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# Genetic, antigenic, and pathogenic characteristics of avian infectious bronchitis viruses genotypically related to 793/B in China



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## ABSTRACT

In this study, 20 infectious bronchitis virus (IBV) strains, which were genotypically related to 793/B, as assessed by an S1 gene comparison and a complete genomic sequence analysis, were isolated and identified from 2009 to 2014 in China. Phylogenetic analysis, network tree, similarity plot analysis, Recombination Detection Program 4(RDP4) and sequence comparison revealed that 12 of the 20 isolates were likely the reisolated vaccine virus. One isolate, ck/CH/LSD/110857, was shown to have originated from recombination events between H120- and 4/91-like vaccine strains that did not result in changes of antigenicity and pathogenicity. The remaining seven IBV isolates were shown to have originated from recombination events between a 4/91-like vaccine strain and a GX-LY9-like virus, which were responsible for the emergence of a novel serotype. A vaccination-challenge test found that vaccination with the 4/91 vaccine strain did not provide protection against challenge with the recombinant viruses. In addition, the results showed that the recombination events between the vaccine and field strains resulted in altered genetics, serotype, antigenicity, and pathogenicity compared with those of their deduced parental viruses. The results are important not only because this virus is of economic importance to poultry industry, but also because it is important for elucidating the origin and evolution of other coronaviruses.

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## 1. Introduction

Coronaviruses (CoVs), a genus of the *Coronaviridae* family, order *Nidovirales*, are enveloped, positive-stranded RNA viruses that have been identified in humans and a variety of animals, including pigs, mice, cows, rats, dogs, cats, rabbits, horses, bats, whales, and birds, and they cause highly prevalent diseases, including respiratory, enteric, cardiovascular, and neurological disorders (Masters, 2006). The genomic RNA of CoV is 27–32-kb in size, and it is capped and polyadenylated. The 5' two-thirds of the CoV genome encodes the replicase genes, which are translated into two large polyproteins (1a and 1ab) that are processed into 15 or 16 non-structural proteins (Nsps) via proteolytic cleavage. The 3' one-third of the CoV genome encodes several structural proteins, such as the surface spike glycoprotein (S), small envelop (E), membrane (M), and nucleocapsid (N) proteins. Additional accessory protein genes are located in the 3' part of the genome in a CoV species-specific

position. In most *Beta* and all gammacoronaviruses, the S protein is cleaved into two polypeptides, S1 and S2, by a trypsin-like host protease (Cavanagh et al., 1986). The S1 domain is the most divergent region of the molecule, both across and within coronavirus genera. It is generally believed that the S1 subunit contains the receptor-binding activity of the S protein, carries virus-neutralizing and serotype-specific determinants, and is a determinant of cell tropism. Because of its genomic plasticity, coronaviruses are characterized by a high potential for evolution, adaption, and interspecies jumping (Woo et al., 2009).

Avian coronavirus infectious bronchitis virus (IBV) belongs to the *Gammacoronavirus* genus, and it causes a highly contagious upper respiratory disease, a decrease in egg production, poor eggshell quality, reduced hatchability, nephritis, and, sometimes, false layers in chickens. Since IBV was first described in 1936, many serotypes and variants have been isolated worldwide. They arose as a result of the point mutations, insertions, or/and deletions in the genome, especially in the S1 subunit of the S protein (Cavanagh, 2007). In addition, like other coronaviruses, such as severe acute respiratory syndrome CoV (Stavrínides and Guttman, 2004; Hon

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et al., 2008), human CoV NL63 (Pyrce et al., 2006), human CoV HKU1 (Woo et al., 2006), and the recently identified Middle East respiratory syndrome CoV (Sabir et al., 2016), recombination is another very important feature that is thought to contribute to the emergence of novel IBV pathotypes, serotypes/genotypes, and variants (Kusters et al., 1989; Wang et al., 1993).

IB was first described as a respiratory disease of chicks by Schalk and Hawn in the USA in 1931 (Schalk and Hawn, 1931). In 1956, Jungherr was the first to demonstrate that IBV was heterogeneous by isolating an IBV in Connecticut, USA, that did not cross-protect chickens against challenge with the original Massachusetts (Mass) isolate (Jungherr et al., 1956); since then, dozens of IBV serotypes and variants have been isolated worldwide (de Wit et al., 2011). 793/B (also named 4/91 or CR88) is among the most well characterized and important IBV serotypes that emerged in the UK and France in the 1990s, and it has been associated with major illnesses and economic impacts in apparently well-vaccinated flocks (Gough et al., 1992; Parsons et al., 1992). The pathology caused by 793/B is not typical of other IBV infections, as it was originally associated with mortality in breeders, scouring in broilers, and possibly muscle myopathy under field conditions. It is interesting that unlike some of the IBV serotypes that remain localized, 793/B type viruses quickly spread to many parts of the world (Xu et al., 2007), but we do not know why this occurred. In addition, the 793/B type virus continues to be a problem in many parts of the world, but its structure and antigenicity do not appear to have changed greatly (de Wit et al., 2011).

The 793/B genotype was first isolated 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 under the accession number AY837465. Since then, some other strains of this virus also have been isolated and identified (Xu et al., 2007; Han et al., 2009). Interestingly, we recently isolated a virulent, nephropathogenic 793/B type virus, which was found to have emerged from a recombination event between the 4/91 vaccine strain and an LX4 type (also known as QX-like) virus (Liu et al., 2013). More recently, we isolated another 793/B genotype virus, which also originated from a recombinant event that resulted in a serotype shift (Zhang et al., 2015). These results indicate that the 793/B type might be becoming a major concern to the poultry industry in China, although the origin and evolution of the virus is not clear and little is known about the influence of the genotype on the ecology of other IBVs. In the present study, 20 793/B isolates were selected by screening 418 IBVs (Chen et al., 2015) that were isolated from chickens during our continuous surveillance activities for IBV in China from 2009 to 2014. The molecular characteristics, antigenicity, and pathogenicity of the 20 viruses were investigated to further elucidate the origin and evolution of IBV isolates genotypically related to 793/B viruses in China.

## 2. Materials and methods

### 2.1. Virus strains

Of 418 IBVs that were isolated from chickens that were suspected to have IBV infections during our continuous surveillance activities for IBV in China, 20 IBV strains were positive by a 793/B genotype-specific RT-PCR method that targeted the S1 gene (Chen et al., 2015). All of the chicken flocks from which these IBV strains were isolated were vaccinated against IB with the commercial, live attenuated H120 vaccine, and some of them were boosted with the 4/91 or Ma5 vaccine strains. Clinical signs of respiratory disease, including gasping, coughing, sneezing, and tracheal rales at the onset of disease, were found in some 20–70-day-old chickens in these flocks. Post-mortem examinations were performed, and gross lesions were evaluated for diseased and/or

dead chickens. The gross examinations showed tracheitis in all of the infected chickens, and most of the chickens showed nephritis and/or proventriculitis. Information about the regions and the organs from which the isolates were obtained are listed in Supplemental Table.

Based on the S1 gene and complete genomic sequence analysis results, six of the 20 IBVs genetically related to 793/B, including ck/CH/LSD/110851, ck/CH/LSD/110857, ck/CH/LHB/130575, ck/CH/LGD/090907, and ck/CH/LGD/120723, were used for virus-neutralization and/or vaccination-challenge tests. A commercial 793/B genotype vaccine, the 4/91 strain, was used for the viral genomic sequencing, virus-neutralization, and vaccination-challenge tests. Two LX4 type IBV strains, ck/CH/LHLJ/04 V and ck/CH/LDL/091022, were also used for virus-neutralization and/or vaccination-challenge tests. The viral stocks of these viruses were prepared by inoculating the viruses into embryonated SPF chicken eggs via the allantoic cavity and collecting the infectious allantoic fluid 48 h post-inoculation as previously described (Liu and Kong, 2004). The titers of the viruses were determined using previously described methods (Reed and Muench, 1938).

### 2.2. S1 gene analysis and genomic sequencing

The 20 IBV isolates, along with the 4/91 vaccine strain, were tested by RT-PCR, and they were sequenced subsequently using primers S1Uni2+ and S1Uni1, which target the portion of the S gene that encodes the S1 subunit (Adzhar et al., 1997). A maximum likelihood tree based on a JTT matrix-based model and 100 bootstrap replicates was built using MEGA4 and the S1 nucleotide sequences of our 21 viruses, 27 793/B reference strains that were isolated in different countries, eight Mass genotype strains, and 22 other IBV strains that were isolated in China (Liu et al., 2013). Meanwhile, a network tree was constructed using SplitsTrees, version 4.1.4.3, using the maximum likelihood (F48) distance model, the NeighborNet network algorithm, 1000 bootstrap replicates and an estimated proportion of invariant sites of 0.37 (Quinteros et al., 2016).

