Table 1). All gene fragments were amplified using the RT-PCR kit (Takara, Dalian, China) according to the manufacturer's instructions, and the RT-PCR products were cloned into the pMD-18T vector (Takara). Rapid amplification of cDNA ends (RACE) was conducted to determine the 3'/5'-termini of ck/CH/LHLJ/140906 genomic RNA using the 3'/5' RACE kit (Takara) according to the manufacturer's instructions (Liu et al., 2014). For each amplicon, DNA from at least three independent clones was sequenced to exclude errors that can occur during the RT-PCR.

The complete genomic sequence of ck/CH/LHLJ/140906 reported in this study has been submitted to GenBank and has been assigned accession number KP036502.

### 2.4. Genomic sequence determination and phylogenetic analysis

Assembly of contiguous sequences was performed with the GeneDoc software (Ammayappan and Vakharia, 2009). Comparative sequence analyses of ck/CH/LHLJ/140906 with other IBVs were conducted using BLAST searches of the National Center for Biotechnology Information database and Vector NTI Advance 10 software. Phylogenetic analyses based on both the S1 gene and corresponding gene fragments were conducted using the MEGA4 program (Tamura et al., 2007). Phylogenetic trees were

**Table 1**  
Sequences of primers used in the present study.

Primer	Sequence	Position in genome <sup>#</sup>	Direction	Size (bp)
0906-1F	GAAGACATCTTGGTGTCTCTCC	901–923	Fw	1979
0906-1R	GTCATCATCACTCTCATAATCAC	2858–2880	ReV	
0906-2F	GTGATTATGAGAGTGATGATGAC	2778–2800	Fw	2017
0906-2R	CAATGGCCACTGTTAGTAGAGCC	4763–4795	ReV	
0906-3F	GATGCAAATAGTGGATGAGG	4643–4664	Fw	2153
0906-3R	CAGGTTCCACTACTAATGTCTC	6775–6796	ReV	
0906-4F	CACAGAGTGGCATGATTAGAG	6677–6699	Fw	2109
0906-4R	GAGTAACGTGGTGGTGATATAC	8766–8786	ReV	
0906-5F	CTTGACGCGTATGCTAGAC	8641–8659	Fw	2229
0906-5R	GAGTAACTGACTGTAAAACAG	10850–10870	ReV	
0906-6F	CTTGACATCGATGATGTTGGAG	10729–10750	Fw	2036
0906-6R	CCTTATTATCTTCAAACCAC	12746–12765	ReV	
0906-7F	CTACGCCTTGAGGCATTTTGAC	12656–12677	Fw	2207
0906-7R	GCGATATAACGCTCCATAACAG	14842–14863	ReV	
0906-8F	GCTAGTGGAGTTGATGGTGAGC	14735–14757	Fw	2089
0906-8R	CATCACGCTGACGCATGACAAC	16803–16824	ReV	
0906-9F	CTGCGGATTTCGAGCATGCGCTG	16729–16751	Fw	2150
0906-9R	CTACATATGAGTACCGCTTATAAC	18856–18879	ReV	
0906-10F	CACAGATATCGAGCCAAATGGCC	18752–18774	Fw	1564
0906-10R	CATCTTTACCAGTAACTTAC	20296–20316	ReV	
S1Oligo3'	TGAAAACCTGAACAAAAGAC	20248–20266	Fw	1719
S1Oligo5'	TTGCCCTTATGTTAGTTATG	21948–21967	ReV	
0906-11F	CAGTTTGTAGTCTCTGGTGGC	21802–21822	Fw	2147
0906-11R	GTCTAGACTGTAAGTTACTATT	23928–23949	ReV	
0906-12F	GTAACCTGAACAATACAGACC	23767–23786	Fw	2266
0906-12R	CTTAGGCTGGGTGAATTAAG	26002–26033	ReV	

<sup>#</sup> Based on the H120 strain genome (FJ888351).

constructed from aligned nucleotide and amino acid sequences using the neighbor-joining method with 1000 bootstraps.

### 2.5. Similarity plot and sequence comparison

Similarity and breakpoint analyses of the complete genomic sequence of ck/CH/LHLJ/140906 were aligned with those of 4/91 and H120, and the multiple alignment results were introduced into SimPlot version 3.5.1 to identify likely recombination breakpoints (Lole et al., 1999). Furthermore, pairwise comparison of the complete genomic sequence of ck/CH/LHLJ/140906 was performed with those of the 4/91 and H120 strains to confirm the precise recombination breakpoints, and a phylogenetic tree was reconstructed for each recombinant fragment to avoid phylogenetic biases derived from ignoring recombination events.

### 2.6. Cross virus-neutralization tests

Cross virus-neutralization tests were performed using constant ( $10^2$  EID<sub>50</sub>) viral titers and diluted serum against each of the three viruses, ck/CH/LHLJ/140906, 4/91 and H120, in SPF chickens embryos for serotyping (Liu et al., 2013). The end-point of each serum sample was calculated using the methods of Reed and Muench (1938). The cross-reactivity *R* values were calculated according to the formula published by Archetti and Horsfall (1950). *R* value less than 50% constitutes a different serotype.

### 2.7. Experimental design

Forty, 1-day-old SPF White Leghorn chicks were housed in different isolators and divided into four groups, each containing 10 birds. Chickens in groups 1 and 2 were vaccinated with the 4/91 and H120 viruses, respectively, by ocular administration at 1-day-old of age with a dose of  $10^4$  EID<sub>50</sub> per chick. Birds in groups 3 and 4 were mock-inoculated with sterile allantoic fluid. Blood samples were collected from all birds in each group at post-inoculation days 4, 8, 12, 16 and 20. At 20 days post-inoculation, birds

in groups 1–3 were challenged by ocular application of  $10^6$  EID<sub>50</sub>/0.1 ml of ck/CH/LHLJ/140906 virus. Birds in group 4 were mock-inoculated with sterile allantoic fluid and served as the negative control. Nasopharyngeal swab and blood samples were collected from all birds at 4, 8, 12, 16 and 20 days after challenge. The nasopharyngeal swabs were placed into separate tubes containing 0.6 ml of phosphate-buffered saline (PBS) containing antibiotics (2000 U/ml penicillin G, 200 µg/ml gentamicin sulfate, 4 µg/ml amphotericin B; Sigma–Aldrich, St Louis, MO, USA) and stored at  $-80$  °C until virus recovery. The serum collected in this study was stored at  $-70$  °C until ELISA testing. Chicks were examined daily for signs of infection for 30 days after inoculation.

