



# Genetic and biological characteristics of four novel recombinant avian infectious bronchitis viruses isolated in China

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## ABSTRACT

Infectious bronchitis viruses (IBVs) of GI-13 (793/B) and GI-19 (QX/LX4) lineages have been frequently detected in China in recent years. Naturally recombinant IBVs originating from the GI-13 and GI-19 lineages have also been isolated from chicken flocks with respiratory and renal problems in China. Thorough genetic and biological investigations of these recombinant viruses have led to speculation regarding their origin, evolution, and control. In order to confirm the previous results and further extend our understanding about the characteristics of the four recombinant IBV strains we had previously identified (I0718/17, I0722/17, I0724/17, and I0737/17), we conducted phylogenetic analysis by comparing their complete S1 gene sequences with those of 71 reference strains of different genotypes and lineages. We identified a close relationship between the S1 sequences of the four strains and those of GI-13 strains. The results of complete genome sequence analysis confirmed the previously identified recombination events in the four IBV strains and revealed additional recombination events in different genomic regions of strains I0718/17 and I0724/17, suggesting that the two strains originated from multiple recombination events between 4/91-like and YX10-like viruses. We comparatively evaluated the antigenicity, pathogenicity, and affinity of the four recombinant viruses and their deduced parental strains in the trachea and kidneys. Some of the strains showed comparable antigenic relatedness, pathogenicity, and affinity for the trachea and kidneys among each other and with their parental viruses; however, some of them showed varying biological characteristics. Point mutations observed in the receptor-binding domain and hypervariable region of the S1 subunit of the spike protein likely have an effect on these differences in biological characteristics, although the influence of other factors—such as host innate-immune responses and changes in genomic regions beyond the S1 protein—might also be responsible for such changes.

## 1. Introduction

Avian infectious bronchitis (IB) is an acute and highly contagious disease caused by the IB virus (IBV), which causes respiratory diseases, interstitial nephritis, and reproductive disorders in chickens of all ages (Cavanagh, 2007). The disease can induce severe economic losses, especially in terms of decreased egg production, poor eggshell quality, reduced hatchability, increased feed conversion, and carcass condemnation at slaughterhouses (Santos Fernando et al., 2017). IBV variants are continuously emerging in different regions of the world and continue to be a major problem for chicken production worldwide (Jackwood, 2012). Vaccination is considered to be the most reliable approach for IBV control; however, existing vaccines have proved to be inadequate due to the constant emergence of new virus variants (De

Wit, 2000; de Wit et al., 2011). In addition, co-infection of a chicken by different IBV types carry the risk of recombination, accounting for the emergence of novel IBV strains with different antigenic properties, tissue tropism, and pathogenicity; such strains have been identified in many parts of the world (Franzo et al., 2015; Hewson et al., 2014; Hong et al., 2012; Kottier et al., 1995; Lee and Jackwood, 2000; Mardani et al., 2010; Naguib et al., 2016; Quinteros et al., 2016; Pohuang et al., 2011; Thor et al., 2011; Zanaty et al., 2016; Zhang et al., 2015), and this presents a major challenge to current IBV prevention and control strategies.

IBV belongs to the *Coronaviridae* family, and its virion contains a single copy of a positive-sense, single-stranded, 27.6-kb RNA, which encodes polypeptides 1a and 1b (which are further processed into non-structural proteins 2–16), four structural proteins (the spike [S],

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envelope [E], membrane [M], and nucleocapsid [N] proteins), and several accessory proteins (3a, 3b, 5a, and 5b). The S protein, especially the S1 subunit formed by post-translational cleavage, is critical for antigenic neutralization, hemagglutination, and determining cell tropism (Casais et al., 2001; Cavanagh, 2007; 1988; 1997; Kant et al., 1992; Promkuntod et al., 2014). The high mutation rate of the S1 gene during IBV replication and evolution generates extensive genotypic, antigenic, and pathogenic variability in the progeny viruses. This implies the existence of multiple genotypes/serotypes and variants, against which the existing IB vaccines might not offer complete cross-protection (Cavanagh et al., 1997; Gelb et al., 2005), thus hampering the control of IBV infection by vaccination.

Many researchers have typically focused on analysis of the IBV S gene; most of such studies have analyzed the S1 subunit of the S protein for genotyping IBV strains, unifying IBV classification, and clarifying IBV evolution. For example, 7 genotypes and 35 distinct lineages of IBV have been defined on the basis of the sequence analysis findings of the complete S1 gene of IBV strains isolated from different countries or geographic regions worldwide (Chen et al., 2017; Jiang et al., 2018; Valastro et al., 2016). However, the results of S1 sequence analysis might not be solely sufficient to explain the changes observed in IBV antigenicity, tissue tropism, and pathogenicity. In recent years, several studies have noted that viral replication, pathogenicity, and immune escape might be modulated by non-structural and accessory viral proteins in IBV and other coronaviruses (Armesto et al., 2009; Chen et al., 2009; Laconi et al., 2018; Zheng et al., 2008; van Beurden et al., 2018). These findings emphasize the importance of studying the characteristics of the complete genomes of coronaviruses and their associations with antigenicity, tissue tropism, and pathogenicity.

More recently, phylogenetic studies have reported that—in addition to the well-established lineages/genotypes such as GI-1, GI-7, GI-13, GI-19, GI-22, and GVI-1—a number of novel IBV variants have been isolated in China (Chen et al., 2015, 2017; Han et al., 2017; Xu et al., 2016, 2018; Zhao et al., 2017). These variants have been proved to be distinct from other reference strains on the basis of genetic characteristics and phylogenetic differences in the S1 gene (Xu et al., 2018). To provide a comprehensive outlook on the genetic characteristics of these IBV variants, we sequenced and comparatively analyzed the complete genome sequences of four novel IBV variants with those of other IBV strains in the present study. We also characterized the antigenicity, pathogenicity, and tissue tropism of these IBV strains and looked for possible associations between their genes and their phenotypic properties.