Based on these results, our 20 viruses, along with the 4/91 vaccine strain, were subjected to complete genomic sequencing. A previously described RT-PCR protocol was used to amplify the complete genomes of our 20 viruses (Liu et al., 2013). Briefly, viral RNA was extracted from 200  $\mu$ l of viral stocks using TRIzol reagent (Invitrogen, Carlsbad, CA, USA) and re-dissolved in 80  $\mu$ l of RNase-free water. RT and PCR were performed using a PrimeScript II High Fidelity One Step RT-PCR kit (TaKaRa, Shiga, Japan) with twenty overlapping primers (Zhang et al., 2015). Primers sequences, amplification and sequencing conditions are available from the author upon request. The 3' and 5' ends of the viral genomes were confirmed by rapid amplification of cDNA ends using a 3'/5' RACE kit (TaKaRa) as previously described (Liu et al., 2013). The PCR products were detected by electrophoresis of a 1% agarose gel and visualization under UV light after ethidium bromide staining. Then, the PCR products were cloned into a pMD 18-T vector (TaKaRa). Sanger sequencing was performed (Big Dye Terminator) and three to five clones were sequenced to determine a consensus sequence for any given genomic region.

### 2.3. The complete genomic sequence comparison and analysis

A maximum likelihood phylogenetic tree based on the JTT matrix-based model and 100 bootstrap replicates was built using MEGA4 and the complete genomic nucleotide sequences of our 21 viruses and the other reference strains (Liu et al., 2013). A total of 17 IBV reference strains were selected based on the results of the phylogenetic analysis of S1 genes, as well as the availability of their complete genomic sequences in the GenBank database. The

selected avian coronavirus reference strains and their accession numbers are shown in Fig. 1. To detect possible recombination events between our 20 isolates and the other viruses, a sliding window analysis was performed using a nucleotide alignment of the available genome sequences that was generated by ClustalX version 1.83 and edited manually with BioEdit version 7.1.3 (Lasergene Corp, Madison, WI, USA). A network tree was constructed using the aforementioned methods (Quinteros et al., 2016). Furthermore, a SimPlot analysis was performed using SimPlot version 3.5.1 (Liu et al., 2013) (window size, 1000 bp; step, 200 bp) with all 20 isolates and the 4/91 vaccine strain, using the H120 vaccine strain as a query. Then, representatives of the individual strains, ck/CH/LSD/110857 and ck/CH/LGD/120723, were further selected based on the aforementioned results and used for the SimPlot analysis again with the 4/91 vaccine strain as a query. To further confirm the recombination events and detect the recombination breakpoints, the representatives of the individual strains were examined using Recombination Detection Program 4 (RDP4, version 4.56) as previously described (Quinteros et al., 2016). In addition, the nucleotide sequences encoding an RNA-dependent polymerase and the S protein of the representatives of the individual strains were pairwise compared with those of the 4/91 vaccine strain and the identified parental virus strains, respectively, to confirm the recombination breakpoints.

#### 2.4. Accession numbers

The genomic sequences of the 21 IBV strains have been submitted to the GenBank database under the accession numbers illustrated in Fig. 1.

#### 2.5. Cross virus-neutralization tests

Six IBV strains, the 4/91 vaccine, ck/CH/LSD/110851, ck/CH/LSD/110857, ck/CH/LGD/090907, ck/CH/LGD/120723, and ck/CH/LDL/091022, were used for antiserum preparation and cross-neutralization tests as described previously (Liu et al., 2013). To determine the antigenic relationships among these IBV strains, reciprocal beta virus neutralization (VN) assays, with a fixed concentration of virus (100 median embryo infectious doses (EID<sub>50</sub>)) and serial dilutions of serum (two-fold serial dilutions), were conducted. The VN tests were performed as described previously (Cavanagh et al., 1999). End-point titers corresponded to the serum dilutions that neutralized 50% of the virus were calculated by the method of Reed and Muench (Reed and Muench, 1938).

#### 2.6. Pathotyping in 1 day-old chicks

All experiments were conducted using standard procedures with the formal approval of the Ethical and Animal Welfare Committee of the Harbin Veterinary Research Institute, China. Based on the results of the phylogenetic analysis of the S1 gene and the complete genomic sequence analysis, as well as the year that the virus was isolated and the amino acid residue at position 95, five 793/B genotype viruses were selected for pathotyping; the 4/91 vaccine strain was used as a control. Seven groups of 10 1-day-old SPF layer chickens were placed in isolators and challenged oculo-nasally on the day of hatching with 10<sup>5</sup> EID<sub>50</sub> of the selected strains, including the 4/91 vaccine, the ck/CH/LSD/110851, ck/CH/LSD/110857, ck/CH/LHB/130575, ck/CH/LGD/090907, and ck/CH/LGD/120723 isolates; mock infected chickens served as controls. Clinical signs, including gasping, coughing, sneezing, tracheal rales, and nasal discharge, were carefully examined for 25 dpc. Gross lesions of dead chickens were carefully examined and recorded. IHC using the monoclonal antibody 6D10 was employed to detect the nucleocapsid in the kidneys of the dead chickens that were

challenged with IBV isolate ck/CH/LDG/120723 (Han et al., 2015). The IHC assay was conducted as described previously (Mariette et al., 2009).

#### 2.7. Vaccination-challenge experiment

One hundred and sixty 1-day-old SPF White Leghorn chicks were housed in different isolators and divided into sixteen groups, each containing 10 birds. Birds in group 1 were only vaccinated with the 4/91 vaccine strain. Birds (1-day-old) in groups 3, 5, 7, 9, 11, 13, and 15 were vaccinated with the 4/91 vaccine strain by ocular administration with 10<sup>4</sup> EID<sub>50</sub> in 0.1 ml of diluent, and they were challenged with 10<sup>6</sup> EID<sub>50</sub> in 0.1 ml of diluent of the ck/CH/LSD/110851, ck/CH/LSD/110857, ck/CH/LHB/130575, ck/CH/LGD/090907, ck/CH/LGD/120723, ck/CH/LHLJ/04V, and ck/CH/LDL/091022 isolates, respectively, by ocular administration at d 20. Birds in groups 2, 4, 6, 8, 10, 12, and 14 were only challenged with IBV isolates ck/CH/LSD/110851, ck/CH/LSD/110857, ck/CH/LHB/130575, ck/CH/LGD/090907, ck/CH/LGD/120723, and ck/CH/LHLJ/04V and ck/CH/LDL/091022 at d 20, respectively. Birds in group 16 were not exposed to any viruses and served as negative controls. Nasopharyngeal and cloacal swabs and blood samples were collected from all of the birds in each treatment group at 4, 8, 12, 16 and 20 d after vaccination and at 4, 8, 12, 16, 20, 24, 28, 32, 36, 40, 44, and 48 dpc. Nasopharyngeal and cloacal swabs were used for virus recovery, and serum was stored at -70 °C until enzyme-linked immunosorbent assay (ELISA) testing was performed.

#### 2.8. Virus recovery and RT-PCR detection

The level of protection provided by the 4/91 vaccine strain was determined by virus recovery from nasopharyngeal swabs that were collected from 10 chickens in each group. Nasopharyngeal swab tubes were centrifuged at 6000 × g for 10 min, and the supernatant samples were inoculated into three to five SPF embryonated eggs via the allantoic cavity (0.1 ml per egg). Allantoic fluid from two of the inoculated embryos was collected for RT-PCR amplification targeting the N gene of IBVs as described previously (Liu et al., 2013). The PCR products were sequenced directly to verify the challenge virus. A positive sample was recorded if specific lesions, such as dwarfing, stunting, or curling of embryos, were observed and the RT-PCR amplification was positive.

