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Characterization of the complete genome, antigenicity, pathogenicity, tissue tropism, and shedding of a recombinant avian infectious bronchitis virus with a ck/CH/LJL/140901-like backbone and an S2 fragment from a 4/91-like virus

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ABSTRACT

In this study, we isolated an infectious bronchitis virus, designated I1101/16, from broiler breeders in China. Analysis of the S1 gene showed that isolate I1101/16 was genetically close to strain ck/CH/LJL/140901, which belongs to the TW I genotype (also known as lineage GI-7 based on the recent IBV classification), however the S2 gene showed genetic diversity comparing to that of S1 gene. Comparison of the genomic sequences showed that the genome of isolate I1101/16 was similar to that of strain ck/CH/LJL/140901 from the 5′ end of the genome to the 5′ end of the S2 gene and from the 5′ end of the 3a gene to the end of the genome, whereas the remaining parts of the genome sequences were more closely related to those of strain 4/91 than those of ck/CH/LJL/140901, thereby suggesting that recombination might have occurred during the origin of the virus. SimPlot and Bootscan analysis of the complete genomic sequence confirmed this hypothesis, where it showed that isolate I1101/16 arose through recombination events between ck/CH/LJL/140901- and 4/91-like viruses. Isolate I1101/16 and strain ck/CH/LJL/140901 shared identical amino acids in almost all five of their B cell epitopes, but the two viruses had a serotype relatedness value of 65, which is well below 80, i.e., the lower cutoff value for viruses of the same serotype. In addition, pathogenicity tests demonstrated that isolate I1101/16 was more pathogenic to 1-day-old specific-pathogen-free chickens than strain ck/CH/LJL/140901, according to analysis of the clinical signs, whereas strain ck/CH/LJL/140901 exhibited prolonged replication and shedding after challenge compared with isolate I1101/16. This study did not provide evidence that recombination can directly alter the antigenicity, virulence, replication, shedding, and tissue tropism of a virus, but it did show that recombination events are likely to be major determinants of viral evolution.

1. Introduction

Avian infectious bronchitis virus (IBV), the prototype *Gammacoronavirus* species in the family Coronaviridae, is a positive-sense, single-stranded RNA virus with a genome of approximately 27.6 kb (5′-untranslated region (UTR)-1a/1ab-S-3a-3b-E-M-5a-5b-N-3′-UTR). It causes an acute and highly contagious disease in chickens, which is responsible for high economic losses in the poultry industry. The current control strategies are based mainly on mass vaccination strategies. Nevertheless, vaccine-induced immunity generally give poor protection because the current vaccine offers only limited cross protection among strains (Cook et al., 2012; de Wit et al., 2011) mainly due to the antigenic diversity caused by the variability of the S1 protein. Recently, numerous IBV strains have been identified and new genotypes/serotypes have emerged from existing viruses via point mutations, insertions, and deletions in the viral genome, especially in the S1 subunit of the spike protein gene. At least six IBV genotypes together comprise 34 distinct viral lineages and a number of unassigned inter-lineage recombinants have been recognized worldwide according to a simple and repeatable phylogeny-based classification system that uses the complete nucleotide sequence of the S1 gene and an unambiguous and rationale lineage nomenclature for the assignment of IBVs (Valastro et al., 2016; Chen et al., 2017; Jiang et al., 2017).

There is also considerable variation in the virulence and tropism of IBVs, and in some cases the novel IBV strains emerged from point mutations, insertions, and/or deletions in the S1 gene. Another

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important mechanism that underlies the emergence of novel IBV strains is genomic recombination. In Australia, three recently isolated novel subgroup IBV strains were shown to be derived from recombination between subgroup 1 and 2 strains (Mardani et al., 2010). More recently, the complete genome analysis of newly emerged strains found multiple recombination events throughout the genome between wild-type viruses and vaccine strains (Quinteros et al., 2016). In the USA, the emergent virulent strain Ark DPI appears to have originated from recombination among four different IBV strains (Ammayappan et al., 2008). In addition, the emergence of new strains in the USA has been reported to have arisen from recombination events between the Massachusetts (Mass), Connecticut (Conn), and Holte strains as well as field strains (Thor et al., 2011). Recently in Europe, it has been shown that emergent viruses, such as the XDN-like virus in Spain and Italy (Moreno et al., 2016), and the γCoV/Ck/Italy/I2022/13 virus in Italy (Franzo et al., 2015), may have arisen from recombination events.

There have been several episodes of infectious bronchitis (IB) in Chinese chicken flocks, and the genotypes/serotypes of IBVs were previously classified based mainly on the nucleotide sequences of genes encoding the S1 subunit of the spike protein (Han et al., 2011), and in some cases based on cross virus-neutralization (Gao et al., 2016; Chen et al., 2017) in China. Since 1995, the predominant IBV type in China has been LX4 (also known as QX-like viruses), but molecular studies have shown that new types and variants are emerging continually (Liu et al., 2013; Mo et al., 2013; Zhao et al., 2013; Liu et al., 2014; Zhou et al., 2014; Chen et al., 2015; Xu et al., 2016; Zhang et al., 2015; Leghari et al., 2016; Chen et al., 2017; Zhao et al., 2017; Zhou et al., 2017). It has been suggested that the emergent IBV strains in China may have different origins. It is considered that some of the IBV types circulating in China, such as Mass and 793/B, are the most widely distributed types worldwide and they may have infected Chinese chickens from an exogenous source, probably due to the use of live vaccines (Chen et al., 2015; Han et al., 2017). By contrast, some IBV types, such as LX4 and ck/CH/LDL/97I (Q1-like), are believed to have originated in China and spread to other regions of the world (Valastro et al., 2016). The remaining IBV types, such as ck/CH/LSC/99I (Liu et al., 2006a,b), nrTW I (Xu et al., 2016), and GI-28 (Chen et al., 2017), are considered to be indigenous to China. The origins of most of the IBV types in China are still unknown, although it has been shown that some of these types have arisen from recombination events (Chen et al., 2015; Xu et al., 2016; Chen et al., 2017). The results of many of these previous studies are based on analyses of the available S1 gene sequences, but it is impossible to fully understand the origins and evolutionary processes related to these emerging viruses by only analyzing this small part of the genome.

In this study, we isolated and identified an nrTW I type IBV from an H120 and 4/91 vaccinated chicken flock with respiratory signs of IB. We sequenced the complete genome and compared it with other IBV sequences available in GenBank, and we then subjected these sequences to phylogenetic, molecular, and recombination analyses. We also investigated the antigenicity, pathogenicity, replication, and shedding of the nrTW I type IBV in chickens.

2. Materials and methods

2.1. Clinical samples, virus isolation, and viral stock preparation

Trachea samples were collected from five suspected IBV-infected broiler breeders and they were submitted to our laboratory in 2016 for routine diagnosis. Chickens in this flock had been vaccinated against IBV using the live attenuated H120 vaccine at 7 days of age, and the chickens were then boosted at 25 and 60 days with the live attenuated 4/91 and H120 vaccines, respectively. The birds were also vaccinated with a bivalent, inactivated vaccine, Newcastle disease virus La Sota/IBV M41, at 120 days. Some of the birds exhibited respiratory signs of IB at 150 days, which was accompanied by decreased egg production and abnormal shell quality in the breeding hens, and the clinical signs disappeared approximately 20 days later. The morbidity was approximately 5% and only a few birds died during this outbreak. Gross lesions were mainly associated with tracheitis and proventriculitis, and hepatitis and hydropericardium were observed in some chickens. Kidney lesions were not observed in the chickens examined.