## 2. Materials and methods

### 2.1. IBV strains

This study used five IBV field isolates—including ck/CH/LGX/100508 (LGX/100508) (Gao et al., 2016), I0718/17, I0722/17, I0724/17, and I0737/17—and the 4/91 vaccine strain. Strains I0718/17, I0722/17, I0724/17, and I0737/17 were isolated from the trachea of broilers with respiratory signs in Fujian province, China, in 2017 (Xu et al., 2018). The six IBV strains were propagated in specific-pathogen-free (SPF) embryonated chicken eggs (Harbin Veterinary Research Institute, Chinese Academy of Agricultural Sciences, China) as described previously (Liu and Kong, 2004). IBV-containing allantoic fluid was harvested, and vials of the virus stocks were stored at  $-70^{\circ}\text{C}$  until use. Virus identification was performed by the reverse transcriptase-PCR (RT-PCR) as previously described (Liu et al., 2009). The virus was titrated by the end-point dilution method in 9-day-old SPF chicken eggs, and the titers were expressed as 50% egg infective dose ( $\text{EID}_{50}$ ) in accordance with the Reed and Muench method (1938).

### 2.2. Cloning and sequencing of the complete genomes of the IBV strains

The complete genomes of strains I0718/17, I0722/17, I0724/17, and I0737/17 were cloned and sequenced in this study. Viral RNA was extracted from allantoic fluid by using an RNAiso Plus kit (Takara Bio Inc., Shiga, Japan) in accordance with the manufacturer's instructions. RT-PCR was performed using a one-step RT-PCR kit (Takara Bio Inc.) in accordance with the manufacturer's instructions. The primers and strategies used for cloning the complete genomes and the 3'/5' termini of the four IBV strains were as previously described (Zhang et al., 2015). Each PCR amplicon was cloned into a PMD-18 T vector system (Takara Bio Inc.), and three to five independent clones were sequenced for each amplicon.

### 2.3. Comparison and analysis of the sequences of S1 gene

The complete sequences of the S1 gene of the four IBV strains were assembled from both forward and reverse sequences by using the Clustal W method (available in the Bioedit software package; <http://www.mbio.ncsu.edu/bioedit>). Phylogenetic trees were constructed with the MEGA 6.0 software (<http://www.megasoftware.net/>) by using the S1 gene sequences of 75 IBV strains, including the four novel strains described in this study and 71 reference strains. The trees were constructed by the neighbor-joining method with 1000 bootstrap replicates. The S1 gene sequences of the reference strains, which represented the 7 well-established genotypes and 30 well-established lineages, were retrieved from the GenBank database.

Alignment of the deduced amino acid sequences of the S1 subunit of the spike protein was performed on our four IBV strains by comparing with those of the vaccine strain, 4/91, which is used commonly in chicken flocks in China, and two IBV field strains, LGX/100508 and YX10, that represented the main IBV type circulating in chicken flocks in China. We compared the amino acid sequences of the three hypervariable regions (HVRs), five conformation-dependent neutralizing antigenic sites (epitopes) mapped to the S1 subunit of the spike protein (Kusters et al., 1989; Lenstra et al., 1989; Koch et al., 1990), as well as the minimal receptor-binding domain (RBD) (Promkuntod et al., 2014) in a pairwise manner.

### 2.4. Characteristics of the complete genome

The consensus sequence of each PCR product was assembled from both forward and reverse sequences by using MEGA 6.0 (<http://www.megasoftware.net/>). The complete genome sequences of the four IBV strains were carefully edited and were submitted for a BLAST search in the NCBI database (<https://blast.ncbi.nlm.nih.gov/Blast.cgi>) for identifying homologous IBV sequences. The complete genome sequence of IBV strain 4/91 was used to compare with those of our four IBV strains. The open reading frames (ORFs) of the four IBV strains were identified by comparison with those of the 4/91 and YX10 strains. The nucleotide sequence data reported in this study were deposited in the GenBank database (I0718/17: [MK032178](#); I0722/17: [MK032179](#); I0724/17: [MK032177](#); and I0737/17: [MK032180](#)).

To further confirm the recombination events previously observed in the S1 gene (Xu et al., 2018) and investigate possible recombination events in other regions of the genome, the complete genome sequences of our four IBV strains were compared with that of the 4/91 vaccine strain by using the Multiple Alignment with Fast Fourier Transformation (MAFFT) tool (version 6; <http://mafft.cbrc.jp/alignment/software/>). Then, sequence fragments that were different from those of the 4/91 strain were submitted for a BLAST search in the NCBI database for identifying homologous IBV sequences. On the basis of the search results, the complete genomic sequences of our four strains were again compared with those of the 4/91 and YX10 strains (GenBank no. [JX840411](#)) by using MAFFT. The recombination events in the genomes of each of our four strains were further confirmed by bootscan analysis

using the Simplot software (Lole et al., 1999). This analysis was conducted using a sliding window of 1000 nucleotides (nt) moving in 50-nt steps, with the genome sequence of strain ck/CH/LJL/101150 as the query.

To identify potential recombination switch sites, multiple sequences of the potential parental viruses were compared pairwise with those of each of our IBV strains on the basis of the MAFFT and SimPlot results.

## 2.5. Virus cross-neutralization tests

On the basis of sequence analysis results, strains I0718/17, I0722/17, I0724/17, and I0737/17 were selected for virus cross-neutralization tests with each other and with strains LGX/100508 and 4/91. Six groups of five 1-day-old SPF layers were housed in six isolators and challenged with strains LGX/100508, 4/91, I0718/17, I0722/17, I0724/17, and I0737/17 by the oculonasal route. At 3 weeks of age, the birds in the six groups were boosted with each of the corresponding viruses. At 35 days of age, all birds were bled. The blood samples were pooled per group, and the serum samples of each group were stored at  $-70^{\circ}\text{C}$  until use. The serum of SPF chickens was also used in this test.