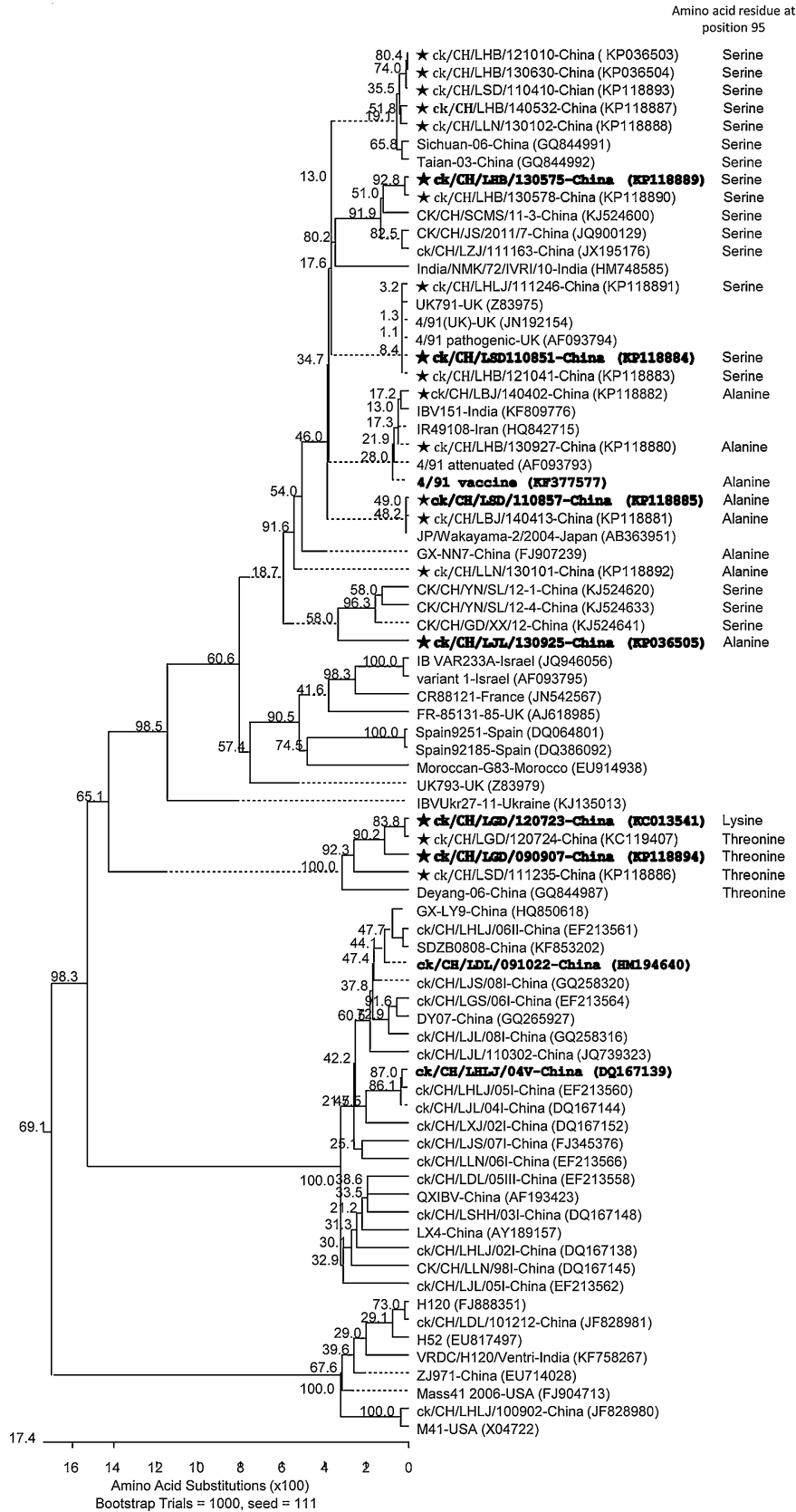
#### 2.9. Serum antibody detection

A commercial, total antibody ELISA (IDEXX Corporation, Westbrook, ME, USA) was used to detect anti-IBV antibodies. Each serum sample was tested in triplicate. Individual serum titers were calculated from the sample to positive (S/P) ratios (Liu et al., 2013) and expressed as the optical density at 650 nm values according to the manufacturer's instructions.

### 3. Results

#### 3.1. The IBV isolates were closely related to 793/B type based on the S1 gene analysis

In line with the results from 793/B genotype-specific reverse transcription-polymerase chain reaction (RT-PCR) (Chen et al., 2015), phylogenetic analysis using the complete sequence of the S1 gene showed that all 20 IBV isolates were genotypically related to the 793/B (Fig. 1). However, some isolates, including ck/CH/LGD/120723, ck/CH/LGD/120724, ck/CH/LGD/090907, and ck/CH/LSD/111235, as well as the reference strain Deyang-06, showed various topologies compared with those of other 793/B viruses, although



**Fig. 1.** The neighbor-joining phylogenetic trees of the complete S1 nucleotide sequence of our 20 isolates (black stars) and reference strains, respectively. The IBV strains used for serotyping and/or vaccination-challenge are shown in bold. In addition, the amino acid residues at position 95, at which a Ser or Ala residue are believed to favor virus replication in chickens and embryonated eggs, are also shown. GenBank accession numbers are indicated in parentheses.

they still clustered together with other 793/B strains in the phylogenetic analysis (Fig. 1). Nucleotide and amino acid similarities also confirmed these results (data not shown). Furthermore, insertions and deletions were also found at different positions between amino acid residues 10 and 135 in the S1 subunit of the S protein in the different viruses. In addition, 19 of the 30 Chinese 793/B-like strains have a Ser residue at position 95, seven have an Ala, and five have other residues at this position (Fig. 1). It was reported that virus replication in chickens and embryonated eggs was favored by a Ser or Ala residue at this position (Xu et al., 2015). These results indicate that IBV isolates genotypically related to 793/B that are circulating in chicken flocks in China likely originated from different ancestors and/or evolved differently as they adapted to, and spread among, different chicken flocks. In addition, the recombination events have been detected in the S1 genes by the network tree constructed between some of the IBV isolates and reference strains (Supplemental Fig. 1).

### 3.2. Complete genomic sequence analysis

A sequence analysis revealed that the full genomic RNA sequences of our 20 IBV isolates contain conserved open reading frames with an overall genome organization that is similar to known IBVs (Boursnell et al., 1987). Twelve of the 20 isolates (designed as type I), including ck/CH/LBJ/140413, ck/CH/LHB/130927, ck/CH/LLN/130102, ck/CH/LSD/110851, ck/CH/LBJ/140402, ck/CH/LLN/130101, HLJ/111246, ck/CH/LSD/110410, ck/CH/LHB/121010, ck/CH/LHB/130630, ck/CH/LHB/120141, and ck/CH/LHB/140532, showed at least 99.8% nucleotide identities to the 4/91 vaccine strain, as well as between each other, as assessed by pairwise sequence alignments. Of the 11 mutations in the S gene that differentiate the pathogenic 4/91 strain and the 4/91 vaccine strain, the 12 isolates shared eight mutations with the 4/91 vaccine strain and only one with the pathogenic 4/91 strain (Table 1), suggesting that this region of the 12 isolates was more similar to that of the 4/91 vaccine strain than that of the pathogenic 4/91 strain. The ck/CH/LSD/110857 isolate (designed as type II), which differed from the other 793/B genotype viruses, had at least 98.8% nucleotide homology to the 4/91 vaccine strain and the type I viruses. Of the 11

mentioned mutations, ck/CH/LSD/110857 shared 10 mutations with the 4/91 vaccine strain and only one mutation with the pathogenic 4/91 strain (Table 1). In contrast, the remaining seven isolates (designed as type III), including ck/CH/LGD/090907, ck/CH/LGD/120723, ck/CH/LGD/120724, ck/CH/LSD/111235, ck/CH/LHB/130575, ck/CH/LHB/130578, and ck/CH/LJL/130925, showed overall genome similarities to the 4/91 strain that ranged from 86.3 to 87.4%, compared with the 94.9–99.9% similarities that they shared with each other. A phylogenetic analysis of the complete genomic sequences confirmed the results, as the 13 type I and II isolates clustered with the 4/91 strain, and the remaining seven type III isolates grouped with other strains isolated in China (Fig. 2).

The network tree constructed using our 20 isolates and reference strains detected the recombination events in the complete genomes between some of our isolates and reference strains (Fig. 3). Therefore, representative isolates ck/CH/LSD/110857 and ck/CH/LGD/120723 were selected for further recombination examination and analysis using RDP4 and SimPlot analysis, and the recombination breakpoints were confirmed by pairwise comparison of the complete genomic sequences. The results suggested that ck/CH/LSD/110857 arose from recombination events between the 4/91 vaccine strain and another vaccine strain, H120 (Fig. 4A and Supplemental Fig. 2A). Two recombination sites in the genome of the ck/CH/LSD/110857 isolate, which are located at nt 9548–9560 and 15594–15602 in the Nsp5- and Nsp13-encoding genes, respectively (Supplemental Fig. 2B), were identified. In addition, as illustrated in Fig. 4B and Supplemental Fig. 3B, isolate ck/CH/LGD/120723 originated from recombination events, and 4/91- and GX-LY9-like viruses served as the parental strains. Two recombination breakpoints (nt 21043–21048 and 22130–22143) were confirmed to be located in the S1 gene of the ck/CH/LGD/120723 isolate (Supplemental Fig. 2B).