To isolate the virus, the tracheal samples from the dead chickens were pooled and inoculated into 9-day-old specific pathogen-free (SPF) embryonated chicken eggs via the allantoic sac route, as described previously (Liu and Kong, 2004). The virus recovered from the third passage was named γCoV/ck/China/I1101/16 (I1101/16) (Ducatez and The European Unit COST Action FA1207, 2016) and it was used in the following experiments. The virus stock was prepared after an additional passage by inoculating the allantoic cavity of 9-day-old embryonated SPF chicken eggs. After incubation for 48 h, the allantoic fluid was collected aseptically and clarified by centrifugation at 2500 × *g* for 20 min, as described previously (Liu and Kong, 2004).

2.2. Eggs and chicks

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

2.3. Viral RNA extraction, reverse transcription–polymerase chain reaction (RT-PCR) amplification, and complete genome sequencing

The complete genomes of the I1101/16 isolate and the ck/CH/LSC/99I strain were sequenced in this study. IBV strain ck/CH/LSC99I was isolated from preventriculus of a layer hen in Sichuan province in China in 1999 (Liu et al., 2006a,b). Genomic RNA was extracted from virus-inoculated allantoic fluids using TRIzol reagent (Invitrogen Corporation, Carlsbad, CA, USA) according to the manufacturer's instructions. First-strand cDNA synthesis and subsequent PCR were performed using a PrimeScript™ One-Step RT-PCR Kit ver. 2 (Takara Bio Inc., Shiga, Japan) according to the manufacturer's instructions. The complete genomes of the I1101/16 isolate and ck/CH/LSC/99I strain were amplified with primers used for amplifying the complete genomes of other Chinese IBV strains (Liu et al., 2013). The PCR profiles comprised initial denaturation for 5 min at 94 °C, followed by 30 cycles at 94 °C for 30 s, 50–60 °C (depending on the primer set) for 30 s, and 72 °C for 2 min. A 3′/5′ rapid amplification of cDNA ends kit (Takara Bio Inc.) was used to determine the 3′ and 5′ ends of the viral genomes according to the manufacturer's instructions (Liu et al., 2013).

The amplified products were sequenced directly or cloned into the pMD18-T vector (Takara Bio Inc.) according to the manufacturer's instructions. Each fragment of the viral genome was sequenced at least three times. The complete genomic sequences of the I1101/16 isolate and the ck/CH/LSC/99I strain were mapped using the genome sequences of the Beaudette and ck/CH/LJL/140901 IBV strains, respectively, in order to determine a consensus sequence.

2.4. Comparison and analysis of the S1 subunit of the spike and spike protein genes

The region coding for the S1 subunit of the spike protein is used for genotyping and classification purposes. Comparisons with published sequences were first performed using the nucleotide BLAST search tool in GenBank with the S1 gene nucleotide sequence from the I1101/16 isolate. The S1 nucleotide sequences from 70 IBV reference strains were downloaded from GenBank, where most of these IBVs were isolated in China and they represented the previously reported types (Valastro et al., 2016; Chen et al., 2017). Phylogenetic analyses were conducted

based on the S1 gene nucleotide sequences using the neighbor-joining method (Jukes-Cantor model) with 1000 bootstrap replicates (MEGA software version 5.0; available at http://www.megasoftware.net/).

To analyze the genetic diversity of the I1101/16 isolate, the percentages of similarity were calculated for both the S1 subunit and the entire spike protein at the nucleotide and amino acid levels using the I1101/16 isolate and seven reference strains, which were selected based on the phylogenetic analysis results. The seven reference strains included two vaccine strains, H120 and 4/91, which are used commonly in chicken flocks in China and they represented two genotypes, and five IBV field strains that circulate in chicken flocks in China, where they represented different genotypes (i.e., ck/CH/LJL/140901 [the nrTW I type] (Xu et al., 2016), ck/CH/LDL/091022 [the LX4 type] (Liu et al., 2013), ck/CH/LGX/111119 [the GI-28 type] (Chen et al., 2017), ck/CH/LSC/99I [the ck/CH/LSC/99I type] (Liu et al., 2006a,b), and ck/CH/LDL/97I [the ck/CH/LDL/97I type] (Liu et al., 2006a)). In addition, we compared the amino acid sequences of the five conformation-dependent neutralizing antigenic sites (epitopes) mapped to the S1 subunit of the spike protein as well as another immunodominant region in the amino-terminal region of S2 (Kusters et al., 1989; Lenstra et al., 1989; Koch et al., 1990) in a pairwise manner in isolate I1101/16 and the seven reference strains.

2.5. Detection of recombination events

Multiple sequence alignment of the complete genome sequences of the I1101/16 isolate and the reference strains was performed with Multiple Alignment with Fast Fourier Transformation (MAFFT) v6 (http://mafft.cbrc.jp/alignment/software/). The alignment included seven sequences from the strains mentioned above.

To refine the recombination event pattern analysis, similarity plots (SimPlots) and Bootscan analysis were used to evaluate the possible recombinant nature of the isolate using the on-line MUltiple sequence Local AligNment (Mulan) tool (Ovcharenko et al., 2005) with the complete genome sequences of the ck/CH/LJL/140901 and 4/91 strains, where we used a window size of 500 bp and a step size of 20 bp. The analyses were performed using SimPlot version 3.5.1 (Lole et al., 1999) where the complete genome sequence of the ck/CH/LSD/110851 strain (accession number KP118884) (Han et al., 2017) was used as the query because it was a representative of 4/91 vaccine sequence and supposed parental recombinant sequence of strain I1101/16.

The deduced sequences of the recombination breakpoints and flanking sequences of the I1101/16, ck/CH/LJL/140901, and 4/91 viruses were compared in a pairwise manner to accurately detect the positions of the recombination breakpoints. In addition, we performed pairwise comparisons of the nucleotide sequences of the S2 subunit of the spike gene between I1101/16 isolate, and 4/91 vaccine (GenBank accession KF377577) and a pathogenic 4/91 strain (GenBank accession JN192154), in order to identify which was the possible parental virus strain of I1101/16 isolate.

2.6. Virus-neutralization test

The IBV I1101/16 isolate and the seven reference IBV strains mentioned above were used in virus-neutralization tests. Stocks of these viruses were prepared by passaging them in the allantoic cavity of 9-day-old embryonated SPF chicken eggs. Viral titers were determined by inoculating 10-fold dilutions into groups of five 9-day-old embryonated chicken eggs and the 50% embryo infectious dose (EID_{50}) was calculated using the method of Reed and Muench (1938).

Serotype-specific antisera against all the viruses listed in Table 1 were prepared in chickens, and cross virus-neutralization tests were conducted in 9-day-old SPF chicken eggs as described previously (Jackwood et al., 2007). The β virus neutralization method with constant virus and diluted serum was employed. Titers were calculated using the method of Reed and Muench (1938), and relatedness values were calculated using the method of Archetti and Horsfall (1950). Viruses with an Archetti and Horsfall relatedness value greater than 50 were considered to be related serotypes.