### 2.8. Virus recovery

Nasopharyngeal swab tubes were centrifuged at 6000g for 10 min, and the supernatant was collected for virus recovery. The supernatant samples were inoculated into two to five SPF embryonated eggs via the allantoic cavity (0.1 ml per egg). The eggs were candled daily to record embryo mortality. 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. Allantoic fluid from two of the inoculated embryos was collected for RT-PCR amplification as previously described (Liu and Kong, 2004) and the PCR productions were used for sequencing directly to verify the challenged virus. A positive sample was recorded if specific lesions were observed and the RT-PCR amplification was positive.

### 2.9. Serum antibody detection

Serum samples were tested for IBV antibodies using the IDEXX IBV AB test kit (IDEXX Corporation, Westbrook, ME, USA), which is an indirect enzyme-linked immunosorbent assay. The kit was used according to the manufacturer's instructions. Each sample was tested in triplicate. Serum-to-positive ratios (S/P ratios) were

calculated and evaluated as positive or negative according to the kit directions.

### 3. Results

#### 3.1. Genome organization and phylogenetic analysis of IBV isolate ck/CH/LHLJ/140906

Complete genome sequence data for the IBV isolate ck/CH/LHLJ/140906 was obtained by sequence assembly. The size of the genome was 27,600 bases, excluding the poly-A tail at the 3' end. The genome of the virus was similar overall in its coding capacities and genomic organizations to those of other IBVs. BLAST searches with the complete genomic sequence of ck/CH/LHLJ/140906 revealed that ck/CH/LHLJ/140906 was most closely related to the 4/91 vaccine and IBVUkr27-11-Ukraine strains, sharing 98% nucleotide identity with these IBV strains.

The phylogenetic trees constructed using the nucleotide sequences of the S1 subunit of the spike gene are shown in Fig. 1. On the basis of the phylogenetic trees, isolate ck/CH/LHLJ/140906 was clustered with 793/B strains from different countries of the world, despite the fact that it showed diversity and had a close relationship with the 793/B-type strain IBVUkr27-11-Ukraine, which was isolated in the Ukraine in 2011. Similarly to the results of the phylogenetic trees, the BLAST searches that were conducted using the entire S1 gene revealed that ck/CH/LHLJ/140906 was most closely related to strain IBVUkr27-11-Ukraine (95%) (KJ135013); the other strains, including the 4/91 vaccine, did not share more than 91% nucleotide identity with ck/CH/LHLJ/140906.

The predicted S1 amino acid sequence of the S1 gene of IBV isolate ck/CH/LHLJ/140906 was pairwise compared with those of six 793/B-type IBV strains: the prototype 793/B-type strain 4/91 (UK), 4/91 pathogenic and attenuated strains, the 4/91 vaccine strain, IBVUkr27-11-Ukraine, and Taian-03, which was the first isolated 793/B-type IBV in China, and two Massachusetts-type strains, H120 and M41 (Fig. 2). Isolate ck/CH/LHLJ/140906 had 88.2% amino acid sequence identity to the 4/91 vaccine strain and 86% identity when compared to the H120 vaccine strain. The amino acid sequence of the S1 domain at residues 1–192 of isolate ck/CH/LHLJ/140906, in which the hypervariable regions I (HVR I) and II (HVR II) were located, was closely related to those of H120 and M41 (it was more similar to that of H120 than M41) compared with that of the 793/B-type IBV. However, the virus was more similar to 793/B-type IBVs than to H120 at residues 199–573 of the S1 subunit of the spike protein, likely indicating that a recombinant event occurred during the origin and evolution of the ck/CH/LHLJ/140906 isolate.

#### 3.2. Identification of recombination within the genome of IBV isolate ck/CH/LHLJ/140906

To identify the possible recombination events that occurred in the genome of IBV isolate ck/CH/LHLJ/140906 and the regions likely to have been involved in the recombination, similarity plots were performed using strains 4/91 and H120 as representatives of the two main groups of IBVs, while the ck/CH/LDL/101212 strain served as a query. SimPlot analysis was used to display the consecutive nucleotide identity and to illustrate the crossover events among the queried strain and the parental strains. As illustrated in Fig. 3A, four crossover events were suggested by the similarity plot, and 4/91 and H120 served as the parental strains.

To obtain a precise picture of these possible crossover points, the genomic sequence of the ck/CH/LHLJ/140906 isolate was carefully pairwise compared with those of the 4/91, H120 and

M41viruses. In line with the results obtained from the SimPlot analysis, four recombination events with four crossover points in the genome of IBV isolate ck/CH/LHLJ/140906 were found, as shown in Fig. 4. The first recombination breakpoint (nt 9178–9188) was located in the Nsp5 gene (Fig. 4A), the second was located in the Nsp14 gene (nt 17163–17188) (Fig. 4B), the third was located in the Nsp16 gene (nt 20371–20329), and the last was located 5' of the S1 subunit coding sequences of the spike gene (Fig. 4C) (nt 20900–20917) of isolate ck/CH/LHLJ/140906. In addition, data from nucleotide similarities using the corresponding gene fragments supported the above-mentioned results (Fig. 3B), and indicated that H120 showed higher similarity with isolate ck/CH/LHLJ/140906 than M41 in the corresponding gene fragments (Fig. 3B). Phylogenetic trees constructed using the corresponding gene fragments also confirmed the result (Fig. 3C). Taken together, our results clearly indicated that isolate ck/CH/LHLJ/140906 is a naturally recombinant virus that emerged from recombination between 4/91- and H120-like viruses.