### 3.3. Serotypes of the isolates genotypically related to 793/B in China

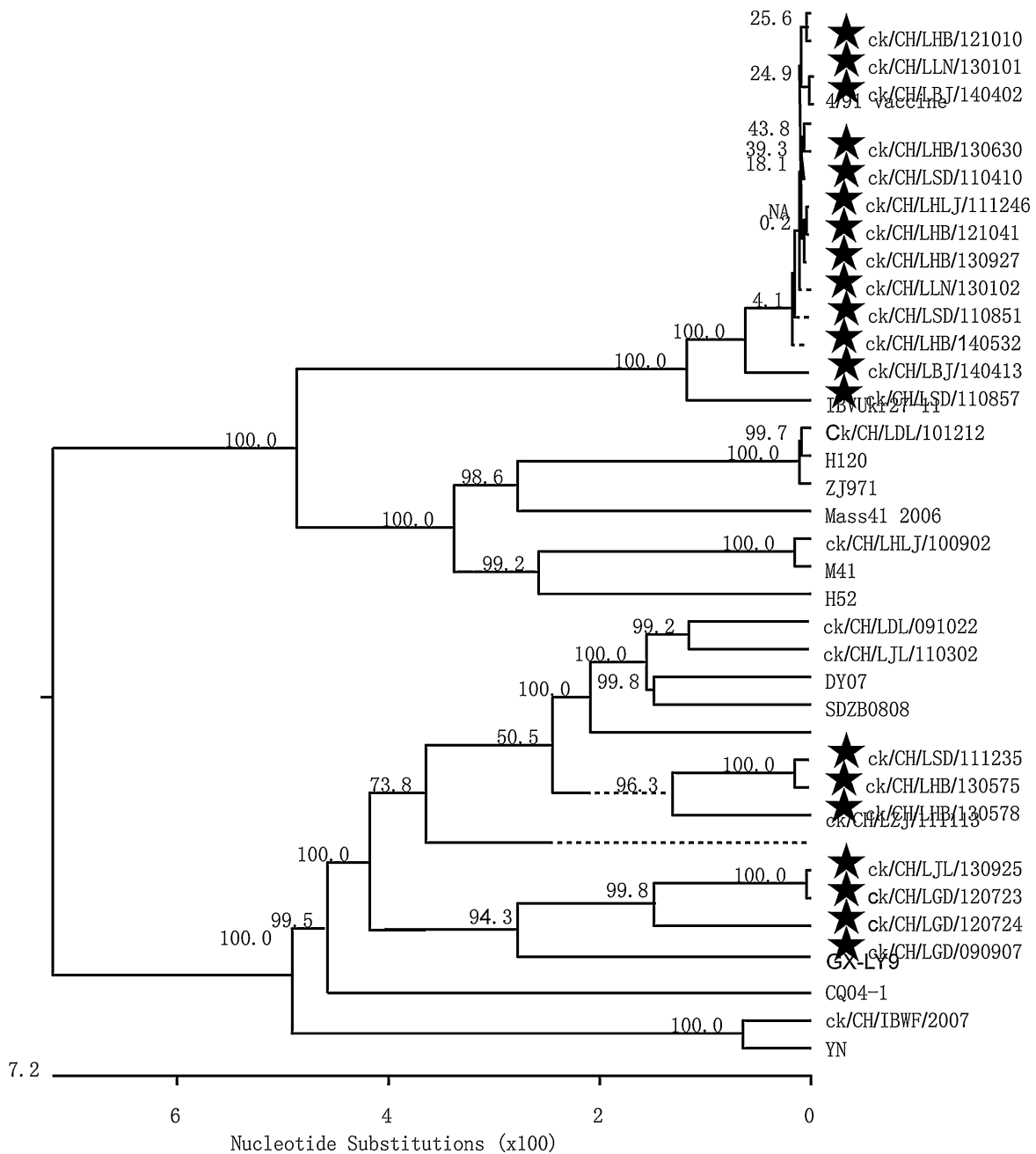
Based on the molecular characterization results, six IBV strains, including our four isolates, and the 4/91 vaccine and LX4-type ck/CH/LDL/091022 strains (Liu et al., 2013), were examined by cross virus-neutralization tests. The results are listed in Table 2. Two

**Table 1**

Pairwise comparisons of the nucleotide sequences of the RNA polymerase (RDRP) and spike (S) genes of 12 4/91-like isolates and the ck/CH/LSD/110857 isolate with those of the 4/91 vaccine and 4/91 pathogenic strains<sup>a</sup>.

| Strain            | Genome |          |       |          |          |          |          |          |          |          |          |          |
|-------------------|--------|----------|-------|----------|----------|----------|----------|----------|----------|----------|----------|----------|
|                   | 14502  | 20599    | 20851 | 22748    | 23327    | 23700    | 23706    | 23715    | 23715    | 23739    | 23751    | 23769    |
|                   | RDRP   |          |       |          |          |          | S        |          |          |          |          |          |
|                   | 322    | 283      | 535   | 2432     | 3011     | 3384     | 3390     | 3399     | 3403     | 3423     | 3435     | 3453     |
| 4/91 vaccine      | T      | G        | C     | <b>A</b> | T        | <b>G</b> | <b>C</b> | <b>T</b> | <b>T</b> | <b>A</b> | <b>C</b> | <b>T</b> |
| ck/CH/LSD/110851  | T      | T        | T     | <b>A</b> | C        | <b>G</b> | <b>C</b> | <b>T</b> | <b>T</b> | <b>A</b> | <b>C</b> | <b>T</b> |
| ck/CH/LBJ/140413  | T      | G        | T     | <b>A</b> | T        | <b>G</b> | <b>C</b> | <b>T</b> | <b>T</b> | <b>A</b> | <b>C</b> | <b>T</b> |
| ck/CH/LSD/110410  | G      | T        | T     | <b>A</b> | C        | <b>G</b> | <b>C</b> | <b>T</b> | <b>T</b> | <b>A</b> | <b>C</b> | <b>T</b> |
| ck/CH/LBJ/140402  | G      | G        | T     | <b>A</b> | C        | <b>G</b> | <b>C</b> | <b>T</b> | <b>T</b> | <b>A</b> | <b>C</b> | <b>T</b> |
| ck/CH/LHB/121010  | G      | T        | T     | <b>A</b> | C        | <b>G</b> | <b>C</b> | <b>T</b> | <b>T</b> | <b>A</b> | <b>C</b> | <b>T</b> |
| ck/CH/LHB/121041  | G      | T        | T     | <b>A</b> | T        | <b>G</b> | <b>C</b> | <b>T</b> | <b>T</b> | <b>A</b> | <b>C</b> | <b>T</b> |
| ck/CH/LHB/130630  | G      | T        | T     | <b>A</b> | T        | <b>G</b> | <b>C</b> | <b>T</b> | <b>T</b> | <b>A</b> | <b>C</b> | <b>T</b> |
| ck/CH/LHB/130927  | G      | G        | T     | <b>A</b> | T        | <b>G</b> | <b>C</b> | <b>T</b> | <b>T</b> | <b>A</b> | <b>C</b> | <b>T</b> |
| ck/CH/LHB/140532  | G      | T        | T     | <b>A</b> | T        | <b>G</b> | <b>C</b> | <b>T</b> | <b>T</b> | <b>A</b> | <b>C</b> | <b>T</b> |
| ck/CH/LHLJ/111246 | G      | T        | T     | <b>A</b> | T        | <b>G</b> | <b>C</b> | <b>T</b> | <b>T</b> | <b>A</b> | <b>C</b> | <b>T</b> |
| ck/CH/LLN/130101  | G      | G        | T     | <b>A</b> | C        | <b>G</b> | <b>C</b> | <b>T</b> | <b>T</b> | <b>A</b> | <b>C</b> | <b>T</b> |
| ck/CH/LLN/130102  | G      | T        | T     | <b>A</b> | T        | <b>G</b> | <b>C</b> | <b>T</b> | <b>T</b> | <b>A</b> | <b>C</b> | <b>T</b> |
| 4/91 pathogenic   | G      | T        | T     | G        | C        | A        | T        | G        | C        | G        | T        | C        |
| ck/CH/LSD/110857  | G      | <b>G</b> | T     | <b>A</b> | <b>T</b> | <b>G</b> | <b>C</b> | <b>T</b> | <b>T</b> | <b>A</b> | <b>C</b> | <b>T</b> |

<sup>a</sup> Only the nucleotide sequences of the RNA-dependent polymerase (RDRP), spike (S) and nucleocapsid (N) genes of a pathogenic 4/91 strain were available in the GenBank database. The nucleotide sequences of the three genes of our 12 4/91-like isolates were compared with those of the 4/91 vaccine and 4/91 pathogenic strains. One and eleven changes were observed among our 12 isolates, and the 4/91 vaccine and pathogenic 4/91 strains, respectively, and no change was found in the N gene. Nucleotides that are shared between our isolates and the 4/91 vaccine strain are shown in bold. Nucleotide positions correspond to those in the sequence of the 4/91 vaccine genome. GenBank accession numbers of the 4/91 pathogenic strain are FN811147 (RDRP), JN192154 (S), and EU780081 (N). The GenBank accession numbers of other viruses are the same as those shown in Fig. 1A.