2.7. Pathogenicity studies

Ethical approval for the animal experiments was granted by the Ethical and Animal Welfare Committee of the Harbin Veterinary Research Institute, China. Forty-five 1-day-old SPF layer chickens were assigned randomly to three groups each containing 15 birds. The I1101/16 isolate and the ck/CH/LJL/140901 strain were administered by eyedrop inoculation to 1-day-old birds in groups 1 and 2, respectively, at a dosage of $1 \times 10^{5.5}$ EID_{50} in a 0.1-ml volume, and the remaining groups served as negative controls. Five days after challenge all chickens were evaluated blindly for respiratory rales, such as nasal and/or tracheal, by closely listening to each bird. Signs were scored as 0 (absent), 1 (mild), 2 (moderate), or 3 (severe). At 5 days post-challenge, five birds from each group were killed and necropsied, and the trachea and kidneys were collected. Measurements of infectivity and viral replication levels in the chicks were performed by determining the viral titers in 9-day-old SPF embryonated chicken eggs. In addition, the IBV antigen was detected by immunohistochemistry using monoclonal antibody 6D10 (Han et al., 2013). Blood samples and nasopharyngeal and cloacal swabs were collected from the birds in each of the groups on days 4, 8, 12, 16, 20, 24, 28, 32, 36, 40, and 44 post-challenge. The blood samples were used to detect antibodies against IBV with a commercial enzyme-linked immunosorbent assay (IDEXX Corporation, Westbrook, ME, USA) according to the manufacturer's instructions (Liu et al., 2013). The swabs were placed in individual tubes containing sterile phosphate-buffered saline (pH 7.4) with antibiotics and stored at −80 °C for virus recovery, as described previously (Han et al., 2017). The chickens were observed for disease symptoms, death, and kidney lesions for 45 days.

3. Results

3.1. Analysis of the spike gene

The S1 gene phylogenetic tree contained seven clusters (Fig. 1), which corresponded to the seven types of IBV, i.e., the GI-28 (Chen et al., 2017), LX4 (QX or GI-19) (Liu and Kong, 2004), ck/CH/LSC/99I (GI-22) (Liu et al., 2006a,b), 793/B (GI-13) (Han et al., 2017), ck/CH/LDL/97I (Q1 or GI-16) (Liu et al., 2006a), Mass (GI-1), and TW I (GI-7) types (Xu et al., 2016). Among these, two vaccine strains, H120 and 4/91, are used commonly in China and they belong to the Mass (GI-1) and 793/B (GI-13) types. The remaining five IBV types are field strains that circulate in chicken flocks in China. The I1101/16 isolate clustered into the TW I group (GI-7), which was genetically close to the ck/CH/LJL/140901 strain and slightly distant from other strains in the TW I group.

In agreement with the results of the phylogenetic analysis, the I1101/16 isolate shared the highest genetic similarity (95.7% and 95.4% at the nucleotide and amino acid levels, respectively) with the ck/CH/LJL/140901 stain. By contrast, the I1101/16 isolate shared low genetic similarity with the other types of viruses, with nucleotide and amino acid identities ranging among 76.1–80.7% and 76.7–80.6%, respectively. Similarly, the I1101/16 isolate also shared the highest similarity with the ck/CH/LJL/140901 strain at both the nucleotide (90.9%) and amino acid (93.5%) levels based on the spike protein gene, whereas it shared no more than 90% similarity with the other strains at either the nucleotide or amino acid levels. Interestingly, the genetic similarities between the I1101/16 isolate and the ck/CH/LJL/140901 strain based on the S1 gene were obviously higher than those based on the spike protein gene, which is the opposite result compared with those obtained between the I1101/16 isolate and most of the reference strains (Table 1).

Pairwise comparisons were performed of the amino acid sequences

Table 1
Antigenic relatedness[a] and S1/S gene similarities between the I1101/16 isolate and seven reference strains.

Virus		Percentage of relatedness/S1 or S similarities							
		I1101/16	H120	4/91	ck/CH/LDL/97I	ck/CH/LSC/99I	ck/CH/LGX/111119	ck/CH/LDL/091022	ck/CH/LJL/140901
I1101/16	*r* value	100	6.8	5	17	12	26	12	65
	S1 NT[b]	100	79.3	77.2	77.3	80.7	79.9	76.1	95.7
	S1 AA[c]	100	77.8	77.0	74.5	78.2	76.7	80.6	95.4
	S NT[d]	100	83.9	89.7	81.2	83.1	82.6	83.7	90.9
	S AA[e]	100	84.4	88.1	82.5	85.2	84.2	86.5	93.5

[a] Antigenic relatedness values (%) were calculated using the method of Archetti and Horsfall (1950) with neutralization indices derived from the results of reciprocal virus-neutralization tests performed in 9-day-old embryonated eggs.

[b] The percentages of relatedness for the first 1665 nucleotides starting at the AUG translation start codon in the S1 protein genes between the I1101/16 isolate and seven IBV reference strains.

[c] The percentages of relatedness for the amino acid of the S1 subunit in the spike protein between the I1101/16 isolate and seven IBV reference strains.

[d] The percentages of relatedness of the nucleotides encoding the spike protein between the I1101/16 isolated and the seven IBV reference strains.

[e] The percentages of relatedness of amino acids in the spike protein between the I1101/16 isolate and seven IBV reference strains. The selected IBV reference strains and their accession numbers are as follows: H120, FJ888351; 4/91, AF093793; ck/CH/LDL/97I, DQ068701; ck/CH/LGX/111119, KX640829; ck/CH/LDL/091022, HM194640; and ck/CH/LJL/140901, KP790146.

of B cell epitopes D (residues 24–61), E (residues 132–149), C/A/B (residues 291–398), and F (residues 497–543) in the S1 subunit and the G region (residues 548–574) of the S2 subunit of the spike protein, and the results are summarized in Fig. 2. The I1101/16 isolate shared similar amino acids in all epitopes with the ck/CH/LJL/140901 strain, whereas it exhibited amino acid differences compared with the ck/CH/LGX/111119, ck/CH/LSC/99I, ck/CH/LDL/97I, Conn, 4/91, and H120 strains. The I1101/16 isolate shared similar amino acids with the ck/CH/LDL/091022 strain in the F and G epitopes, but the amino acid residues differed in the D, E, and C/A/B epitopes of these strains.

3.2. Genome sequence

The genome sequences of the I1101/16 isolate and the ck/CH/LSC/99I strain were determined and deposited in GenBank under accession numbers KY620116 and KY799582, respectively. The genomes of the viruses comprised 27,674 bp and 27,665 bp, respectively, excluding the poly (A) tail. The complete genome sequences of the I1101/16 isolate and the ck/CH/LSC/99I strain had the typical IBV gene order of 5′-UTR-1a-1b-S-3a-3b-E-M-5a-5b-N-3′-UTR, and the gene lengths are shown in Table 2. The nucleotide similarities between the complete genomic sequence of the I1101/16 isolate shared with the representative IBV types circulating in China and with the vaccine strains were: ck/CH/LJL/140901, 96.9%; ck/CH/LDL/091022, 94.9%; ck/CH/LGX/111119, 94.5%; ck/CH/LSC/99I, 87.9%; ck/CH/LDL/97I, 86.5%; Conn, 86.8%; 4/91, 86.7%; and H120, 86.1%.