#### 3.3. Serotype of IBV isolate ck/CH/LHLJ/140906

Based on the molecular characterization results, cross virus-neutralization tests were employed to serotype the recombinant virus ck/CH/LHLJ/140906 and its deduced parental viruses, 4/91 and H120. The results showed that the R value between virus ck/CH/LHLJ/140906 and the 4/91 vaccine strain was 15%, indicating that ck/CH/LHLJ/140906 possessed a serotype distinct from that of the 4/91 strain (793/B serotype); however, ck/CH/LHLJ/140906 was antigenically closer to Massachusetts strain H120, which resulted in an R value of 32% (Table 2), than to strain 4/91. In addition, the results in this study demonstrated that isolate ck/CH/LHLJ/140906 was serotypically distinct from either of the parental viruses and represented a new serotype.

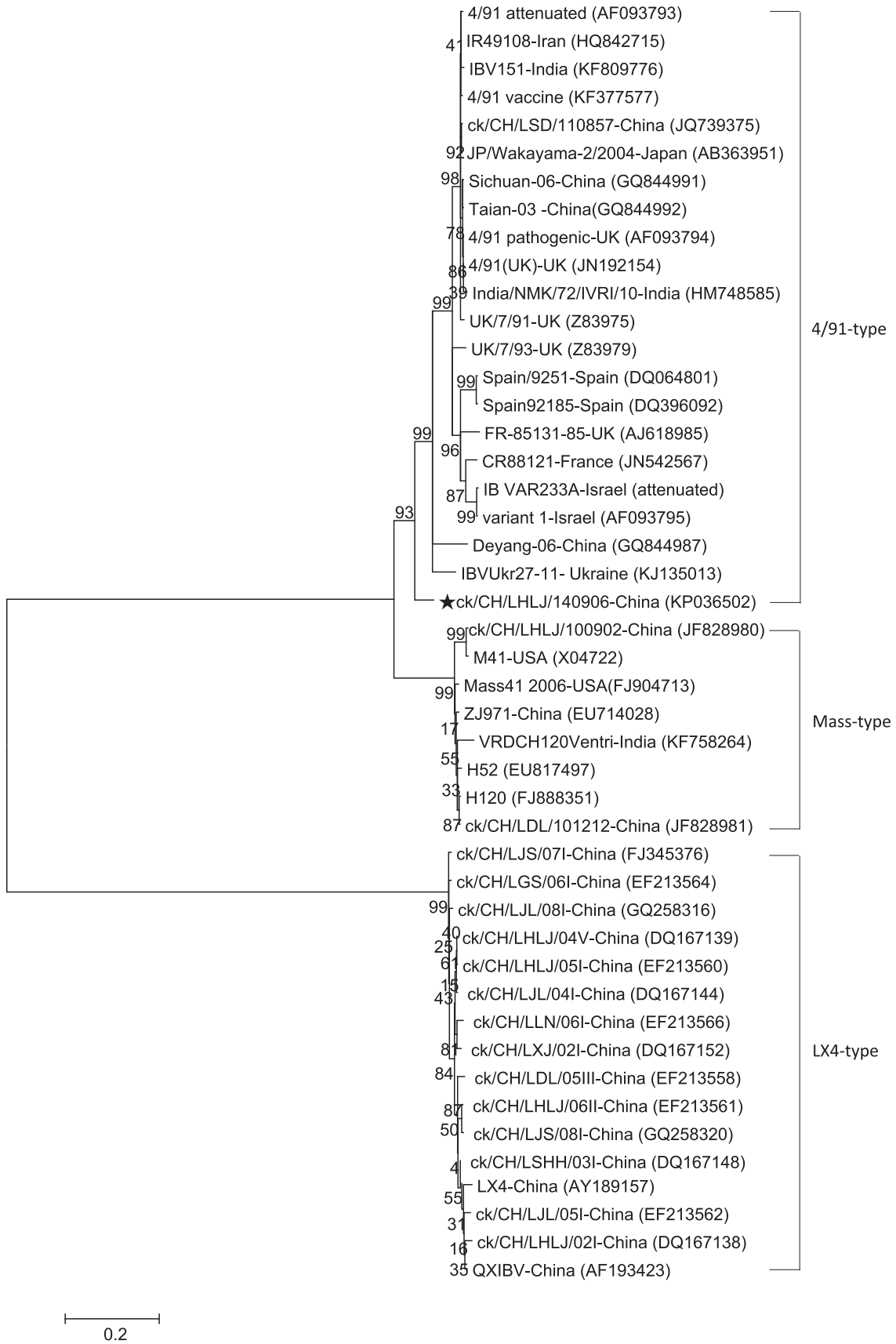
#### 3.4. Protection provided by vaccination with the 4/91 and H120 strains

None of the chickens showed clinical signs when challenged with the IBV isolate ck/CH/LHLJ/140906 when they were 20 days old, indicating that this IBV strain is apathogenic to 20-day-old SPF chickens. Similarly, chickens vaccinated with either 4/91 or the H120 strains did not show clinical signs after challenge with ck/CH/LHLJ/140906. As listed in Table 3, the challenged virus was re-isolated from the tracheas of 30% of the birds in the group vaccinated with the 4/91 vaccine at 4 days post-challenge, whereas 50% of the birds were positive for virus recovery in the group vaccinated with the H120 vaccine. In contrast, the challenged virus was recovered from nasopharyngeal swabs of nearly all birds in the non-vaccinated group at 4 and 8 days post-challenge. As expected, none of the chickens were positive for virus isolation in the negative control group. The serological responses induced by the IBV vaccines and challenge viruses are presented in Table 3. Most of the birds (70%) showed seroconversion at 8 days post-challenge. However, only 50% of the birds in each vaccinated group had seroconverted by 12 days post-vaccination with the 4/91 or H120 vaccine strains; by 20 days post-vaccination, all of the birds vaccinated with either strain 4/91 or the H120 vaccine strain had seroconverted.