Virus cross-neutralization tests were conducted by the  $\beta$  method. The virus ( $100\text{ EID}_{50}$ ) was added to a twofold dilution of the serum and incubated for 1 h at  $37^{\circ}\text{C}$ . Subsequently, the virus-serum mixtures were injected into the allantoic cavity of five SPF chicken embryos, which were then observed for 7 days. Virus replication was determined by amplifying the IBV N gene from allantoic fluid by RT-PCR (Liu et al., 2009). The tests were performed in the presence of appropriate controls.

## 2.6. Pathogenicity tests

### 2.6.1. Challenge viruses

On the basis of the results of genome sequence analysis, six strains of IBV (LGX/100508, 4/91, I0718/17, I0722/17, I0724/17, and I0737/17) were selected for further testing by pathotyping. These challenge strains were inoculated by the oculonasal route, and this procedure yielded  $10^4\text{ EID}_{50}$  per bird at the age of 1 day.

### 2.6.2. Experiment tests

The Ethical and Animal Welfare Committee of the Harbin Veterinary Research Institute, China, granted ethical approval for the animal experiments. One-day-old SPF layer chickens ( $N = 105$ ) were randomly divided into seven groups ( $n = 15$ , each), and housed in isolators. Each group of chickens was challenged with one of the challenge IBV strains (LGX/100508, 4/91, I0718/17, I0722/17, I0724/17, or I0737/17). One group of 15 SPF birds was mock-infected with phosphate-buffered saline in the same manner, and this functioned as the negative control group. Signs, such as eye irritation and/or scratching of the inoculated eye, lethargy, mild coughing and/or “snicking,” were scored as 0 (absent), 1 (mild), 2 (moderate), and 3 (severe). To determine the pathogenicity of the challenge viruses in the respiratory and urinary tracts at 5 days post-challenge (dpc) and to evaluate their tissue tropism in SPF chickens, five birds in each group were humanely sacrificed, and samples of the trachea and kidneys were carefully collected for titrating the viruses in 9-day-old SPF chicken eggs. The remaining ten birds were monitored daily for clinical signs and death for a period of 32 days. Blood samples and oropharyngeal and cloacal swabs were collected on days 4, 8, 12, 16, 20, 24, 28, and 32 dpc. The serum samples were analyzed for the presence of IBV-specific antibodies by using a commercial enzyme-linked immunosorbent assay kit (IDEXX, Inc., Westbrook, ME, USA) in accordance with the manufacturer's instructions (Han et al., 2013). The oropharyngeal and cloacal swabs were used for virus recovery in 9-day-old SPF chicken eggs (Zhang et al., 2015).

## 2.7. Statistical analysis

Data are expressed as mean  $\pm$  standard deviation. Virus titers were analyzed by a Student's *t*-test using GraphPad Prism for Windows version 5 (GraphPad Software, La Jolla, CA, USA). Differences were considered significant if the *p* value was  $< 0.05$  (\**p*  $< 0.05$ , \*\**p*  $< 0.01$ , \*\*\**p*  $< 0.001$ ).

## 3. Results

### 3.1. Phylogenetic analysis and sequence comparison based on the complete S1 gene

Phylogenetic analysis was performed by using the complete nucleotide sequences of the S1 gene of our four IBV strains and 71 reference IBVs strains, which were representative of 35 well-established lineages and 7 genotypes. The results demonstrated that our four IBV strains were phylogenetically clustered among IBV strains with the GI-13 genotype (Valastro et al., 2016), including the 4/91 vaccine strain and Moroccan/G/83 (El-Houadfi et al., 1986). Our results also showed that our four strains formed two phylogenetic subclusters, in which strains I0737/17, 4/91, and Moroccan/G/83 occurred in the same subcluster, and strains I0718/17, I0722/17, and I0724/17 were grouped together (Fig. 1).

The results of alignment of the complete S1 gene sequences of our four viruses with those of the 4/91 strain (the currently used vaccine strain) and the LGX/100508 and YX10 strains (GI-19-lineage viruses) revealed the presence of multiple mutations (Fig. 2). The majority of variations between I0737/17 and 4/91 were observed at the far N terminus of the S1 subunit of the S protein, in which three substitutions were located in HVRI. The nucleotide sequence of this region in strain I0737/17 was same as those in strains LGX/100508 and YX10. In contrast, the nucleotide sequences of the remaining regions in I0737/17 were nearly the same as those in strain 4/91 and different from those in strains LGX/100508 and YX10. These remaining regions included several important domains, such as the deduced minimal RBD, most parts of HVRI, all parts of HVRs II and III, and the five epitopes mapped to the S1 subunit of the S protein (Kusters et al., 1989; Koch et al., 1990). Strains I0718/17, I0722/17, and I0724/17 possessed similar nucleotide sequences and showed differences with the amino acid sequence of 4/91 at the N terminus of the S1 subunit, which included HVRs I and II, epitopes D and E, half of the RBD, and four critical amino acids in the RBD. The nucleotide sequences of the remaining regions in the three viruses were the same as those in 4/91 and different from those in strains LGX/100508 and YX10. In addition, strains LGX/100508 and YX10 differed only by two amino acid substitutions (F193Y and I252T).

### 3.2. Complete genome sequence and recombination analysis

The complete genomes of strains I0718/17, I0722/17, I0724/17, and I0737/17 are 27677, 27666, 27669, and 27666 nt in length, respectively, excluding the poly-A tail. The genomes include ten ORFs and are organized as follows: 5'-UTR-1a-1b-S<sup>3</sup>-a-3b-E-M-5a-5b-N-3'-UTR (UTR: untranslated region). The ORFs, genes, or genomic regions present in our four IBV strains and two reference strains (4/91 and YX10) are listed in Table 1. The complete genome sequences have been deposited in GenBank, and the accession number of each virus is listed in Fig. 1.