3.3. Recombination analysis

After comparing the genomic sequence of the I1101/16 isolate with those of the reference strains using the MAFFT alignment, we found that the genome of the I1101/16 isolate was similar to that of the ck/CH/LJL/140901 strain from the 5′ end of the genome to the 5′ end of the S2 gene, whereas the sequences differed significantly from that of the ck/CH/LJL/140901 strain from the 5′ end of the S2 gene to the end of the S2 gene. In the I1101/16 isolate, this region was most closely related to the 4/91 strain. Interestingly, the sequence of the I1101/16 isolate was more closely related to that of the ck/CH/LJL/140901 strain than that of the 4/91 strain from the 5′ end of the 3a gene to the end of the genome (Fig. 3). SimPlot and Bootscan analyses also confirmed these results (Fig. 4). Similarly, sequence comparison showed that the I1101/16 isolate shared 98.1% similarity at nt 1–22,091, 85.6% similarity at nt 22,093–23,836 (nearly all of the S2 gene), and 95.4% similarity at nt 23,872–27,674 with the ck/CH/LJL/140901 strain, whereas it shared 85.8% similarity at nt 1–22,091, 99.7% similarity at nt 22,093–23,836, and 86.4% similarity at nt 23,872–27,674 with the 4/91 strain, thereby confirming that the I1101/16 isolate is a new recombinant strain with a ck/CH/LJL/140901-like backbone and an S2 subunit of the spike protein gene that is similar to that of a 4/91-like virus. Thus, two recombination breakpoints at nt 22091–22093 and 23836–23872 were identified in the genome of the I1101/16 isolate (Fig. 5).

In addition, 12 nucleotides differences were identified in the S2 subunit of the spike protein gene of isolate I1101/16 and the two 4/91 strains; pairwise comparisons demonstrated that isolate I1101/16 shared seven nucleotide identities with the 4/91 vaccine strain and only two with the pathogenic 4/91 strain (Table 3), thereby indicating that this region of the I1101/16 isolate is more similar to that of 4/91 vaccine strain than that of the pathogenic strain.

3.4. Virus-neutralization test

Based on the method of Archetti and Horsfall (1950), serotype-relatedness values were calculated in cross virus-neutralization studies using embryonating chicken eggs (Table 1), which showed the I1101/16 isolate and the ck/CH/LJL/140901 strain were related serotypes with a serotype relatedness value of 65. None of the other IBV strains tested in this study were related serotypically to isolate I1101/16.

3.5. Pathogenicity studies

The pathogenicity of the I1101/16 isolate was tested in 1-day-old SPF chickens and compared with that of the ck/CH/LJL/140901 strain, and the results are presented in Table 4. At 5 days post-challenge, clinical signs were observed in 2/10 birds and 1/10 birds inoculated with the I1101/16 isolate and the ck/CH/LJL/140901 strain, respectively. At 11 days post-challenge, 10/10 birds inoculated with the I1101/16 isolate had severe clinical signs including tracheal rales, watery eyes, nasal mucus, and occasional sneezing, whereas 9/10 birds inoculated with the ck/CH/LJL/140901 strain had only mild clinical signs. The mean scores of the clinical signs for the birds challenged with the I1101/16 isolate and the ck/CH/LJL/140901 strain were listed in Table 4. No birds died in any of the groups during the experiments. No clinical signs were observed in the non-challenged birds.

At 8 days post-challenge, antibodies against IBV were detectable by an enzyme-linked immunosorbent assay in seven and four birds challenged with the I1101/16 isolate and the ck/CH/LJL/140901 strain, respectively. Antibodies were detected in all the challenged birds at 12 days post-challenge. Antibodies were not detected in the non-challenged birds.

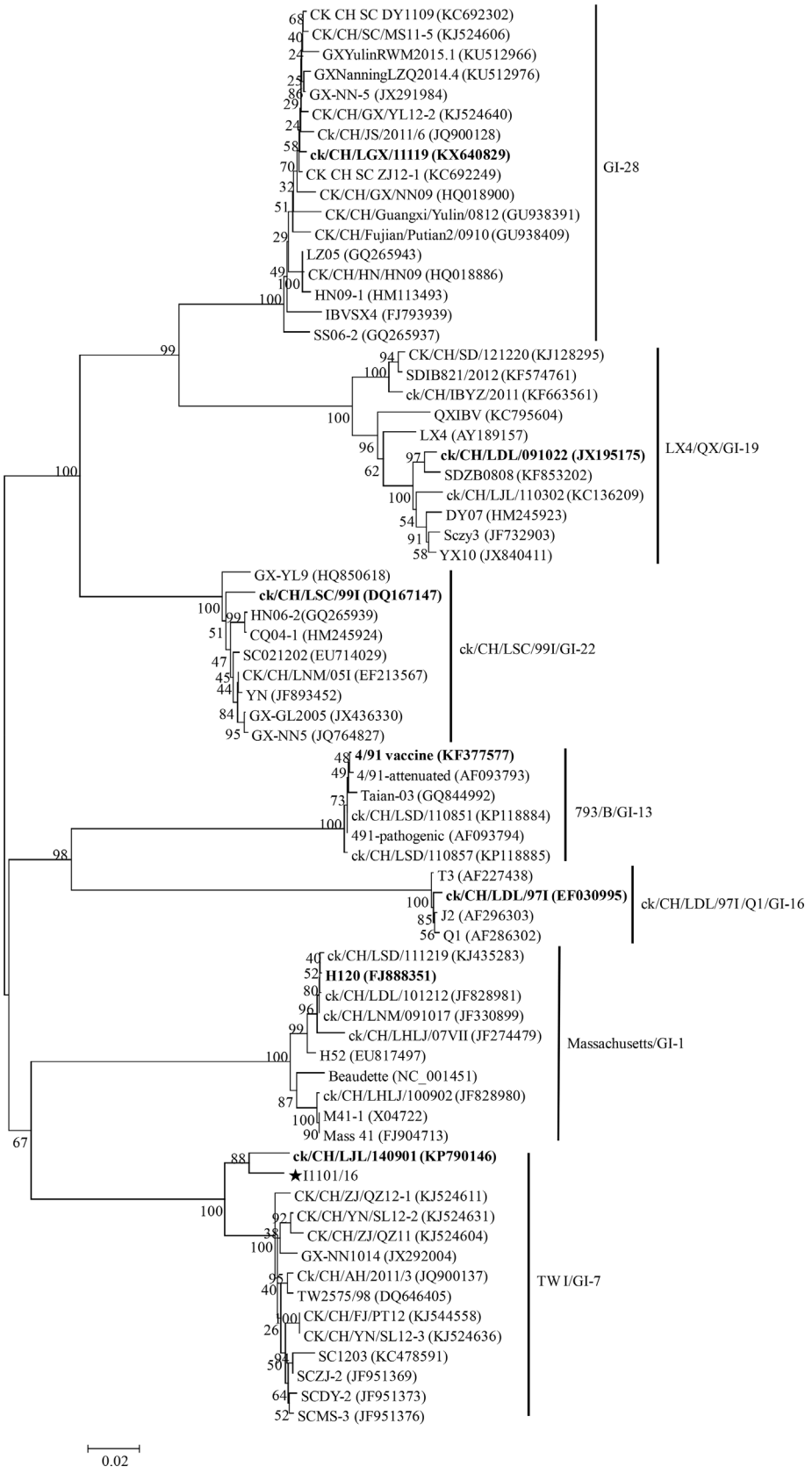

Fig. 1. Phylogenetic tree based on the S1 nucleotide sequences (from the ATG start codon to the cleavage site of the spike protein). The phylogeny contains a total of 71 IBV strains, including the I110116 isolate and 70 IBV reference strains representing seven genotypes from China. Bars denoting the genotype and lineage designations are shown. The IBV strain and GenBank accession number are given for each strain. The scale bar represents the number of nucleotide substitutions per site. The IBV strains used for further comparisons and virus-neutralization testing are highlighted in bold.