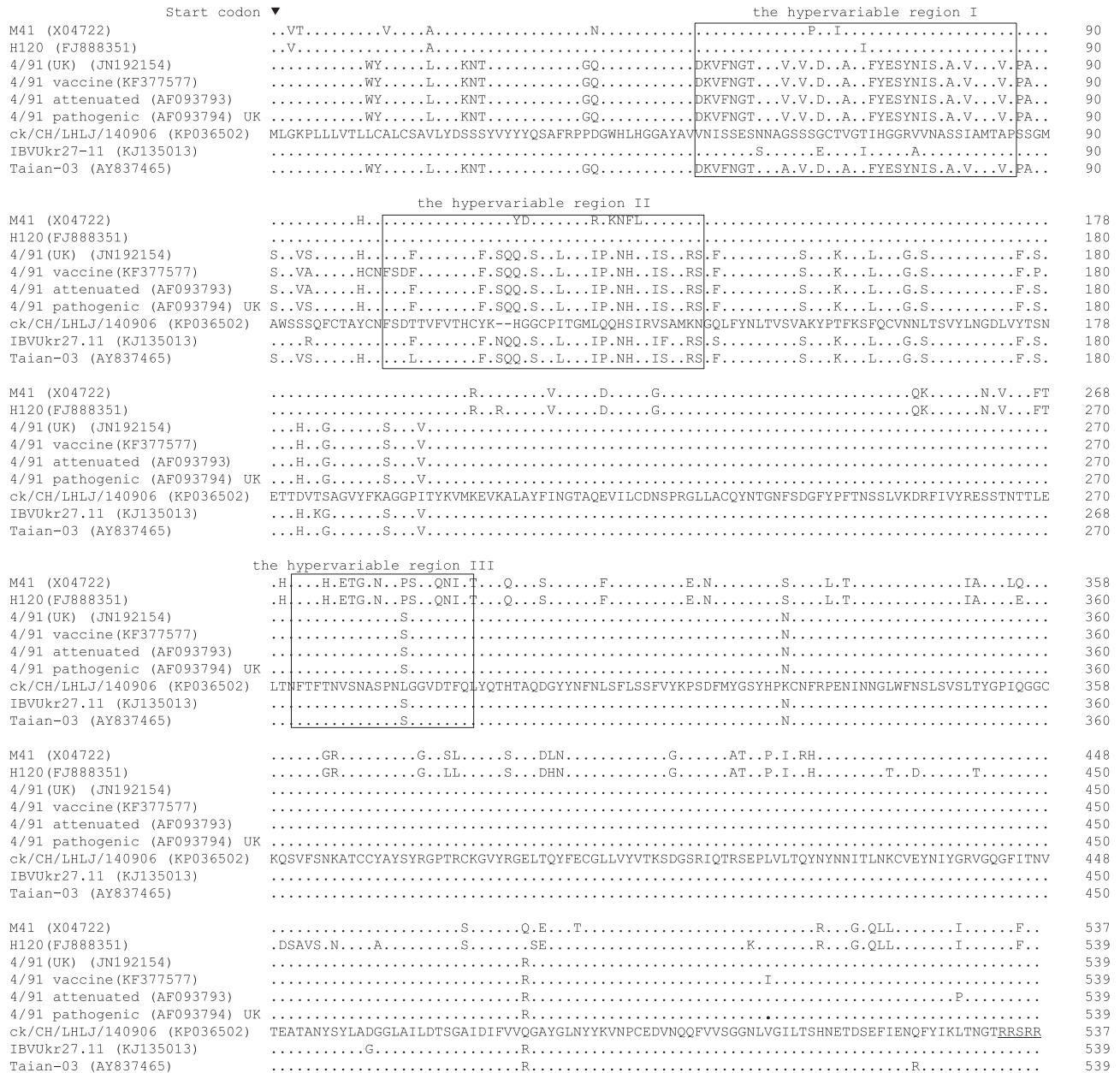
### 4. Discussion

Recombination is a common phenomenon among coronaviruses. The high frequency of homologous recombination, together with the high mutation rates of the genome may lead to the adaptation of coronaviruses and allow the generation of new strains and genotypes (Jackwood et al., 2012; Pasternak et al.,





**Fig. 1.** Consensus phylogenetic tree resulting from the analysis of the nucleotide sequences of the S1 gene of IBV isolate ck/CH/LHLJ/140906 (black star), other 793/B reference strains, LX4- and Massachusetts-type viruses (accession numbers in parentheses). The trees were computed using the neighbor-joining method with 1000 bootstrap replicates using the MEGA4 program.