The complete genome sequences of our four strains were compared with that of strain 4/91 by using MAFFT. The results showed differences in the sequence of 1ab gene between our four IBV strains and 4/91 (data not shown). Therefore, the 1ab gene sequences were submitted to a BLAST search in the NCBI database. The search results demonstrated that the 1ab gene of our four strains was highly homologous with that of IBV strain YX10. Then, the complete genome sequences of our four IBV strains were compared with those of 4/91 and YX10 by



**Fig. 1.** Phylogenetic trees of IBV strains based on the S1 nucleotide sequences of four strains and 71 reference strains. The trees were constructed by the neighbor-joining method with 1000 bootstrap replicates (bootstrap values are shown on the trees). The four novel IBV strains sequenced in this study are highlighted by a black circle. Two IBV strains, LGX/100508 and 4/91, were used for further investigation and are highlighted by a triangle. IBV, infectious bronchitis virus.

using MAFFT, and the results suggested the occurrence of recombination events in the genomes of our four strains (Fig. 3).

In both bootscan analysis and pairwise comparison, strain I0717/18 exhibited a greater similarity to strain YX10 in three fragments (from 5' UTR–nt10546; nt10,743–21,014; and nt23899–26,166) and to strain 4/91 in three other fragments (nt10546–10,704; 21,017–23,893; and

26,174–37,677) (Fig. 4A). The nucleotide sequence of strain I0724/17 was similar to that of strain YX10 in two fragments (nt1–10,276 and 11,715–20,796) and strain 4/91 in two other fragments (nt10293–11,712 and 20,800–27,669) (Fig. 4B). These results implied that strains I0718/17 and I0724/17 both originated from multiple (five and three, respectively) recombination events between a 4/91-like



	Epitope D										Hypervariable region I										Hypervariable regionII																				
4/91 vaccine	MLGKPLLLVTLWYALCSAL										LYDK-NTYVYVYQSAFRPGQGWHLGGAYAVDKVFNGTNNVSVSDCTAGTFYESYNISAASVAMTVPPAGMSWSVAQFCTAHCNFSDFTVFVTHCFKSQSSCPLTGMIPQNHIR																				134										
I0737/17	.....S.F.....ILC.....N										.....F.SA.....N										.....VNST.Y.....G.A.G.V.VIKDV..Q.....I.....A.Q.A.A.KS.....S.....EI.....YS.GA.....I.....ARDY.....																				135
I0724/17	.....S.F.....ILC.....N										.....F.SA.N.....PN.....Q.....										.....VNST.Y.....G.A.G.V.VIKDV..Q.....I.....A.Q.A.A.KS.....S.....EI.....YS.GA.....I.....ARDY.....																				135
I0722/17	.....S.F.....ILC.....N										.....F.SA.N.....PN.....Q.....										.....VNST.Y.....G.A.G.V.VIKDV..Q.....I.....A.Q.A.A.KS.....S.....EI.....YS.GA.....I.....ARDY.....																				135
I0718/17	.....S.F.....ILC.....N										.....F.SA.N.....PN.....Q.....										.....VNST.Y.....G.A.G.V.VIKDV..Q.....I.....A.Q.A.A.KS.....S.....EI.....YS.GA.....I.....ARDY.....																				135
LGX/100508	.....S.F.....ILC.....N										.....F.SA.N.....PN.....Q.....										.....VNST.Y.....G.A.G.V.VIKDV..Q.....I.....A.Q.A.A.KS.....S.....EI.....YS.GA.....I.....ARDY.....																				135
YX10	.....S.F.....ILC.....N										.....F.SA.N.....PN.....Q.....										.....VNST.Y.....G.A.G.V.VIKDV..Q.....I.....A.Q.A.A.KS.....S.....EI.....YS.GA.....I.....ARDY.....																				135
Epitope E																																									
4/91 vaccine	ISAMRSGLFENLVISVSKYFKFKSLQCVGNSTSVYLNGDLVFTPNETHVTGAGVYFKSGGPVTVYKMKVKALAYFINGTAQEVILCDNSPRGLLACQYNTGNFSDGFYFTNSSLVKDRFIVYRESSTNTTL																														269										
I0737/17	.....KN.S.....T.....F.....N.....S.....																														270										
I0724/17	.....KN.S.....T.....F.....N.....S.....																														270										
I0722/17	.....KN.S.....T.....F.....N.....S.....																														270										
I0718/17	.....KN.S.....T.....F.....N.....S.K.AD.S.....A.....																														270										
LGX/100508	.....KN.S.....T.....F.....N.....S.K.AD.S.....A.....N.S.....F.V.....V.....D.....K.....T.R.K.....V.....																														270										
YX10	.....KN.S.....T.....F.....N.....S.K.AD.S.....Y.A.....N.S.....F.V.....V.....D.....K.....T.R.K.....V.....																														270										
Hypervariable region III																														Epitope B/C/A											
4/91 vaccine	ELTNFTFTTNVSNASPNSSGGVDYDFQLTQHTAQAQGYNFNLSFLSSFFVYKPSDFMGYSHPNCFRPNINNGLWFNLSLSVSLTYGPIQGGCKQSVFSNKATCCYAYSIRGPTCKCKVYRGRLITQYFECGLIVVYT																				404																				
I0737/17	.....										.....																				405										
I0724/17	.....										.....																				405										
I0722/17	.....										.....																				405										
I0718/17	.....										.....																				405										
LGX/100508	.....T.....T.Q.....S.H.....Q.S.....Q.....A.....R.S.....T.S.....L.....NG.....N.RA.....SG.SKT.....																				405																				
YX10	.....T.....T.Q.....S.H.....Q.S.....Q.....A.....R.S.....T.S.....L.....NG.....N.RA.....SG.SKT.....																				405																				
Epitope F																																									
4/91 vaccine	KSDGSRQITRSEPLVLTQYNNYNTLNKVCYENIYGRVQGFTINVTETATANYSLADGGLAILDTSAGDIDFVVRGAYGLNYYKNPCEDVNQQFVVSNGNLVGLTSHNETDSEFTENQFYIKLTNGTRRSRR																														539										
I0737/17	.....																														540										
I0724/17	.....																														540										
I0722/17	.....																														540										
I0718/17	.....																														540										
LGX/100508	.....T.....T.....M.H.....D.....D.....DSA.F.....V.....																														540										
YX10	.....T.....T.....M.H.....D.....D.....DSA.F.....V.....																														540										