3.6. Tissue tropisms and viral shedding

As illustrated in Fig. 6, replication of the ck/CH/LJL/140901 strain was detected in the trachea and kidneys of chickens, with titers of 3.83–4.83 $\log_{10}$ EID_{50} and 3.5–4 $\log_{10}$ EID_{50}, respectively. By contrast, replication of the I1101/16 isolate was detected in the trachea and kidneys, with titers of 1.7–2.83 $\log_{10}$ EID_{50} and 1.38–1.68 $\log_{10}$ EID_{50}, respectively. In addition, viral antigens were detected by

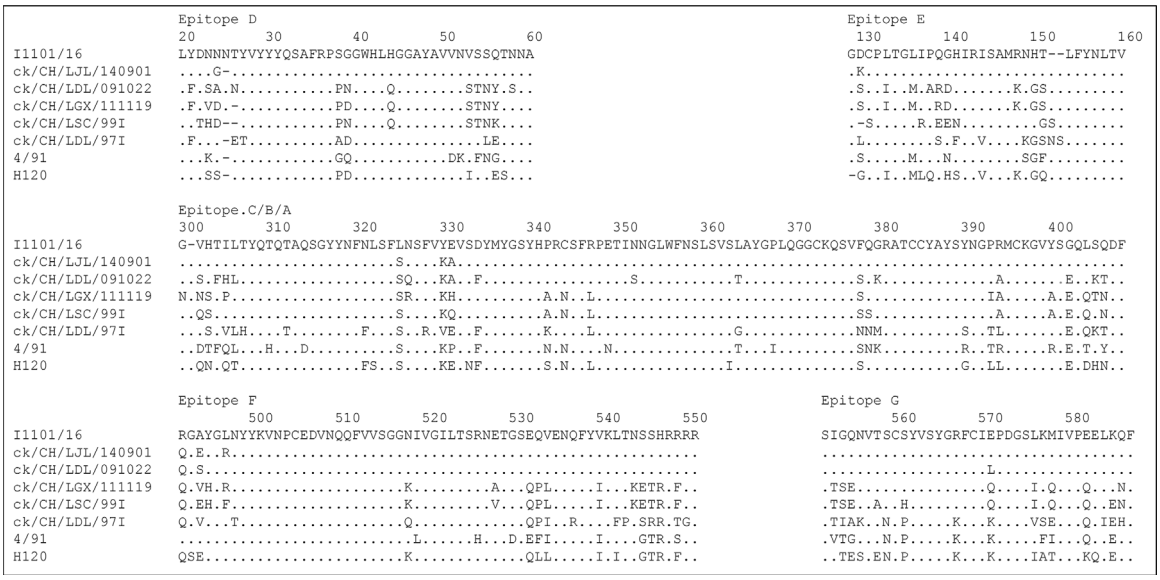

Fig. 2. Multiple alignment of the deduced amino acid sequences of the five conformation-dependent neutralizing antigenic sites (epitopes) mapped to the S1 subunit of the spike protein and another immunodominant region in the amino-terminal region of the S2 protein using the I1101/16 isolate and the seven reference strains. The sequences of the I1101/16 isolate are listed; only the amino acids that differed from those of the I1101/16 isolate are depicted and the amino acids that were same as those of the I1101/16 isolate are represented by dots. Deleted amino acid residues are represented by dashes.

Table 2
Gene (nucleotide) lengths of the I1101/16 isolate and ck/CH/LSC/99I strain.

Gene	1ab	Spike	3a	3b	3c	M	5a	5b	N
I1101/16	19,892	3501	174	189	327	678	198	249	1230
ck/CH/LSC/99I	19,892	3504	174	189	327	678	198	249	1230

immunohistochemistry in the trachea and kidneys of the birds challenged with both viruses (Fig. 7). No virus was detected in the trachea and kidneys of birds in the control group.

Shedding of the challenge viruses in the respiratory and digestive tracts was determined by virus recovery using nasopharyngeal and cloacal swabs from 9-day-old SPF chicken eggs. As illustrated in Table 4, the challenge viruses were recovered from the nasopharyngeal swabs of all the birds at 4 days post-challenge, whereas viruses were not detectable in some of the birds at 8 and 12 days post-challenge with the I1101/16 isolate and the ck/CH/LJL/140901 strain, respectively. Viral shedding was not detectable in the nasopharyngeal swabs of all the birds at 24 days post-challenge with the ck/CH/LJL/140901 strain. By contrast, viral shedding was still detectable at 28 days post-challenge in the nasopharyngeal swabs from one bird challenged with the I1101/16 isolate. Based on the cloacal swabs, viral shedding from some of the birds lasted until 40 and 28 days post-challenge with the I1101/16 isolate and the ck/CH/LJL/140901 strain, respectively. Virus was not recovered from the non-challenged control birds.

4. Discussion

IB is among the most important diseases in both vaccinated and non-vaccinated chickens in China, and it has been monitored continuously in chicken flocks since it was first identified. An outbreak possibly caused by a respiratory pathogen occurred at the end of 2016 in a broiler breeder flock in Neimenggu Province in China, and only a very small number of diseased chickens died during the outbreak. However, the egg production decreased and the egg quality was adversely affected. The main lesions were tracheitis and proventriculitis at the early stage of the outbreak, and severe bacterial infections were observed at the late stage, but no obvious kidney lesions were observed in the dead chickens throughout the outbreak. An IBV strain designated as I1101/16 was isolated from the tracheas of the dead chickens. Both the IB live vaccines H120 and 4/91 as well as an inactivated vaccine were used to vaccinate the chicken flock before the occurrence of the IB outbreak, so we initially sequenced the S1 gene to identify, differentiate, and genotype the IBV isolate. Based on the S1 gene analysis, the I1101/16 isolate clustered most closely with reference strains of the nTW I type (Xu et al., 2016), and it has been diverging as an independent genetic clade with the ck/CH/LJL/140901 strain (Fig. 1). Our analysis showed that the I1101/16 isolate and the ck/CH/LJL/140901 strain were more closely related serologically compared with other IBV serotypes (Table 1), but the serotype relatedness value was only 65 between these strains, which is greater than 50% but well below 80%, i.e., the lower cutoff value for the same serotype and the

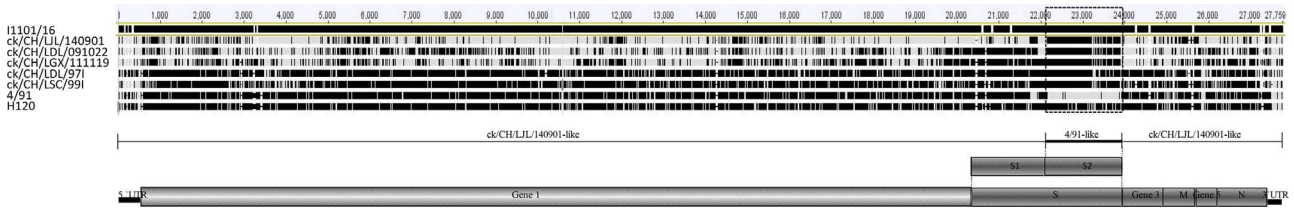

Fig. 3. Alignment of the complete genome sequences of the I1101/16 isolate and the seven reference strains performed using MAFFT. The genome sequence of the IBV I1101/16 isolate was set as the reference sequence. The reference sequence was represented by black and gray gaps in the reference sequence indicated the nucleotide insertion occurred in the genomes sequence of other viruses. The nucleotide sequences of other viruses which disagreement to the reference sequence at indicated positions were represented by black, while the nucleotide sequences of other viruses which agreement to the reference sequence at indicated positions were represented by gray. The GenBank accession numbers for these genome sequences are the same as those in Fig. 1.