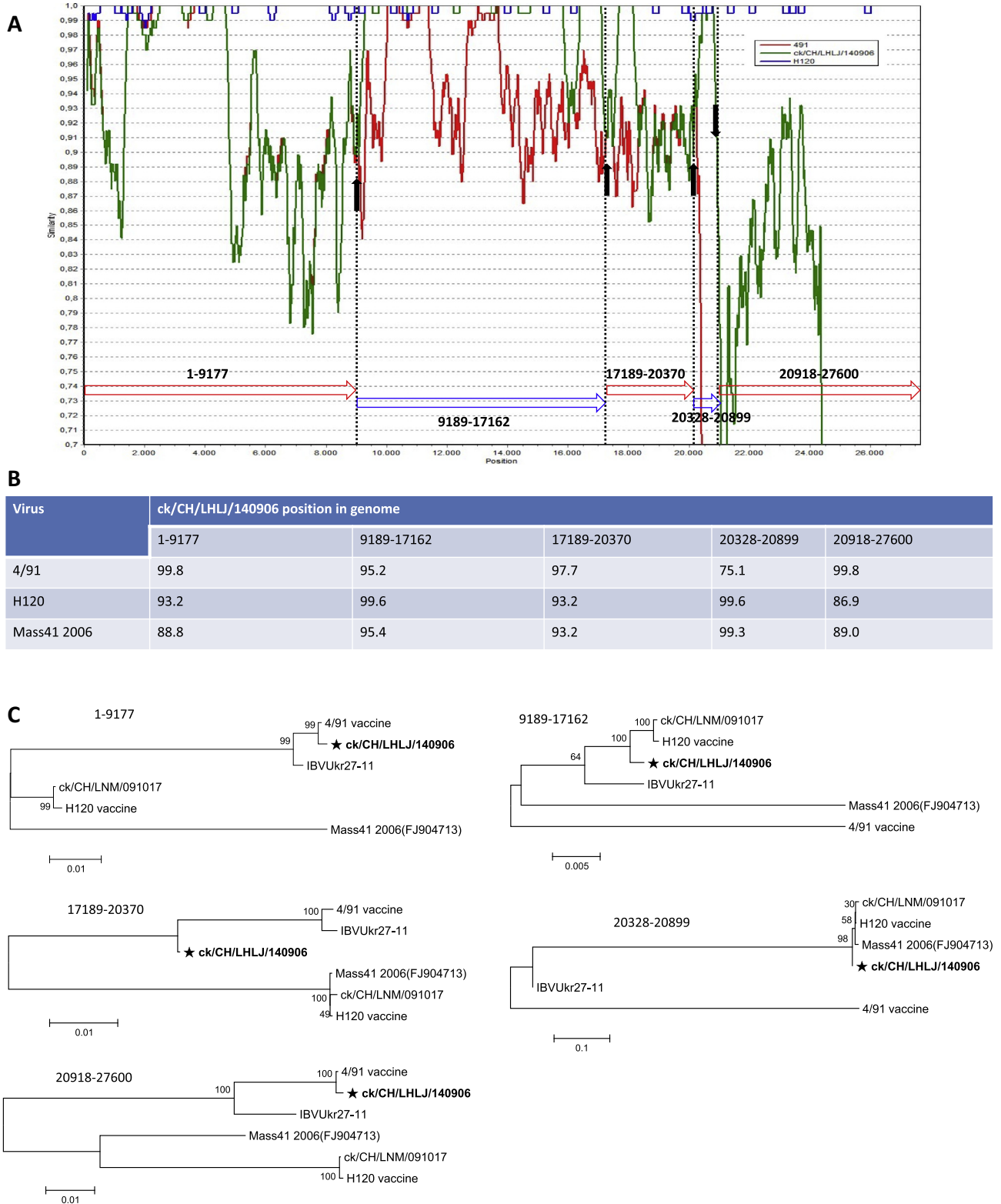


**Fig. 2.** Multiple sequence alignment of S1 amino acid sequences from six 4/91- and two Massachusetts-type IBVs. The numbers on the right of each alignment showed the nucleotide positions in S1 subunit of spike protein of each virus. The sequences of ck/CH/LHLJ/140906 are listed and the only amino acids differing from those of ck/CH/LHLJ/140906 are depicted. The three hypervariable regions (HVRs) are boxed. The deleted nucleotides are represented as -. The cleavage sites of S1 subunit of spike protein are underlined. The GenBank accession numbers are the same as those in Fig. 1.

2006). In this study, based on the SimPlot analysis, which was confirmed by the pairwise nucleotide identity comparison and phylogenetic analysis of different corresponding gene fragments, our results provide convincing evidence that recombination events occurred during the origin and evolution of the IBV isolate ck/CH/LHLJ/140906. Recombination between IBVs and its role in the emergence of new IBV variants have been reported previously (Cavanagh, 1992; Jia et al., 1995; Lee and Jackwood, 2000) and may occur at multiple sites (Jia et al., 1995; Lee and Jackwood, 2000). This is the case in the genome for IBV isolate ck/CH/LHLJ/140906, in which template switches may be occurred between H120- and 4/91-like viruses. It has been shown that crossover events in IBVs occur more frequently at the 3' end of the S gene (Chen et al., 2009; Jia et al., 1995; Mondal and Cardona, 2007; Mardani et al., 2010). However, of the four recombination events

investigated in this study, three presented in the ORF1 region (Nsp genes 5, 14 and 16), and the last was located at the 5' end of the spike gene. It is particularly notable here that the recombination event in the S1 gene of the IBV isolate ck/CH/LHLJ/140906 made this virus a novel strain of IBV that is somewhat distinct from both parental viruses, H120 and 4/91 (Fig. 1), although it is 793/B genotype according to the phylogenetic analysis.

The emergence of the new ck/CH/LHLJ/140906 isolate was considered to be explained, as was the earlier emergence of novel viruses, by recombination between H120- and 4/91-like viruses. In China, only Massachusetts- and LDT3-A-based live vaccines are licensed for use. Based on field experience in large establishments, Massachusetts-based commercial IBV vaccines, especially the H120 strain, are extensively used and can persist in the field



**Fig. 3.** Recombination analysis of the IBV isolate ck/CH/LHLJ/140906. Similarity plot using ck/CH/LDL/101212 as the query sequence (A). The solid arrows showed the deduced recombination breakpoints. The hollow arrows showed the different fragments and their colors were the same as those of the parental viruses. The numbers showed the nucleotide positions of the corresponding fragments in the genome of isolate ck/CH/LHLJ/140906. The y-axis shows the percentage similarity within a sliding window of 200 bp centered on the position plotted, with a step size between plots of 20 bp. IBV isolate ck/CH/LHLJ/140906 was compared to 4/91 and H120 vaccine strains. Percentages of nucleotide sequence identity among ck/CH/LHLJ/140906, 4/91, H120 and Mass41 2006 (B). Percentages of nucleotide sequence identity of corresponding gene fragments are indicated. Phylogenetic analysis of genome positions 1-9177, 9189-17162, 17189-20370, 20328-20899 and 20918-27600 among ck/CH/LHLJ/140906, two 793/B-type (4/91 and IBVUkr27-11) and three Massachusetts-type (ck/CH/LNM/091017, H120 and Mass41 2006) strains (C). The trees were constructed using the neighbor-joining method. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