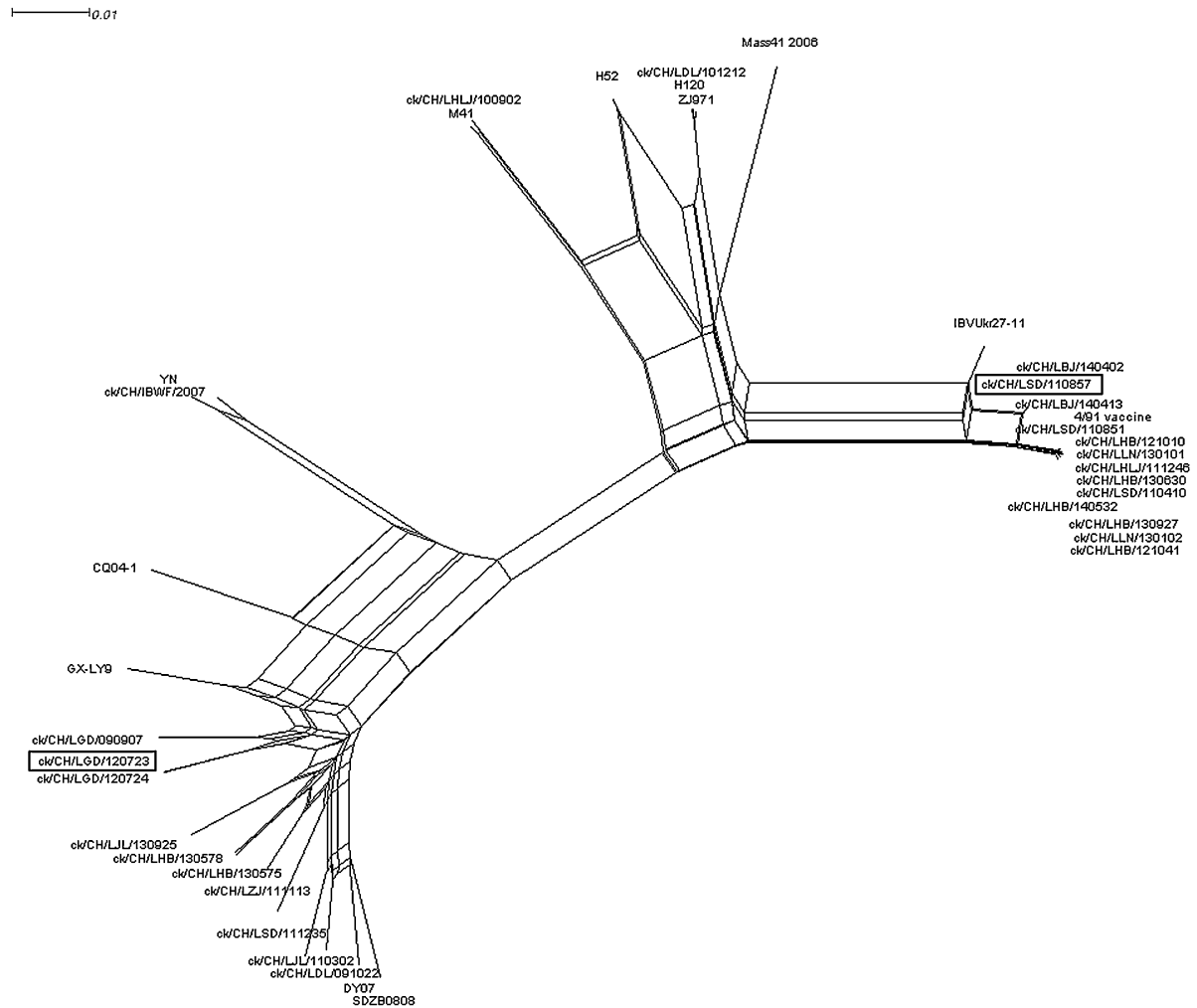
**Fig. 2.** The neighbor-joining phylogenetic trees of the full-length genomic sequence of our 20 isolates (black stars) and reference strains, respectively. GenBank accession numbers are same as those in Fig. 1.

isolates, ck/CH/LSD/110851 and ck/CH/LSD/110857, shared the same serotype as the 4/91 strain (the 793/B serotype); however, two other isolates, ck/CH/LGD/090907 and ck/CH/LGD/120723, antigenically differed from the other strains in this study and were more similar to each; thus, they represent a novel serotype.

**3.4. Pathotyping in 1-day-old chicks**

The pathogenicity of five selected IBV strains in 1-day-old specific pathogen-free (SPF) layers was assessed as described previously (Cavanagh et al., 2005); the 4/91 vaccine strain was used as a control. Chicks were monitored daily for clinical signs. Abnormal respiratory sounds, called snicks, made by all of the birds were counted by three people over a 2-min period. Birds were

checked individually for tracheal rales, nasal discharge, watery eyes, and wheezing (Cavanagh et al., 2005). The results are shown in Table 3. No clinical signs were observed in the chickens that were challenged with the ck/CH/LSD/110851 and ck/CH/LSD/110857 isolates, which are similar to the 4/91 vaccine strain, or in the negative control group. In contrast, the remaining three isolates, ck/CH/LHB/130575, ck/CH/LGD/090907, and ck/CH/LGD/120723, induced obvious respiratory signs in some or all of the challenged chickens. Some challenged chickens died during the experiment, with gross lesions mainly confined to the kidneys. The kidney parenchyma was pale, swollen, and mottled, and tubules and urethras were distended and contained uric acid crystals (Supplemental Fig. 4A). Immunohistochemistry (IHC) results revealed that IBV-positive cells were present in the kidneys of



**Fig. 3.** A network tree (SplitsTree4, Quinteros (2016), version 4.14.3) was constructed with the complete genome sequences of our 20 IBV isolates and other reference strains. The networked relationships indicate the presence of recombination events. Boxes indicate the likelihood of recombination. The representative strains used for further analysis of recombination events were boxed. A phi test for each of the network trees indicated that there was a significant likelihood of recombination ( $P < 0.001$ ), using a window size of 100.

the dead chickens (Supplemental Fig. 4B). These results identified these three IBV isolates as nephropathogenic strains. The clinical signs in inoculated birds tended to disappear gradually after 15 days post-infection (dpi). The seroconversion in chickens inoculated with ck/CH/LSD/110851 and ck/CH/LSD/110857 was similar to that in chickens inoculated with the 4/91 vaccine strain, which was later than that induced by the remaining three isolates (Table 3). We did not observe seroconversion in any chickens in the negative control group.