**Fig. 2.** Alignment of the deduced amino acid sequences of the S1 subunit of the spike protein of IBV strains LGX/100508, I0718/17, I0722/17, I0724/17, I0737/17, and 4/91. Amino acid sequences encoding the three hypervariable regions are shown in bold. The sequences of the five conformation-dependent neutralizing antigenic sites (epitopes) mapped to the S1 subunit are underlined. The minimal receptor-binding domains corresponding to the most N-terminal residues 19–272 of the spike protein of IBV M41 are boxed, and the critical amino acids 38, 43, 63, and 69 are highlighted in red. The GenBank accession numbers are the same as those in Fig. 1. IBV, infectious bronchitis virus.

virus and a YX10-like virus. The results of bootscan analysis of the complete genome revealed that the 5' ends of strains I0717/22 and I0717/37 were highly similar in sequence to that of YX10 and less similar to that of 4/91; in contrast, their 3' ends were highly related to that of 4/91 and different from that of YX10 (Supplemental Figure). These results correspond to those of the previous report (Xu et al., 2018).

3.3. Antigenic characteristics

Twofold dilutions of the serum were tested for the presence and quantity of cross-neutralizing antibodies (against homologous and heterogeneous novel IBV strains as well as the deduced parental IBV strains 4/91 and LGX/100508) against a constant virus titer of 100 EID<sub>50</sub>. The results are shown in Table 2. The antigenic relatedness value (r, 64.4) calculated in this experiment confirmed the presence of an antigenic relationship between strains I0718/17 and I0724/17. The value of r obtained by comparing strains I0737/17 and 4/91 was relatively high (r, 48.6) but did not reach the 50% threshold for antigenic relatedness between strains. Strains 4/91 and LGX/100508 were not cross-neutralized by LGX/100508 and 4/91 antisera, respectively. The antisera against our four novel viruses did not neutralize LGX/100508, although LGX/100508 antiserum was highly neutralizing against our four IBV strains. Although some cross-reactivity was observed among the other viruses, the r values did not reach the 50% threshold, which suggested the absence of an antigenic relationship among these strains.

3.4. Pathogenicity in SPF chickens

Ten birds in each challenge group were observed for clinical signs until 32 dpc. None of the mock- or 4/91-infected birds exhibited any signs of disease. However, SPF chickens challenged with different IBV strains exhibited variable mortality rates and clinical manifestations. These birds exhibited respiratory signs starting at 3 dpc and continuing until 12 dpc, after which all birds recovered. Birds challenged with strains I0722/17, I0724/17, and I0737/17 showed only mild clinical signs, ranging from sneezing and coughing to head shaking; these signs were limited to the respiratory tract. In contrast, SPF chickens experimentally infected with strains I0718/17 and LGX/100508 exhibited

relatively severe respiratory signs represented by occasional mouth breathing. The duration and severity of the clinical responses induced by the six IBV strains were evaluated by scoring the respiratory signs (Fig. 5).

The results of analysis of the oropharyngeal and cloacal swabs collected at regular intervals showed that the IBV-challenged birds shed the challenge viruses from the respiratory tract between 4 and 24 dpc and from the cloaca between 4 and 32 dpc. Birds generally shed viruses for a longer duration from the cloaca than from the respiratory tract. In the present study, birds challenged with strains I0722/17, I0724/17, and LGX/100508 exhibited similar patterns of oropharyngeal and cloacal shedding. However, the durations of tracheal shedding of I0718/17 and both tracheal and cloacal shedding of I0737/17 were shorter than those of other viruses. At 32 dpc, the viruses were still detected in the cloacal swabs of some birds challenged with strains I0718/17, I0722/17, and I0724/17. Birds inoculated with I0724/17 shed the virus for a longer duration than did those inoculated with other viruses. No IBVs were detected in any of the swabs collected from the mock-infected birds at any time point. The results of virus recovery are presented in Fig. 6.

At 4 days of age, all birds in all challenge groups exhibited antibody titers below the cutoff values. At 12 days of age, IBV-specific antibodies were detected in nearly half of the birds in the groups challenged with strains I0718/17, I0722/17, I0724/17, and I0737/17. In the LGX/100508-challenge group, IBV seroconversion was detected in some and all birds at 8 and 12 days of age, respectively. No IBV-specific antibodies were detected in birds in the negative control group during the experiment.

3.5. Tissue tropism

Fig. 7A and B show the virus titers observed in the trachea and kidneys of IBV-challenged birds at 5 dpc. Overall, strains I0718/17, I0722/17, I0724/17, and LGX/100508 showed similar virus titers and replication efficiency in the trachea of experimentally infected chickens; the virus titers in these groups were relatively high, exceeding 3.2 log<sub>10</sub> in the trachea. In contrast, birds inoculated with IBV strains I0737/17 and 4/91 showed an obviously lower production of infectious virus particles in the trachea at 5 dpc. Strains I0718/17 and LGX/

**Table 1**  
Comparison of putative genes/genomic regions between the four novel IBV strains and two reference strains (4/91 and YX10).