## A

4/91 vaccine	.....	9036
ck/CH/LHLJ/140906	TCAGGTGACCAATGGAATGATGTACTTAACCTTGCTAATAATACAGAGTTTGAATACAACTCAAATGGTGTGCTTTGAATGTTGTC	9036
H120	.....T.....G.....GT.....AA.....A.....	9036
Mass41 2006-USA	.....T.....G.....T.....T.....G.....G.....G.....A.....T	9096
4/91 vaccine	.....	9126
ck/CH/LHLJ/140906	AGTAGGCGGCTAAGAGGTGCACTTTTGGTATTTACAACTGCTGTCGCTAATGCTGAACTCCAAAGTATAAGTTTATTAAAGCAAATGTT	9126
H120	.....A.....A.....T.....C.....G.....C.....	9126
Mass41 2006-USA	.....T.....G.....C.....A.....C.....C.....T.....G.....T.....	9186
4/91 vaccine	.....G..A.....A.....T.....A..C	9216
ck/CH/LHLJ/140906	GGTGATAGTTTTACCATAGCTTGTCTTATGGTGTACAGTTGTAGGACTTACCCTGTTACTATGCGCTCTAATGGTACTATTAGGGCG	9216
H120	.....A.....G.....T.....T.....C.....G.....T.....C.....	9216
Mass41 2006-USA	.....T.....T.....T.....G.....C.....T.....T.....A..T	9276
4/91 vaccine	..A..TCFT.....T.....C...GTT.....T..C.TC.....	9306
ck/CH/LHLJ/140906	TCTTCTTAGCAGGAGCGTGTGGTTCCTCCAGGTTTAAATATAGAAAAGGGTGTAGTTAACTTTTATTATATGCACCATCTTGTAGTTGCCT	9306
H120	.....	9306
Mass41 2006-USA	.....T.....T.....C..AGTT.....T..C.T.....	9366
4/91 vaccine	.....C.....T.....	9396
ck/CH/LHLJ/140906	AATGCATTACACACAGAACTGACCTAATGGGAGAGTCTATGGTGGTTATGTGGACGAAGAGGTTGCACAAAGGGTGCCACCAGATAAT	9396
H120	.....	9396
Mass41 2006-USA	.....T.....	9456

## B

4/91 vaccine	.....C..C.....A.....T..A..C.....T..G..G.....C.....TC	17046
ck/CH/LHLJ/140906	CTGCAACTTATAAAGTTAATGATGAAGTGGCTGCACTTGTAAACGTAGAATCTGGTTCAGAAATAACATATAAACATCTTATTCTCTGT	17046
H120	.....	17046
Mass41 2006-USA	.....C.....	17103
4/91 vaccine	.T..G.....G.....T.....T.....A..T.....	17136
ck/CH/LHLJ/140906	TAGGATTTAAGATGAGTGTAAATGTTGAAGGTTGCCACAACATGTTTATAACACGTGATGAGGCCATCCGCAATGTAAGAGGTTGGGTAG	17136
H120	.....	17136
Mass41 2006-USA	.....	17193
4/91 vaccine	.....C.....T.....C.....	17226
ck/CH/LHLJ/140906	GTTTTGATGTGGAAGCAACACATGCTTGTGGTACTAACCATTGGTACTAACCTCCCTTTCAAGTGGGCTTCTCGACAGGTGCAGATTTTG	17226
H120	.....G.....A.....T..T.....T..C.....	17226
Mass41 2006-USA	.....G.....A.....T..T.....T..C.....	17283
4/91 vaccine	.....	17316
ck/CH/LHLJ/140906	TAGTCACACCTGAGGACTTGTAGACACTTCAATAGGCAATAATTTGAGCCTGTGAATTCTAAAGCACCTCCAGGTGAACAATTTAATC	17316
H120	.....T.....A..A..G..C.....T.....A.....G.....C.....	17316
Mass41 2006-USA	.....T.....A..A..G..C.....T.....T.....A.....G.....C.....	17373
4/91 vaccine	.....	17406
ck/CH/LHLJ/140906	ACTTGAGAGCTTTATTTAAGAGTGCTAAACCTTGGCATGTTATAAGACCAAGGATAGTGCAAATGTAGCAGATAACCTATGCAATGTTT	17406
H120	.....C..T.....	17406
Mass41 2006-USA	.....C..T.....	17463

**Fig. 4.** Analysis of recombination among IBV ck/CH/LHLJ/140906, 4/91, H120 and Mass41 2006 strains. The numbers on the right of each alignment showed the nucleotide positions in genome of each virus. The sequences of ck/CH/LHLJ/140906 are listed and only the nucleotides differing from those of ck/CH/LHLJ/140906 are depicted. The region where the template switches (breakpoint) have taken place is underlined. The deleted nucleotides are indicated by -. The GenBank accession numbers are the same as those in Fig. 1.

for long periods. In regard to the 4/91-like viruses, this type of vaccine was introduced to China in the late 1990s before the detection of this genotype in the field. Increased numbers of this type of virus have been isolated from chicken flocks of various bird farms in recent years in China. In addition, based on partial sequencing of the S1 gene, it appears that IBV strain 4/91 can change rapidly or that multiple introductions have occurred, e.g., the S1 genes of the 4/91 isolates were 97.8–98.6% identical to that of the IBV 4/91 vaccine in Canada in 2012; however, in 2013, the identity was 94.3–98.8% (Martin et al., 2014). The 4/91-like sequence from isolate ck/CH/LHLJ/140906 was more than 99.6% identical to the S1 gene of the 4/91 vaccine strain, suggesting that the vaccine was the likely source of the 4/91-like sequence. Therefore, it is noteworthy that the natural recombination event demonstrated here suggests that sometimes live vaccines can be involved in recombination.