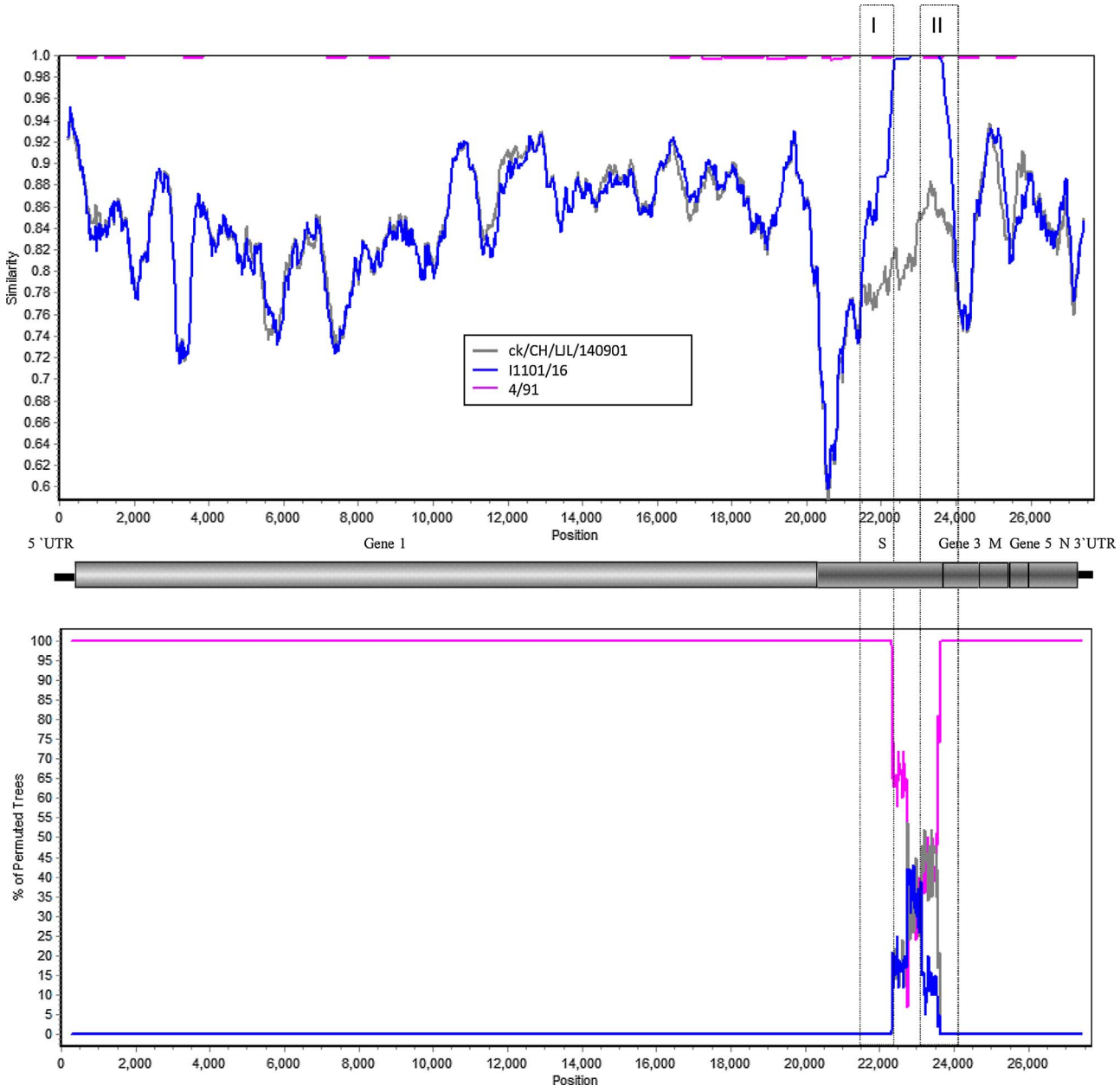

Fig. 4. Recombination analysis by SimPlot (A) and Bootscan (B) of the complete genomic sequence of the I1101/16 isolate. Breakpoints (indicated by I and II, respectively) of the first and second recombination events were detected and highlighted in boxes. The SimPlot was created using a window size of 500 bp and a step size of 20 bp. Strain ck/CH/LSD/110851 was used as the query strain.

maximum value defining serotype relatedness, respectively (Wadey and Faragher, 1981). The low antigenic relatedness between the I1101/16 isolate and the ck/CH/LJL/140901 strain was not due to differences in their B cell epitopes because the sequences of the B cell antigenic epitopes located in the spike protein were nearly identical in both viruses, although it has been reported that only a few amino acid changes in the S1 subunit of the spike protein can result in serologically distinct viruses (Cavanagh et al., 1992a,b). Hence, we focused on the entire spike gene of the two viruses, especially the S2 subunit of the spike gene. Interestingly, there was greater genetic diversity in the spike gene (90.9%) between the I1101/16 isolate and the ck/CH/LJL/140901 strain compared with that of S1 gene (95.7%), thereby indicating the greater divergence of the S2 region, which led us to conclude that possible recombination events may have occurred in the genome of the I1101/16 isolate.

Next, recombination events were analyzed using the complete genomic sequences of these strains. According to MAFFT, only the S2 gene of the I1101/16 isolate was highly similar to that of the 4/91 strain, whereas the remainder of the genome was highly similar to that of the ck/CH/LHLJ/140901 strain. This suggests that for the emergent I1101/16 isolate, the donor of the S2 gene was probably a 4/91-like virus, whereas the remainder of the genome came from a ck/CH/LHLJ/140901-like virus.