Strain	5' UTR	Gene 1		S	Gene 3			M			Gene 5		N	3' UTR
		ORF1a	ORF1ab		3a	3b	3c	5a	5b					
10718/17	1-526 (526) <sup>a</sup>	527-12388 (11,862)	527-20421 (19,895)	20372-23869 (3498)	23869-24042 (174)	24042-24230 (189)	24217-24543 (327)	24515-25192 (678)	25556-25753 (198)	25750-25998 (249)	25941-27170 (1230)	27171-27677 (507)		
10722/17	1-525 (525)	526-12387 (11,862)	526-20420 (19,895)	20371-23868 (3498)	23868-24041 (174)	24041-24235 (195)	24216-24536 (321)	24517-25185 (669)	25545-25742 (198)	25739-25987 (249)	25930-27159 (1230)	27160-27666 (507)		
10724/17	1-528 (528)	529-12390 (11,862)	529-20423 (19,895)	20374-23871 (3498)	23871-24044 (174)	24044-24238 (195)	24219-24539 (321)	24520-25188 (669)	25548-25745 (198)	25742-25990 (249)	25933-27162 (1230)	27163-27669 (507)		
10737/17	1-525 (525)	526-12387 (11,862)	526-20420 (19,895)	20371-23868 (3498)	23868-24041 (174)	24041-24235 (195)	24216-24536 (321)	24517-25185 (669)	25545-25742 (198)	25739-25987 (249)	25930-27159 (1230)	27160-27666 (507)		
YX10	1-525 (525)	526-12387 (11,862)	526-20420 (19,895)	20371-23841 (3471)	23868-24041 (174)	24041-24229 (189)	24216-24542 (327)	24514-25191 (678)	25555-25752 (198)	25749-25997 (249)	25941-27169 (1230)	27170-27674 (505)		
4/91	1-528 (528)	529-12327 (11,799)	529-20363 (19,835)	20314-23808 (3495)	23808-23981 (174)	23981-24175 (195)	24156-24476 (321)	24457-25125 (669)	25485-25682 (198)	25679-25927 (249)	25870-27099 (1230)	27100-27606 (507)		

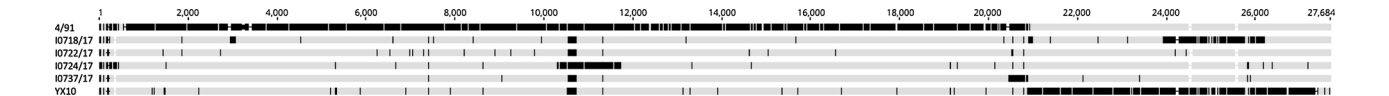
<sup>a</sup> Sizes of the putative genes/genomic regions are shown in parentheses. IBV, infectious bronchitis virus; M, membrane protein; N, nucleocapsid protein; ORF, open reading frame; S, spike protein; UTR, untranslated region.

100508 showed an obviously high affinity for both tracheal and kidney tissues, as reflected by their high titers in these organs. Interestingly, one of the birds infected with I0722/17 showed a relatively low viral titer in the kidneys, while a chicken challenged with I0724/17 showed a higher viral titer in the kidneys relative to the remaining birds in the same group (data not shown). In sharp contrast, in the 4/91-challenge group, chickens showed very lower viral titer in the kidneys.