In contrast to virus species belonging to the alpha- and beta-coronaviruses, which occur as only one or two different serotypes, there are many different serotypes of chicken IBV gamma-

coronaviruses (Jackwood, 2012; de Wit et al., 2011). As the main antigenic viral protein containing epitopes for neutralization (Cavanagh, 2003), the high sequence diversity of the S1 domain accounts for this serotypical variation. Consequently, the analysis of the S1 subunit of the spike glycoprotein to distinguish between genotypes has allowed the IBVs actually present on farms to be readily identified, and this has allowed the vaccine genotype to match that of any current field threat. Furthermore, the technique has allowed the detection of new genotypes or variants, some of which might cause disease. A complicating factor in regard to genotyping IBVs is that when the IBV strain tested is the result of a recombination event in the S1 subunit regions of the spike protein between different IBV genotypes, an examination of different parts of S1 can result in the determination of a different genotype (Wang et al., 1993; Jia et al., 1995; Dolz et al., 2008). In this study, isolate ck/CH/LHLJ/140906 shared similar amino acid sequence identities of the S1 subunit of the spike protein to those of the 4/91 and H120 vaccine strains (88.2% to 4/91 and 86% to H120) due to a recombination event in the S1 gene, although it was



C

4/91 vaccine	.....	20226
ck/CH/LHLJ/140906	ACGCAAATTATATATTTTGGAGGAATTGTAATTATTACAAACCTCTGCTTATAGTGTATTTGACGTTGCAAGTTTGAATTAACAACTAA	20226
H120	.....A.....T.....T.....T.....G.G.T.G.	20226
Mass41 2006-USA	.....A.....T.....T.....T.....G.G.T.G.	20283
4/91 vaccine	.....	20316
ck/CH/LHLJ/140906	AAGCAACGCCAGTTGTTAATTTGAAAACGAACAAAAGACAGACTAGTCTTTAATTTAATTAAGTGTGGTAAAGTTACTGGTAAGAGATG	20316
H120	.....	20316
Mass41 2006-USA	.....	20373
4/91 vaccine	.....G..A.....T.GC.T.....T.AA.A.A.....T.....	20406
ck/CH/LHLJ/140906	TTGGCAAACCGCTTTTACTAGTGACTCTTTTGTGTGCACATAGTGTGCTTTTGTATGACAGTAGTTCTTACGTACTACTACCAA	20406
H120	.....TA.C...T.....C.....	20406
Mass41 2006-USA	.....TA.C...T.....C.....	20463
4/91 vaccine	.....T..G..TGG.C.A.....C.....T....A..A.A...GG...T..A..G.A.C...C.....T.AG.	20496
ck/CH/LHLJ/140906	AGTGCCCTCAGACACCTGATGGTTGGCATTACATGGGGGTGCGTATGCGGTTGTTAATATTTCTAGTGAATCTAATAATGCAGGCTCT	20496
H120	.....	20496
Mass41 2006-USA	.....	20553
4/91 vaccine	GT....AT..C...C.....T...T...AA.CTA.AA.A..TC...G....G....C....A.T...AC.TG.T.....T..	20586
ck/CH/LHLJ/140906	TCATCTGGGTACTGTTGGTACTATTCTATGGTGGTCTGTTGTTAATGCTTCTTATAGCTATGACGGCACCCGTCATCAGGTATGGCT	20586
H120	.....T.....	20586
Mass41 2006-USA	.....T.....	20643
4/91 vaccine	.....AGTTGCA.....A..TC.T.....C.....CTT.....G.....T..AAAGTC....A...A.T	20376
ck/CH/LHLJ/140906	TGGTCTAGCAGTCAGTTTGTACTGCATACTGTAACCTTTCAGATACTACAGTGTGTTGTTACACATTGTTATA-----AACATGGTGGG	20370
H120	.....	20370
Mass41 2006-USA	.....	20727
4/91 vaccine	.....AT.G..A..T...A...CT...A.CA...T...A.....G.TC...ATTTT.G.....C...	20466
ck/CH/LHLJ/140906	TGTCCTATAACTGGCATGCTTCAACAGCATTCTATACGTGTTTCTGCTATGAAAATGGCCAGCTTTTATAATTTAACAGTTAGTGTA	20460
H120	.....	20460
Mass41 2006-USA	.....T...C.....	20817
4/91 vaccine	T...A.....AA.....GC...A.....GGC...CT...T..C.....T...TC.....A	20556
ck/CH/LHLJ/140906	GCTAAGTACCCTACTTTTAAATCATTTCAGTGTGTTAATAATTTAACATCCGTATATTTAAATGGTGATCTTGTTTACACCTCTAATGAG	20550
H120	.....	20550
Mass41 2006-USA	.....	20907
4/91 vaccine	..A..TC.C.....GGG.....C.....AG.....G...G.....	20646
ck/CH/LHLJ/140906	ACCACAGATGTTACATCTGCAGGTGTTATTTTAAAGCTGGTGACCTATAACTTATAAAGTTATGAAAGAAGTTAAAGCCCTAGCCTAC	20640
H120	.....G.....T.....G.....C...T.A...C...T...C.....	20640
Mass41 2006-USA	.....G.....T.....G.....C...T.A...C...T...C.....	20997
4/91 vaccine	.....	20736
ck/CH/LHLJ/140906	TTTATTAATGGTACCGCACAGAGGTTATTTTATGTGATAACTCACCTAGAGGTTGCTTGCATGTCAGTATAACACTGGTAATTTTTC	20730
H120	..G.....T.....T.....G.....GGG.....C...T.A...C...T...C.....	20730
Mass41 2006-USA	..G.....T.....T.....G.....GGA.....C...T.A...C...T...C.....	21087
4/91 vaccine	.....	20826
ck/CH/LHLJ/140906	GATGGATTCTACCCCTTTACTAATCTCTTTAGTTAAGGATAGGTTTATTGTATATCGAGAAAGTAGCACTAACACTACTTTAGAGTTA	20820
H120	.....C..T..T.....AG.AG.....C.G.A.....C...T...A...TGT...T.....TAC....	20820
Mass41 2006-USA	.....C..T..T.....AG.AG.....C.G.A.....C...T...A...TAT...T.....C.TAAG...	21177

Fig. 4 (continued)

Table 2

Titers were obtained in reciprocal  $\beta$  virus neutralization tests (diluted serum, constant virus).