### 3.5. Vaccination-challenge experiment

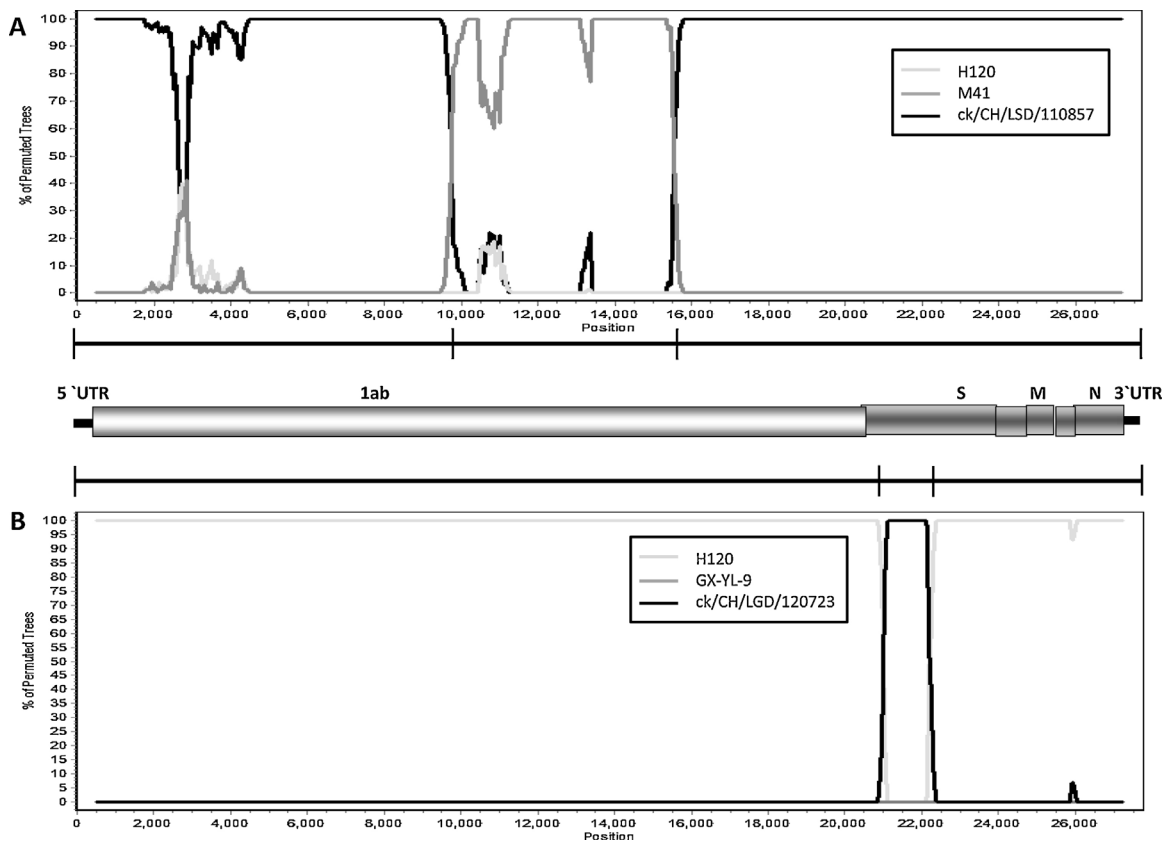
The results of the vaccination-challenge experiment are shown in Table 4. None of the chickens vaccinated with the 4/91 vaccine strain showed clinical signs or mortality when challenged with either the five IBV isolates or the two LX4 type strains (Table 4). None of the vaccinated birds were positive for virus recovery in the respiratory tract when challenged with the ck/CH/LSD/110851 isolate, and only one chicken was positive for ck/CH/LSD/110857 at 4 days post-challenge (dpc), indicating that the 4/91 vaccine strain provided good protection against the two viruses. In contrast, at least 80% of the vaccinated chickens were positive for virus recovery when challenged with the three viruses, ck/CH/LHB/130575, ck/CH/LGD/090907, and ck/CH/LGD/120723, and the two

LX4 type viruses, ck/CH/LHLJ/04V and ck/CH/LDL/091022, demonstrating that the 4/91 vaccine strain provided poor protection against these IBV strains. As expected, none of the chickens in the negative control group were positive for virus isolation. Interestingly, virus shedding in the cloaca of the vaccinated chickens after challenge was different from that in the respiratory tract. The 4/91 vaccine virus could not be recovered from the respiratory tract after 16 dpi. However, it could be recovered from the cloaca after 20 dpi, and it could be recovered at 40 dpi in some chickens (Table 5); the viruses recovered from the cloaca were a mixture of the 4/91 vaccine and the challenge viruses (data not shown). In addition, the 20-day-old chickens showed slight resistance to the challenge viruses compared with 1-day-old chickens, although the pathogenicity of each virus was similar in these two age groups. Nearly all of the 20-day-old chickens seroconverted by 8 dpc with each of the viruses, which was earlier than that of the 1-day-old chickens; none of the birds in the negative control group seroconverted (Table 4).

## 4. Discussion

One of the disadvantages of live vaccines is that they spread easily in the field (Meulemans et al., 2001; Hodgson et al., 2004). While spreading, most RNA viruses, such as IBV, may undergo a





**Fig. 4.** Bootscan analysis to detect recombination and estimate breakpoint within the two representative strains ck/CH/LSD/110857 (A) and ck/CH/LGD/120723 (B). The results showed two recombination breakpoints for each of the representative strains. The IBV 4/91 strain was used as the query. Both analyses were performed with the F84 distance model, window size of 1000 bp and step size of 50 bp.

**Table 2**

Titers were obtained in reciprocal  $\beta$  virus neutralization tests (diluted serum, constant virus).<sup>a</sup>

| Virus            | Serum        |                  |                  |                  |                  |                  |
|------------------|--------------|------------------|------------------|------------------|------------------|------------------|
|                  | 4/91         | ck/CH/LSD/110851 | ck/CH/LSD/110857 | ck/CH/LGD/120723 | ck/CH/LGD/090907 | ck/CH/LDL/091022 |
| 4/91             | <b>668.9</b> | <b>615.2</b>     | <b>474.4</b>     | <2               | <2               | <2               |
| ck/CH/LSD/110851 | <b>591.4</b> | <b>647</b>       | <b>449.7</b>     | <2               | <2               | 18.4             |
| ck/CH/LSD/110857 | <b>548</b>   | <b>475.6</b>     | <b>588.6</b>     | 55.7             | 27.9             | <2               |
| ck/CH/LGD/120723 | <2           | <2               | <2               | <b>445.7</b>     | <b>415.9</b>     | <2               |
| ck/CH/LGD/090907 | 4.3          | <2               | <2               | <b>471</b>       | <b>530</b>       | <2               |
| ck/CH/LDL/091022 | <2           | 12.1             | <2               | <2               | <2               | <b>477.7</b>     |

<sup>a</sup> The end-point values of the viruses that belong to the same serotype are highlighted (bold area).

**Table 3**

Results of the pathotyping tests.

| Virus               | Morbidity | Mortality | Antibody response |      |              |      |              |
|---------------------|-----------|-----------|-------------------|------|--------------|------|--------------|
|                     |           |           | 4 d <sup>a</sup>  | 8 d  | 12 d         | 16 d | 20 d         |
| 4/91 vaccine strain | 0/10      | 0/10      | 0/10              | 0/10 | 2/10         | 5/10 | <b>10/10</b> |
| ck/CH/LSD/110851    | 0/10      | 0/10      | 0/10              | 0/10 | 3/10         | 5/10 | <b>10/10</b> |
| ck/CH/LSD/110857    | 0/10      | 0/10      | 0/10              | 0/10 | 2/10         | 6/10 | <b>10/10</b> |
| ck/CH/LHB/130575    | 7/10      | 0/10      | 0/10              | 8/10 | <b>10/10</b> |      | <b>10/10</b> |
| ck/CH/LGD/090907    | 10/10     | 5/10      | 0/10              | 5/5  | 5/5          | 5/5  | 5/5          |
| ck/CH/LGD/120723    | 10/10     | 8/10      | 0/10              | 2/2  | 2/2          | 2/2  | 2/2          |
| Negative control    | 0/10      | 0/10      | 0/10              | 0/10 | 0/10         | 0/10 | 0/10         |

<sup>a</sup> Days after challenge. The numbers that show that only some chickens seroconverted are indicated in italic, while those that show that all chickens seroconverted are indicated in bold.

high rate of genetic change that contributes to diverse subpopulations of the virus that continually emerge to form new variants of existing strains, as well as completely new types of the virus. For

IBV, it is also believed that the spread of the live vaccine strain may serve as a parental virus, thereby driving the generation of recombinant viruses, especially where multiple IBV genotypes might be involved (Liu et al., 2013), as the results herein suggest. Hence, live vaccine use should be ideally restricted to situations in which there is a known protective benefit (Farsang et al., 2002).