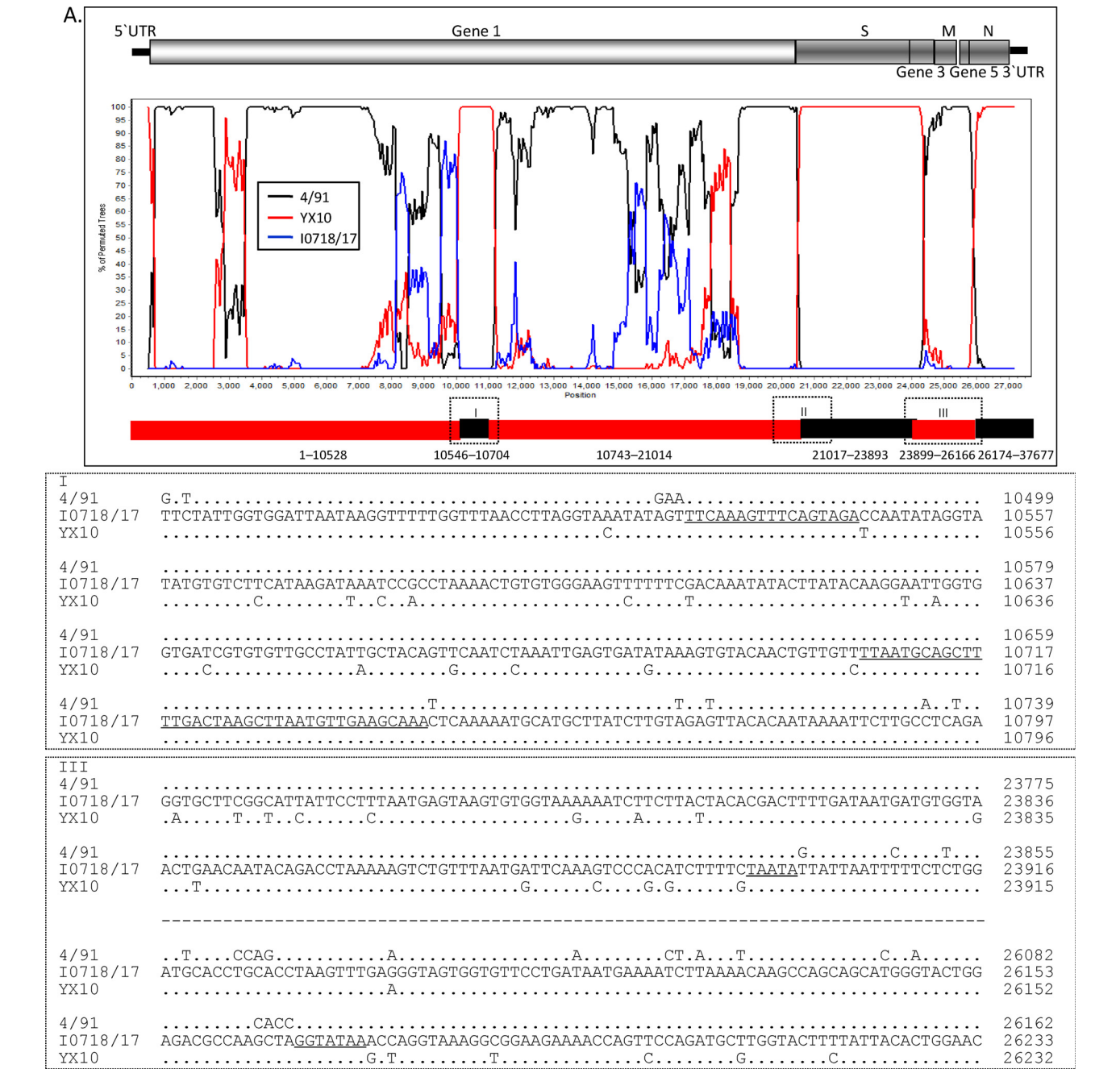
4. Discussion

Understanding the genetic and biological characteristics related to the antigenicity, pathogenicity, tissue tropism, and shedding of IBV isolates in chickens is important for better elucidating how this virus is evolving and contributing to the emergence of IBV variants that can alter the immune status conferred by regular IBV vaccines. It also helps address the processes of virus maintenance and circulation in chicken flocks. We, therefore, studied the genetic characteristics of four IBV isolates by analyzing their full-length genome sequences and also evaluated their persistence and biological properties. It has been reported that the side effects of the 4/91 vaccine might provide the basis for recombination with other strains. This phenomenon has been shown by several studies which have traced the emergence of many novel IBV strains to recombination events between the 4/91 vaccine and field strains (Feng et al., 2018; Han et al., 2017; Jiang et al., 2017, 2018; Liu et al., 2013; Mo et al., 2013; Ovchinnikova et al., 2011; Zhang et al., 2015, 2016; Zhou et al., 2017) in cases where the 4/91 vaccine strain could not provide effective protection against the field strains (Han et al., 2017), leading to coinfection of the vaccine and field strains in the same chickens and ultimately resulting in recombination. In this study, we performed complete genome sequence analysis of strains I0718/17, I0722/17, I0724/17, and I0737/17 and confirmed the occurrence of recombination events at the 5' ends of the S1 gene between a 4/91-like virus (GI-13 lineage) and a YX10-like virus (GI-19) (Xu et al., 2018), which contributed to the emergence of the IBV strains. In addition, we also identified recombination “hotspots” in other regions of the genome in strains I0718/17 and I0724/17. We, therefore, speculate that these two strains originated from multiple recombination events between 4/91-like and YX10-like IBV strains.

A potential concern regarding these recombination events is that the changes occurring in the S1 subunit during recombination have altered the neutralizing epitopes, resulting in the emergence of novel IBV serotypes. The S1 subunit of the S protein contains HVRs associated with serotype-specific and virus-neutralizing epitopes; these HVRs are present within amino acid residues 38–67, 91–141, and 274–387 (Wang and Huang, 2000). In the present study, strains I0718/17, I0722/17, and I0724/17 were found to show a significant clustering of substitutions in HVRs I and II and epitopes D and E in comparison with 4/91 strain; these strains also showed such significant clustering of substitutions in the remaining regions of the S1 domain—including HVR III and epitopes B/C/A—in comparison with strains YX10 and LGX/100508 (which differ by only two amino acids in the S1 region). These regions have previously been identified as the most variable genomic regions among different IBV serotypes (Cavanagh, 2007), and point mutations in these regions might have an effect on protein structure and polarity, possibly leading to changes in protein antigenicity. Such point mutations might also account for the decreased cross-neutralization activity between the two deduced parental strains (4/91 and LGX/100508) and the novel IBV strains evaluated in this study (I0718/17, I0722/17, and I0724/17), as evident from the absence of antigenic relatedness between the two groups. Upon comparing the r values between strains I0718/17, I0722/17, and I0724/17, we found that strain I0718/17 is serologically related to I0724/17. However, it was surprising to find that the other IBV pairs are not serologically related to each other, although the three viruses have very similar S1 gene sequences. IBV strains differing only by a few amino acid substitutions have been reported to show variations in antigenicity (Abdel-Moneim



**Fig. 3.** Alignment of the complete genome sequences of IBV strains I0718/17, I0722/17, I0724/17, I0737/17, YX10, and 4/91 by using MAFFT. Nucleotide sequence disagreement at indicated positions is shown in black, while nucleotide sequence agreement is shown in gray. The GenBank accession numbers for the six IBV strains are the same as those in Fig. 1. IBV, infectious bronchitis virus; MAFFT, Multiple Alignment with Fast Fourier Transformation.



**Fig. 4.** Bootscan analysis of the genomes of IBV strains. The y-axis shows the percentage of permutated trees using a sliding window of 1000-bp width centered on the plotted position, with a step size of 50 bp. IBV strains I0718/17 (A) and I0724/17 (B) were compared to strains 4/91 and YX10, respectively, and ck/CH/LJL/101150 was used as the query strain. The genome map is shown at the top of the plot, and the bars represent the relative positions of the coding regions for gene 1, spike protein, gene 3, membrane protein, gene 5, and nucleocapsid protein. The bars at the bottom of each plot represent the different sources of each of the deduced parental viruses. Alignments of the sequences surrounding the recombination sites are shown at the bottom of each plot. The sequence alignment boxes at the bottom of each Figure correspond to the boxes at the bottom of each bootscan. Nucleotide differences with respect to the genome sequences of I0718/17 (A) and I0724/17 (B) are shown. The given nucleotide positions are numbered with respect to I0718/17 (A) and I0724/17 (B). The sequences of the deduced recombination sites are underlined. bp, base pair; IBV, infectious bronchitis virus.