The first prerequisite for recombination is considered to be co-infection. IBV strain ck/CH/LHLJ/14090 is one of the deduced parental viruses of the I1101/16 isolate and it has an nrTW serotype, where it originated from recombination events between TW I-like and LX4-like viruses in China around 2009 (Xu et al., 2016). In the commercial poultry industry in China, IBV is controlled by the use of live attenuated and inactivated vaccines. Vaccine 4/91 is another deduced parental virus of the I1101/16 isolate and it has been used widely in China for a long time. Previously, we found that vaccination with the 4/91 strain

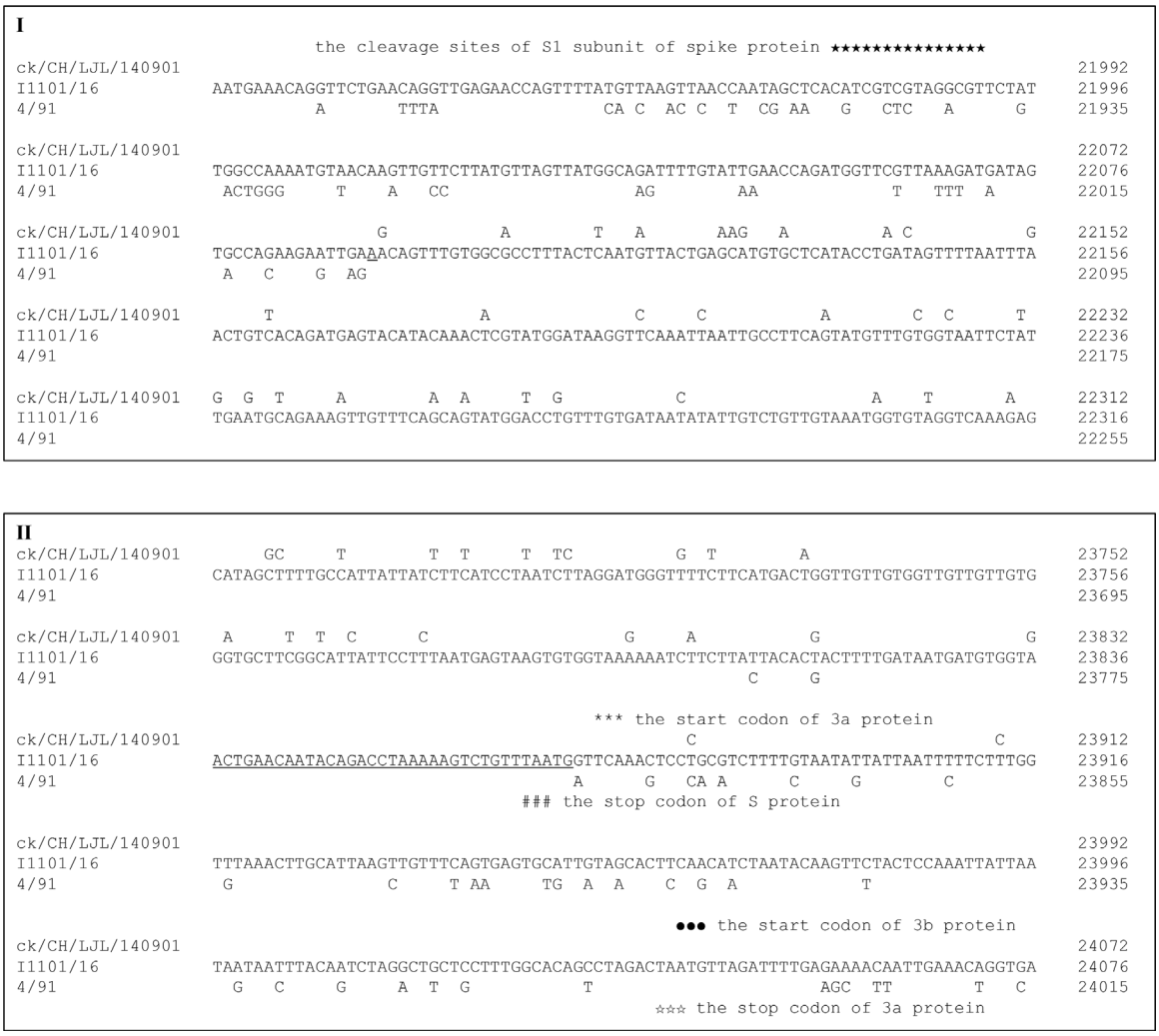

Fig. 5. Multiple sequence alignment of the predicted breakpoint and flanking sequences in the I1101/16 isolate and its putative parental viruses, the ck/CH/LJL/140901 and 4/91 strains. Numbers to the right of each alignment denote the nucleotide positions in the genome of each virus. The sequences of the I1101/16 isolate are listed and only the nucleotides that differed from those in the I1101/16 isolate are depicted. The regions where the template switches (breakpoints) occurred are underlined.

Table 3
Pairwise comparisons of the nucleotide sequences of the S2 subunit of the spike genes between the 4/91 vaccine strain, I1101/16 isolate, and pathogenic 4/91 strain[a].

Strain	Position[b]											
	2,09	2,14	2,43	3,011	3,38	3,39	3,39	3,40	3,44	3,4	3,44	3,453
	2	7	2		4	0	9	3	1	35	1	
4/91 vaccine	T	A	A	T	G	C	T	T	A	C	G	T
I1101/16	G	G	A	C	G	C	T	T	A	T	T	T
pathogenic 4/91	T	A	G	C	A	T	G	C	G	T	G	C

[a]Nucleotides shared between the I1101/16 isolate and 4/91 vaccine strain are shown in gray.
[b]Nucleotide positions corresponding to those in the spike gene in the 4/91 vaccine strain. The GenBank accession number of the pathogenic 4/91 strain is JN192154. The GenBank accession numbers of the other viruses are the same as those shown in Fig. 1.

cannot provide complete protection against nrTW viruses (Gao et al., 2016), but vaccination with the 4/91 strain allows prolonged replication and shedding of the vaccine virus (Han et al., 2017). Hence, it is reasonable to speculate that vaccination with the 4/91 vaccine produces an environment where co-infections between field and vaccine strains can occur, which may enhance the likelihood of recombination. High frequencies of recombination between vaccines (such as the 4/91 strain) and field strains have been reported frequently in China and other parts of the world (Cavanagh et al., 1992a,b; Jackwood et al., 2012; Liu et al., 2013; Han et al., 2017; Jiang et al., 2017).

It has been suggested that recombination may play an important role in viral evolution. The exchange of part of a region of the genome allows viruses to rapidly explore areas of sequence space, potentially leading to the emergence of variants with different features in terms of their virulence, cross-protection, and cell and host tropisms (Simon-Loriere and Holmes, 2011). It is considered that the emergence of the Australian N1/88 strain was driven by antigenic differences between the virus and other circulating IBVs, but also by the enhanced capacity of the recombinant virus to replicate in chickens (Quinteros et al., 2016). We found that the recombinant I1101/16 isolate was serologically related to but not the same as strain ck/CH/LHLJ/140901. We cannot conclude that this antigenic change was due to recombination because the B cell antigenic epitopes located in the spike protein (including that located in the S2 subunit of the spike gene) were nearly identical in the two viruses. In addition, it is very interesting to note that the/I1101/16 isolate exhibited decreased replication levels in both the tracheal and kidney tissues (two target tissues for most IBVs) compared with one of its parental viruses (the ck/CH/LHLJ/140901 strain, which does not cause severe clinical disease in SPF chickens), but it exhibited prolonged replication and shedding post-challenge in a

Table 4
Clinical sign scores and recovery of IBV strains I1101/16 and ck/CH/LJL/140901 from chickens challenged at 1 day of age.