793/B has been the most dominant IBV genotype in European countries, the Middle East, including Saudi Arabia, Israel, Jordan, Oman, and Iran, and Asia in recent years (Franzo et al., 2014; Najafi et al., 2015). In China, this genotype emerged at the end of 20th century; however, use of the 4/91 vaccine strain began by the mid-1990s. Recent studies showed that the 793/B type has become a major concern to the poultry industry in China in recent years (Liu et al., 2013). To elucidate the origin and evolution of IBV isolates genotypically related to 793/B in China, we selected 20 IBVs that were isolated from 2009 to 2014 in China, and we investigated their genetic, antigenic, and pathogenic characteristics by comparing them with those of the 4/91 vaccine strain. Based on a sequence

**Table 4**

Overview of the results of vaccination–challenge test in which five 793/B genotype and two LX4 genotype viruses were tested using the 4/91 vaccine strain.

| Group | Vaccine         | Challenge        | Morbidity         | Mortality | Antibody response |      |      |      |       |       |                  |                    |       |       |       |       |       |       |
|-------|-----------------|------------------|-------------------|-----------|-------------------|------|------|------|-------|-------|------------------|--------------------|-------|-------|-------|-------|-------|-------|
|       |                 |                  |                   |           | Post-vaccination  |      |      |      |       |       | Post-challenge   |                    |       |       |       |       |       |       |
|       |                 |                  |                   |           | 4 d <sup>a</sup>  | 8 d  | 12 d | 16 d | 20 d  | 24 d  | 4 d <sup>a</sup> | 8 d                | 12 d  | 16 d  | 20 d  | 24d   |       |       |
| 1     | 4/91 vaccine    | NC <sup>b</sup>  | 0/10 <sup>c</sup> | 0/10      | 0/10              | 0/10 | 3/10 | 6/10 | 10/10 | 10/10 | 10/10            | 10/10 <sup>d</sup> | 10/10 | 10/10 | 10/10 | 10/10 | 10/10 | 10/10 |
| 2     | NV              | ck/CH/LSD/110851 | 0/10              | 0/10      | –                 | –    | –    | –    | –     | –     | –                | 0/10               | 9/10  | 10/10 | 10/10 | 10/10 | 10/10 | 10/10 |
| 3     | 4/91 vaccine    | ck/CH/LSD/110851 | 0/10              | 0/10      | 0/10              | 0/10 | 3/10 | 5/10 | 10/10 | 10/10 | 10/10            | 10/10              | 10/10 | 10/10 | 10/10 | 10/10 | 10/10 | 10/10 |
| 4     | NV              | ck/CH/LSD/110857 | 0/10              | 0/10      | –                 | –    | –    | –    | –     | –     | –                | 0/10               | 10/10 | 10/10 | 10/10 | 10/10 | 10/10 | 10/10 |
| 5     | 4/91 vaccine    | ck/CH/LSD/110857 | 0/10              | 0/10      | 0/10              | 0/10 | 3/10 | 5/10 | 10/10 | 10/10 | 10/10            | 10/10              | 10/10 | 10/10 | 10/10 | 10/10 | 10/10 | 10/10 |
| 6     | NV <sup>e</sup> | ck/CH/LHB/130575 | 5/10              | 0/10      | –                 | –    | –    | –    | –     | –     | –                | 0/10               | 8/10  | 10/10 | 10/10 | 10/10 | 10/10 | 10/10 |
| 7     | 4/91 vaccine    | ck/CH/LHB/130575 | 0/10              | 0/10      | 0/10              | 0/10 | 4/10 | 5/10 | 10/10 | 10/10 | 10/10            | 10/10              | 10/10 | 10/10 | 10/10 | 10/10 | 10/10 | 10/10 |
| 8     | NV              | ck/CH/LGD/090907 | 10/10             | 2/10      | –                 | –    | –    | –    | –     | –     | –                | 0/10               | 8/8   | 8/8   | 8/8   | 8/8   | 8/8   | 8/8   |
| 9     | 4/91 vaccine    | ck/CH/LGD/090907 | 0/10              | 0/10      | 0/10              | 0/10 | 3/10 | 6/10 | 10/10 | 10/10 | 10/10            | 10/10              | 10/10 | 10/10 | 10/10 | 10/10 | 10/10 | 10/10 |
| 10    | NV              | ck/CH/LGD/120723 | 10/10             | 7/10      | –                 | –    | –    | –    | –     | –     | –                | 0/10               | 3/3   | 3/3   | 3/3   | 3/3   | 3/3   | 3/3   |
| 11    | 4/91 vaccine    | ck/CH/LGD/120723 | 0/10              | 0/10      | 0/10              | 0/10 | 3/10 | 5/10 | 10/10 | 10/10 | 10/10            | 10/10              | 10/10 | 10/10 | 10/10 | 10/10 | 10/10 | 10/10 |
| 12    | NV              | ck/CH/LHLJ/04V   | 10/10             | 3/10      | –                 | –    | –    | –    | –     | –     | –                | 0/10               | 8/8   | 7/7   | 7/7   | 7/7   | 7/7   | 7/7   |
| 13    | 4/91 vaccine    | ck/CH/LHLJ/04V   | 0/10              | 0/10      | 0/10              | 0/10 | 2/10 | 6/10 | 10/10 | 10/10 | 10/10            | 10/10              | 10/10 | 10/10 | 10/10 | 10/10 | 10/10 | 10/10 |
| 14    | NV              | ck/CH/LDL/091022 | 10/10             | 2/10      | –                 | –    | –    | –    | –     | –     | –                | 0/10               | 8/8   | 8/8   | 8/8   | 8/8   | 8/8   | 8/8   |
| 15    | 4/91 vaccine    | ck/CH/LDL/091022 | 0/10              | 0/10      | 0/10              | 0/10 | 3/10 | 6/10 | 10/10 | 10/10 | 10/10            | 10/10              | 10/10 | 10/10 | 10/10 | 10/10 | 10/10 | 10/10 |
| 16    | NV              | NC               | 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> Days after vaccination/challenge.<sup>b</sup> NC = not challenged.<sup>c</sup> Numbers of chickens that seroconverted/the number of chickens tested.<sup>d</sup> These birds were not challenged.<sup>e</sup> NV = not vaccinated.**Table 5**

Virus recovery results of a vaccination–challenge test in which five 793/B genotype and two LX4 genotype viruses were tested using the 4/91 vaccine strain.

| Group <sup>a</sup> | Nasopharyngeal swab |       |      |      |                |      | Cloacal swabs |       |       |       |       |      |      |      |      |      |      |
|--------------------|---------------------|-------|------|------|----------------|------|---------------|-------|-------|-------|-------|------|------|------|------|------|------|
|                    | 4 <sup>b</sup>      | 8     | 12   | 16   | 20             | 24   | 4             | 8     | 12    | 16    | 20    | 24   | 28   | 32   | 36   | 40   | 44   |
| 1                  | 10/10 <sup>c</sup>  | 8/10  | 7/10 | 0/10 | – <sup>d</sup> | –    | 8/10          | 10/10 | 10/10 | 10/10 | 10/10 | 6/10 | 6/10 | 4/10 | 2/10 | 1/10 | 0/10 |
| 2                  | 9/10                | 10/10 | 8/10 | 3/10 | 1/10           | 0/10 | 10/10         | 10/10 | 10/10 | 10/10 | 8/10  | 6/10 | 6/10 | 3/10 | 0/10 | –    | –    |
| 3                  | 0/10                | –     | –    | –    | –              | –    | 8/10          | 2/10  | 0/10  | –     | –     | –    | –    | –    | –    | –    | –    |
| 4                  | 10/10               | 10/10 | 3/10 | 0/10 | –              | –    | 10/10         | 10/10 | 10/10 | 9/10  | 3/10  | 0/10 | –    | –    | –    | –    | –    |
| 5                  | 1/10                | 0/10  | –    | –    | –              | –    | 6/10          | 2/10  | 1/10  | 5/10  | 1/10  | 3/10 | 1/10 | 0/10 | –    | –    | –    |
| 6                  | 10/10               | 9/10  | 7/10 | 0/10 | –              | –    | 10/10         | 10/10 | 10/10 | 10/10 | 10/10 | 5/10 | 5/10 | 3/10 | 2/10 | 1/10 | 0/10 |
| 7                  | 8/10                | 4/10  | 0/10 | –    | –              | –    | 7/10          | 5/10  | 4/10  | 3/10  | 1/10  | 1/10 | 0/10 | –    | –    | –    | –    |
| 8                  | 10/10               | 6/8   | 1/8  | 0/8  | –              | –    | 8/10          | 8/8   | 8/8   | 7/8   | 5/8   | 5/8  | 4/8  | 1/8  | 0/8  | –    | –    |
| 9                  | 9/10                | 4/10  | 0/10 | –    | –              | –    | 4/10          | 9/10  | 8/10  | 4/10  | 2/10  | 2/10 | 2/10 | 2/10 | 2/10 | 1/10 | 0/10 |
| 10                 | 10/10               | 2/2   | 1/2  | 0/2  | –              | –    | 10/10         | 2/2   | 2/2   | 2/2   | 1/2   | 0/2  | –    | –    | –    | –    | –    |
| 11                 | 10/10               | 4/10  | 0/10 | –    | –              | –    | 8/10          | 5/10  | 5/10  | 6/10  | 3/10  | 0/10 | –    | –    | –    | –    | –    |
| 12                 | 10/10               | 9/9   | 4/7  | 0/7  | –              | –    | 10/10         | 8/8   | 5/7   | 4/7   | 4/7   | 0/7  | –    | –    | –    | –    | –    |
| 13                 | 9/10                | 9/10  | 0/10 | –    | –              | –    | 10/10         | 9/10  | 9/10  | 9/10  | 10/10 | 4/10 | 4/10 | 2/10 | 0/10 | –    | –    |
| 14                 | 10/10               | 5/8   | 5/8  | 0/8  | –              | –    | 10/10         | 8/8   | 8/8   | 7/8   | 5/8   | 0/8  | –    | –    | –    | –    | –    |
| 15                 | 8/10                | 6/10  | 0/10 | –    | –              | –    | 9/10          | 10/10 | 10/10 | 10/10 | 3/10  | 3/10 | 2/10 | 1/10 | 1/10 | 0/10 | –    |
| 16                 | 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> The groups are the same as those in Table 4.<sup>b</sup> Days after challenge.<sup>c</sup> Number of chickens that were positive for virus recovery/number of chickens tested.<sup>d</sup> The sample that was not tested for virus recovery is indicated as –.

comparison and a phylogenetic analysis (Fig. 1) of the S1 gene, it appears that the IBV isolates genotypically related to 793/B in China exhibited diversity in recent years, which is consistent with previous findings (Farsang et al., 2002). A sequence comparison and a phylogenetic analysis (Fig. 3) using complete genomic sequences confirmed this result, and a significant signal of phylogenetic incongruence between the S1 gene and the complete genomic sequences in some of the isolates indicated the possible occurrence of recombination events during the origin of these viruses.