Virus	Serum		
	ck/CH/LHLJ/140906	4/91	H120
ck/CH/LHLJ/140906	147.0	40.0	107.6
4/91	18.4	222.9	<2
H120	32	<2	227.5

clustered with 793/B-type viruses when the phylogenetic tree was constructed using S1 genes (Fig. 1). In contrast, the ck/CH/LHLJ/140906 was serologically more closely related to strain H120 than strain 4/91. Using monoclonal antibodies, five conformation-dependent, neutralizing antigenic sites were mapped on S1, as were other immunodominant regions in the N-terminal regions of S2 (Koch et al., 1990; Kusters et al., 1989; Lenstra et al., 1989). The five neutralizing antigenic sites on S1 co-locate within three

hypervariable regions (HVRs) (Cavanagh et al., 1988, 1992; Moore et al., 1997; Niesters et al., 1987), suggesting that the HVRs are involved in antigenicity and, hence, serotypical variation. In this study, the first two HVRs were found to be located in the H120-like sequences in ck/CH/LHLJ/140906, and the third HVR was in the 4/91-like sequence, suggesting that a recombination event in the S1 gene likely accounts for the serotype shift of the 4/91 genotype to a new serotype, although multiple recombination events were identified in the genome of the virus. Hence, genomic information is objective and provides essential information for epidemiological studies and evolutionary analyses in this case.

In general, IBV strains that have been shown to have the same serotype by virus neutralization may be able to induce complete immunity in chickens based on challenge studies, although in some cases, IBV strains of different serotypes can induce partial or complete immunity in chickens (Ladman et al., 2006). The broader degree of cross-protection in birds is likely to be related to cell-mediated immune responses to shared T-cell epitopes

**Table 3**  
Results of vaccination-challenge tests (vaccination with infectious bronchitis virus vaccine strains 4/91 and H120, followed by challenge with isolate ck/CH/LHLJ/140906).

Group <sup>a</sup>	Morbidity	Mortality	Antibody response										Virus recovery <sup>†</sup>				
			Vaccinated					Challenged					4 d <sup>‡</sup>	8 d	12 d	16 d	
			4 d <sup>‡</sup>	8 d	12 d	16 d	20 d	4 d <sup>‡</sup>	8 d	12 d	16 d	20 d					
1	0/10	0/10	0/10	0/10	5/10	8/10	10/10	10/10	10/10	10/10	10/10	10/10	10/10	3/10	1/10	0/10	0/10
2	0/10	0/10	0/10	0/10	5/10	9/10	10/10	10/10	10/10	10/10	10/10	10/10	10/10	5/10	1/10	1/10	0/10
3	0/10	0/10	–	–	–	–	–	0/10	7/10	9/10	10/10	10/10	10/10	9/10	2/10	0/10	0/10
4	0/10	0/10	0/10	0/10	0/10	0/10	0/10	0/10	0/10	0/10	0/10	0/10	0/10	0/10	0/10	0/10	0/10

<sup>a</sup> Birds in groups 1 and 2 were vaccinated with 4/91 and H120 vaccines, respectively, and challenged with isolate ck/CH/LHLJ/140906. Birds in group 3 were only challenged with IBV isolate ck/CH/LHLJ/140906. Birds in group 4 were not exposed to any viruses and served as negative controls.

<sup>†</sup> Two procedures were used for virus recovery after challenge as described previously (Liu et al., 2009). First, embryos that had been inoculated with individual nasopharyngeal swab samples were observed for lesions. Second, reverse transcription PCR (RT-PCR) using a pair of oligonucleotide primers, N(–) and N(+), was conducted on RNA recovered from the allantoic fluid of the same eggs. The results from the two procedures were identical.

<sup>‡</sup> Days after vaccination/challenge.

among some heterologous strains (Collisson et al., 2000; Seo et al., 2000). In this study, 4/91 vaccination conferred better protection against challenge with ck/CH/LHLJ/140906 than did the H120 vaccine in our vaccination-challenge test, which contrasts with the results that showed that the serotype of ck/CH/LHLJ/140906 was more closely related to that of the H120 strain. Generally, there is a higher chance of good cross-protection between strains with a high level of homology than between strains with low homology (de Wit et al., 2011), and a previous report showed that S1 sequence identity values were more strongly correlated with protective relatedness values than antigenic relatedness values (Ladman et al., 2006). In this study, IBV strain ck/CH/LHLJ/140906 had higher S1 amino acid identity with strain 4/91 than with strain H120. However, the regions in the S1 domain that were considered to contribute to the cross-protection and serotypal variation were HVRs, especially HVR I and HVR II (Ladman et al., 2006). These two HVRs in the S1 subunit of the spike protein of ck/CH/LHLJ/140906 were H120-like, which cannot explain why vaccination with strain 4/91 conferred better protection against ck/CH/LHLJ/140906 than did vaccination with strain H120, despite the fact that the serotype of ck/CH/LHLJ/140906 was more closely related to that of the H120 strain. However, isolate ck/CH/LHLJ/140906 shared higher sequence identity in the S2 domain and in the N protein with the 4/91 vaccine than with those of strain H120; these regions are also important for the immune response, especially cell-mediated immune responses (Seo et al., 2000; Wickramasinghe et al., 2014).

The IBV strain ck/CH/LHLJ/140906 in this study was isolated from H120-vaccinated broilers that showed signs of respiratory disease. However, the 20-day-old SPF chickens did not show clinical signs and mortality when challenged with this strain in this study. These results may not be in conflict with the field results because our observations were obtained from SPF chickens over short periods of time and only considered chickens infected with ck/CH/LHLJ/140906 alone. If other factors, such as secondary infections with pathogenic bacteria or co-infections with immunosuppressing viruses, such as infectious bursal disease virus, are thought to be important in commercial broilers in field conditions (Cavanagh and Gelb, 2008), the present results would have to be adjusted and re-evaluated. Otherwise, 20-day-old SPF white leghorn layers may be not susceptible to ck/CH/LHLJ/140906 strain. In addition, it was possible that ck/CH/LHLJ/140906 strain was indeed non-pathogenic giving that the virus emerged from recombination of vaccine strains and some other agent caused or contributed to the clinical signs observed in the field. Hence, challenge studies in younger chickens or in broilers will be needed to verify the possibility that the virus is indeed non-pathogenic.

The IBV strain ck/CH/LHLJ/140906 was isolated in September 2014 in China and, thus far, we do not know whether this virus

has established itself to a significant extent in China. The implication of our results emphasizes, consequently, the importance of IBV surveillance in chicken flocks, although the ability of the virus to change continually by mutation or recombination challenges our ability to both diagnose and control it.

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