Group	Clinical signs	Clinical signs score	Viral shedding[a]																		
			Nasopharyngeal swabs							Cloacal swabs											
			4	8	12	16	20	24	28	4	8	12	16	20	24	28	32	36	40	44
I1101/16	10/10	3	10/10[b]	9/10	7/10	6/10	2/10	1/10	1/10	7/10	8/10	10/10	7/10	7/10	5/10	6/10	6/10	6/10	2/10	0/10
ck/CH/LJL/140901	9/10	0.9	10/10	10/10	6/10	2/10	2/10	0/10	-[c]	10/10	10/10	10/10	10/10	6/10	6/10	6/10	0/10	–	–	–
Negative	0/10	0	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	0/10

[a] Two procedures were used for virus recovery after challenge. First, lesions were observed in individual tissue samples (trachea or kidneys) from inoculated embryos. Second, RT-PCR was conducted using the oligonucleotide primers N(+) and N(−) on RNA recovered from the allantoic fluid in the same eggs. The results obtained using the two procedures were identical.
[b] Number of chickens yielding virus/number of chickens tested.
[c] Not done.

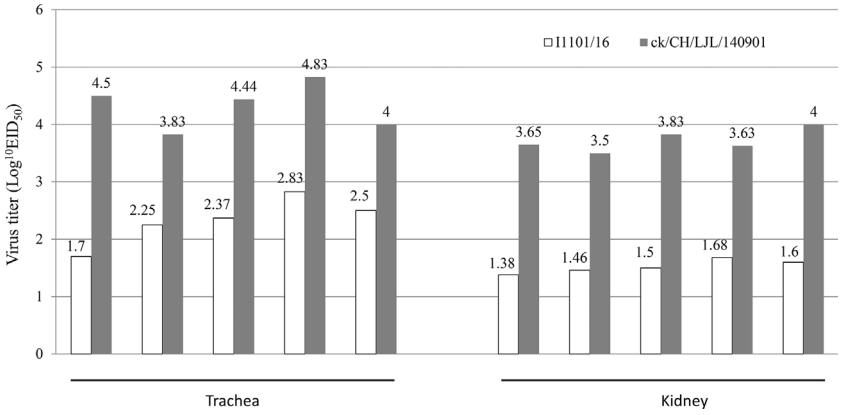

Fig. 6. Replication of the I1101/16 isolate and ck/CH/LJL/140901 strain in the trachea and kidneys of chickens. One-day-old SPF layer chickens were inoculated with a dosage of $1 \times 10^{5.5}$ EID_{50} of the I1101/16 isolate or the ck/CH/LJL/140901 strain in a 0.1-ml volume, and the trachea and kidneys from each bird were collected at 5 days post-challenge for virus titration in eggs. Each bar represents the virus titer from an individual bird.

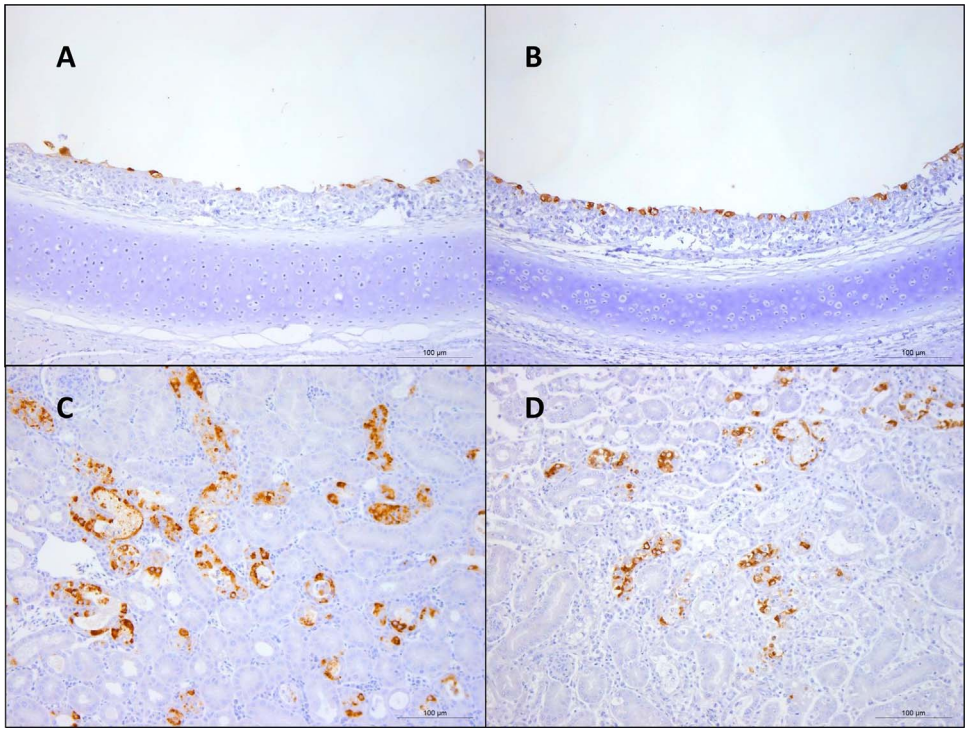

Fig. 7. Detection of IBV antigen by immunohistochemical analysis of the trachea (A and B) and kidneys (C and D) from birds challenged with the I1101/16 isolate (A and C) and the ck/CH/LJL/140901 strain (B and D).

similar manner to one of its parental viruses, the 4/91 vaccine (Han et al., 2017). Meanwhile, the high viral titer but low clinical score of chickens infected with ck/CH/LJL/140901 was also observed in this study although the exact mechanism was unknown and needed to be further investigated.

It is believed that the coronavirus S1 domain is involved with host cell receptor binding and that the conserved S2 domain mediates fusion between the virus and cellular membranes (Bosch et al., 2003). The S2

membrane fusion unit of the ectodomain contains two heptad repeat regions, which interact to form the coiled-coil structure of the stalk (de Groot et al., 1987; Wickramasinghe et al., 2011), and a putative fusion peptide. After endocytosis, conformational changes in the S protein are triggered by exposure to acidic pH in the endosomes (Chu et al., 2006), thereby resulting in fusion of the viral envelope with the cellular membrane. The S2 domain is not involved mainly with binding to the host cell receptor, but the interplay between S1 and S2 might synergistically determine the avidity and specificity of viral attachment (de Hann et al., 2006; Promkuntod et al., 2013). It is unknown whether the changes in the spike protein caused by the recombination event altered the antigenic and pathogenic features of the I1106/16 isolate, or whether it is more likely that these changes as well as other changes in polymerase peptides are jointly rather than individually responsible for the differences in the features of the two IBV strains. However, it has been reported that recombination is likely to have a high fitness cost because of the destruction of optimized intra- and inter-protein interactions (Simon-Loriere and Holmes, 2011), where the fitness of recombinant IBV strains must be superior to that of the parental strains if they are to emerge and spread in the environment. Thus, the fitness of recombinant IBVs requires further investigation.

This study does not provide evidence that recombination can directly alter the antigenicity, virulence, replication, shedding, and tissue tropism of a virus, but because the backbone of the investigated virus differs substantially, we showed that gene(s) exchange between IBVs is likely to be the major determinant of viral evolution. Our previous results (Liu et al., 2013; Liu et al., 2014; Chen et al., 2015) and the results obtained in this study provide evidence that recombination events are occurring frequently in the field in China. High chicken densities in poultry farms and the co-circulation of multiple IBV strains in a given flock as well as the use of different live vaccine strains may explain the high frequency of recombination. Researchers should be aware of the effect of virus recombination, especially recombination events between field and vaccine strains, which may cause changes in viral features. In addition, the currently applied vaccination strategy for protection against the nTW I IBV type should be improved and implemented properly.

Competing interests

The authors declare that they have no competing interests.

Acknowledgments

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