The genetic characteristics of most (12 of the 20) of isolates in this study were similar to those of the 4/91 vaccine strain, as opposed to the pathogenic 4/91 strain, and as demonstrated by similarities of the complete genomes and a sequence comparison of the S gene (Table 1). Hence, based on the mild clinical signs, the virus shedding in SPF chickens, and the seroconversion in the animal experiments, as well as their close genetic relationships,

most of the isolates might be vaccine-derived strains, although a direct comparison has not been conducted because the pathogenic 793/B strain was not available in our laboratory. Furthermore, the fact that, in most samples, the IBVs were isolated in the first egg passage also supports this hypothesis, as vaccine-derived viruses are more easily isolated because they have adapted to grow in embryonated eggs (Martin et al., 2014). It is possible that the use of the 4/91-like vaccine in chickens in China may have contributed to the spread of the isolates genotypically related to 793/B. As expected, the representative strain of the 12 isolates, ck/CH/LSD/110851, had the same serotype as the 4/91 vaccine strain, and vaccination with the 4/91 vaccine strain provided complete protection against a challenge with the ck/CH/LSD/110851 isolate.

The replication of coronavirus genomes, including that of IBV, requires a step in which a set of subgenomic RNAs is generated. This mechanism promotes homologous recombination events (McKinley et al., 2011). Recombination contributes to the genetic

diversity of coronaviruses, and it can lead to the emergence of new viruses and outbreaks of new diseases (Makino et al., 1986; Woo et al., 2009). In this study, two classes of viruses genotypically related to 793/B were shown to have evolved from recombinant viruses. Isolate ck/CH/LSD/110857 was the first class, and it was predicted to have originated from two vaccine strains, the Mass type H120 and 793/B type 4/91 strains, which are the most commonly used vaccines in chicken flocks in China. The genome of this isolate acquired the 5' (5'-untranslated region (UTR) to 5' part of the Nsp6 gene) and 3' parts of its genome (3' part of the Nsp13 gene to the 3'-UTR) from the 4/91-like virus, which includes the S gene. Because the S gene contains the epitopes of the main antigenic viral protein and the diversity of which is thought to account for the level of serotypical variation, the serotype of the ck/CH/LSD/110857 isolate was the same as that of the 4/91-like ck/CH/LSD/110851 isolate and the 4/91 vaccine strain (the 793/B type). Consistent with this result, vaccination with the 4/91 vaccine strain provided complete protection against challenge with the ck/CH/LSD/110857 isolate.

Interestingly, we found that many (seven of the 20) isolates emerged from recombination events between the 4/91 vaccine strain and IBV field strains circulating in China. Although the genomes of the seven isolates were diverse, which suggested that they have a varied history of evolution, they still have a similar phylogeny topology, indicating that they likely have the same origin. Analysis of the representative ck/CH/LGD/120723 isolate revealed that it acquired the 5' (5'-UTR to 5' part of S1) and 3' parts of its genome (most regions of the 3' part of the S2 gene to the 3'-UTR) from an GX-YL9-like virus, while only parts of the S1 gene and small parts of the S2 gene were acquired from a 4/91-like virus. It is reported that serotype differences among genetically distinct IBVs generally correlate with variations in the hypervariable regions of the S1 gene (Cavanagh, 2007), and that differences of as little as 5% between the S1 sequences of IBVs could result in poor cross-protection by currently used vaccines (Hofstad, 1981). In this study, the ck/CH/LGD/120723 isolate acquired HVR I and II of the S1 gene from a GX-YL9-like virus, as well as HVR III from a 4/91-like virus, suggesting that a recombination event in the S1 gene likely accounts for the change in serotype of the ck/CH/LGD/120723 isolate and the poor protection of the 4/91 vaccine strain against challenge with this isolate. IBV strains 4/91 and GX-YL-9 belong to different types and therefore, vaccination with 4/91 likely did not cross-protect chickens against challenge with GX-YL-9-like strain. This indicates that it is possible for a GX-YL9-like virus and the 4/91 vaccine strain to co-exist in a certain chicken flock, which is believed to be an important prerequisite for recombination between IBV strains in the field (Cavanagh, 2007). Importantly, the 4/91 vaccine strain is still commonly used in China, and GX-YL-9-like viruses are still circulating in China for a long time (Zhao et al., 2016); hence, more IB cases associated with ck/CH/LGD/120723-like viruses might be expected in the future. This was the case in the present study, as seven viruses, which emerged from recombination events between the 4/91 vaccine strain and a GX-YL-9-like virus, were isolated in different regions in different years in China. In addition, the results of this study emphasize that conclusions based on a single gene or partial gene segment analysis should be made cautiously, because the true origin and evolutionary history of an IBV can only be evaluated by an extensive, complete genome analysis (Reddy et al., 2015).

The first nephropathogenic IBV strains were reported in the USA and Australia, and later in other parts of the world (Reddy et al., 2015). Over the past 20 years, nephropathogenic IBV strains have emerged as the most predominant IBV strains in the poultry industry, and they were responsible for outbreaks of kidney disease on chicken farms in China (Xu et al., 2007). In this study, the third class of identified recombinant viruses was isolated from

chickens that exhibited kidney diseases (Supplemental Table). Furthermore, two of the selected recombinant isolates were shown to be nephropathogenic to SPF chickens, as IBV-positive cells were observed in the kidneys of the dead chickens following challenge with these IBV isolates, as assessed by IHC, thereby demonstrating that the tissue tropism and pathogenicity of the naturally occurring recombinant viruses that we isolated were similar to those of their parental virus and differed from those of the 793/B type. Thus far, a conclusion had not been made about the pathogenicity determinants of IBV, although it was reported that they might be located in the 1a, S, M, and accessory proteins of some virus strains (Reddy et al., 2015). Only parts of the S1 gene were shared between the two recombinant viruses and the 4/91 vaccine strain, suggesting that the pathogenicity determinants of these strains were not likely to be located in this region of the S1 gene; the molecular basis of the nephropathogenicity of IBV will need to be elucidated by reverse genetics. For the ck/CH/LHB/130575 isolate, no chickens died after challenge, and we did not investigate the kidney tropism of this isolate. It was shown that the replicase gene of IBV is a pathogenicity determinant (Armesto et al., 2009); however, the ck/CH/LHB/130575 isolate shared very high nucleotide similarity to the aforementioned strains, indicating that a mutation(s) in the replicase gene likely accounted for its altered pathogenicity. Alternatively, the pathogenicity determinants of some IBV strains might be located in other regions of the genome (Reddy et al., 2015), although this will require further investigation. For the recombinant strain ck/CH/LSD/110857, the chickens from which the virus was isolated did not exhibit kidney diseases. It was not surprising to find that the ck/CH/LSD/110857 isolate was not pathogenic to SPF chickens, because this strain was predicted to have arisen from recombination events between two vaccine strains, H120 and 4/91.

In summary, we investigated the genetic, antigenic, and pathogenic characteristics of isolates genotypically related to 793/B IBVs in recent years in China. We found that most of the viruses might be 4/91 vaccine-derived strains; however, some of the viruses originated from recombination events between the 4/91 vaccine strain and other vaccine or field strains. The recombination that occurred between the 4/91 vaccine strain and field strains resulted in the emergence of a novel serotype, and vaccination using the 4/91 vaccine strain did not provide complete protection against the new serotype. The spread of novel type IBVs and their effect on the poultry industry will be evaluated in future investigations.

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## Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at <http://dx.doi.org/10.1016/j.vetmic.2017.01.039>.

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