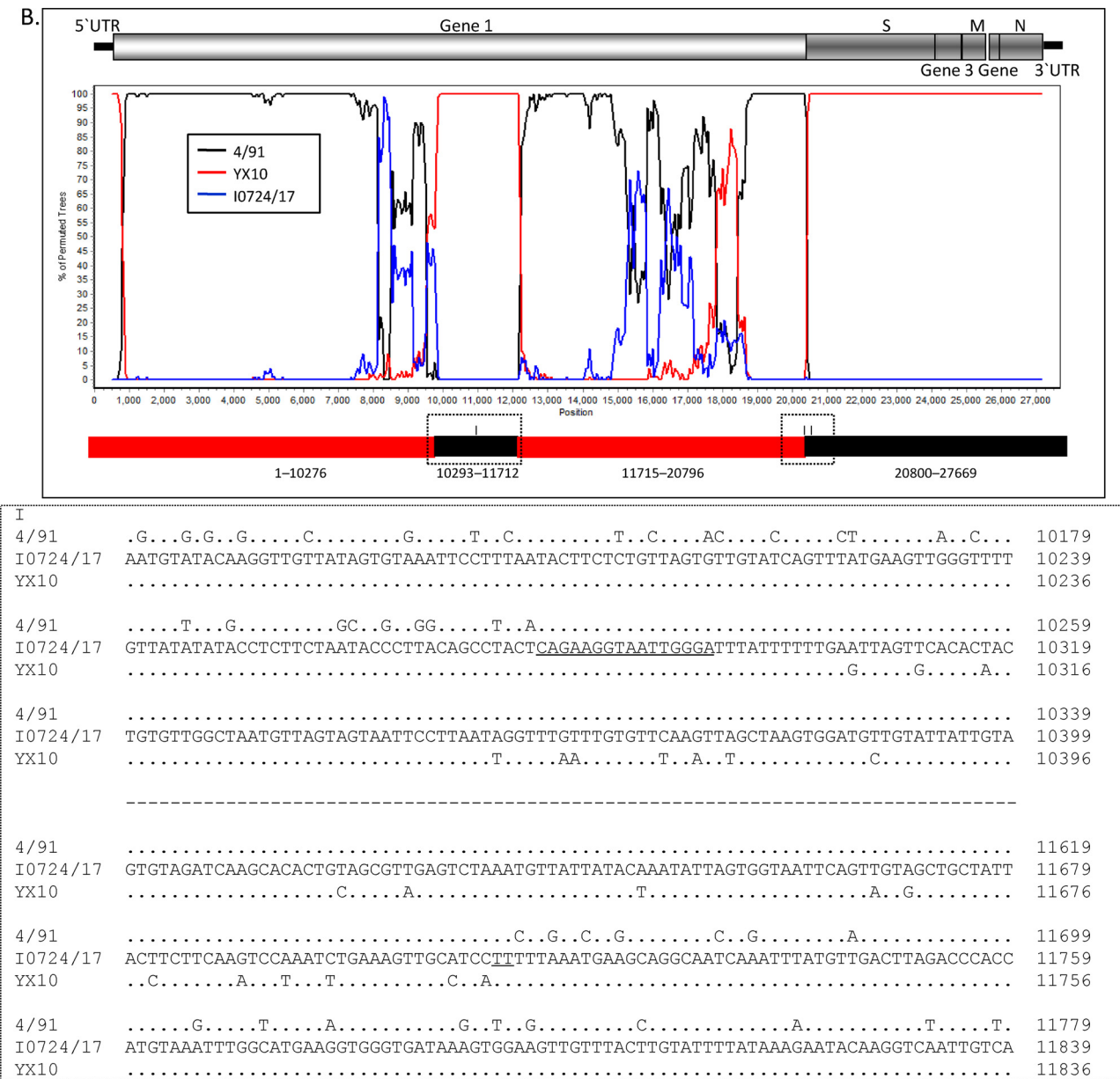


Fig. 4. (continued)

**Table 2**  
Serum titers in the reciprocal  $\beta$  virus neutralization tests (diluted serum, constant virus concentration).

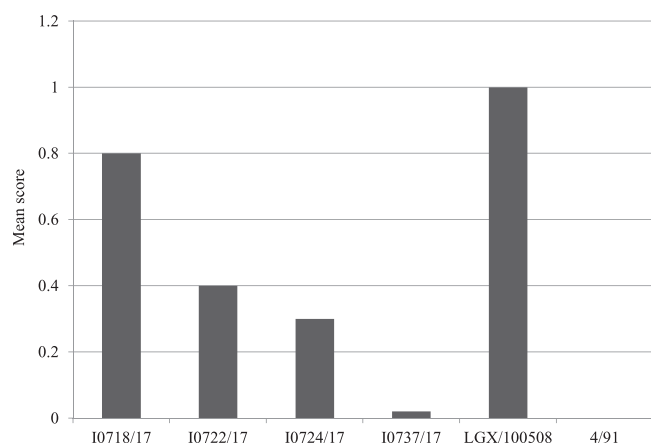
Strain	Serum					
	I0718/17	I0722/17	I0737/17	I0724/17	4/91	LGX/100508
I0718/17	205.1	60.5	20.7	83.3	51.3	90.5
I0722/17	45.3	455.1	35.8	45.3	90.5	149.1
I0737/17	181	142.2	724.1	198	396.2	113.8
I0724/17	209.4	104	64	227.5	183.5	183.5
4/91	4.5	45.3	88.6	45.3	205.1	< 2
LGX/100508	< 2	< 2	< 2	< 2	< 2	910.2

et al., 2006). However, strains I0722 and I0724/17 have identical S1 gene sequences. Similarly, we found a difference of only ten substitutions between strains I0737/17 and 4/91, nine of which were located at the N terminus of the S1 subunit (with only three being located in HVR I). The  $r$  value calculated from the cross-neutralization test was less

than 50, which implied that strain I0737/17 is not serologically related to 4/91. Contrary to this result, we have previously demonstrated that replacing the three HVRs of the avirulent Beaudette strain of IBV separately or simultaneously with the corresponding regions from a GI-19-lineage nephropathogenic strain by reverse genetics does not produce any changes in the serotype of the Beaudette strain (Shan et al., 2018). These results might suggest that the amino acid sequences of the HVRs were not the only factor accounting for the variations in antigenicity of IBVs.

Another potential concern regarding recombination events between vaccine and field strains is that recombination events leading to reversion to virulence might produce nephropathogenic and highly pathogenic recombinant strains. In the present study, all four IBV strains were pathogenic in comparison with the 4/91 vaccine strain, although they showed varying levels of pathogenicity in SPF chickens. Some of the strains showed a comparable affinity for replication in the kidneys as the GI-19 LGX/100508 strain. Coronavirus binding and entry into susceptible host cells requires interactions between cell-surface receptors and the viral attachment protein (S protein). As in other

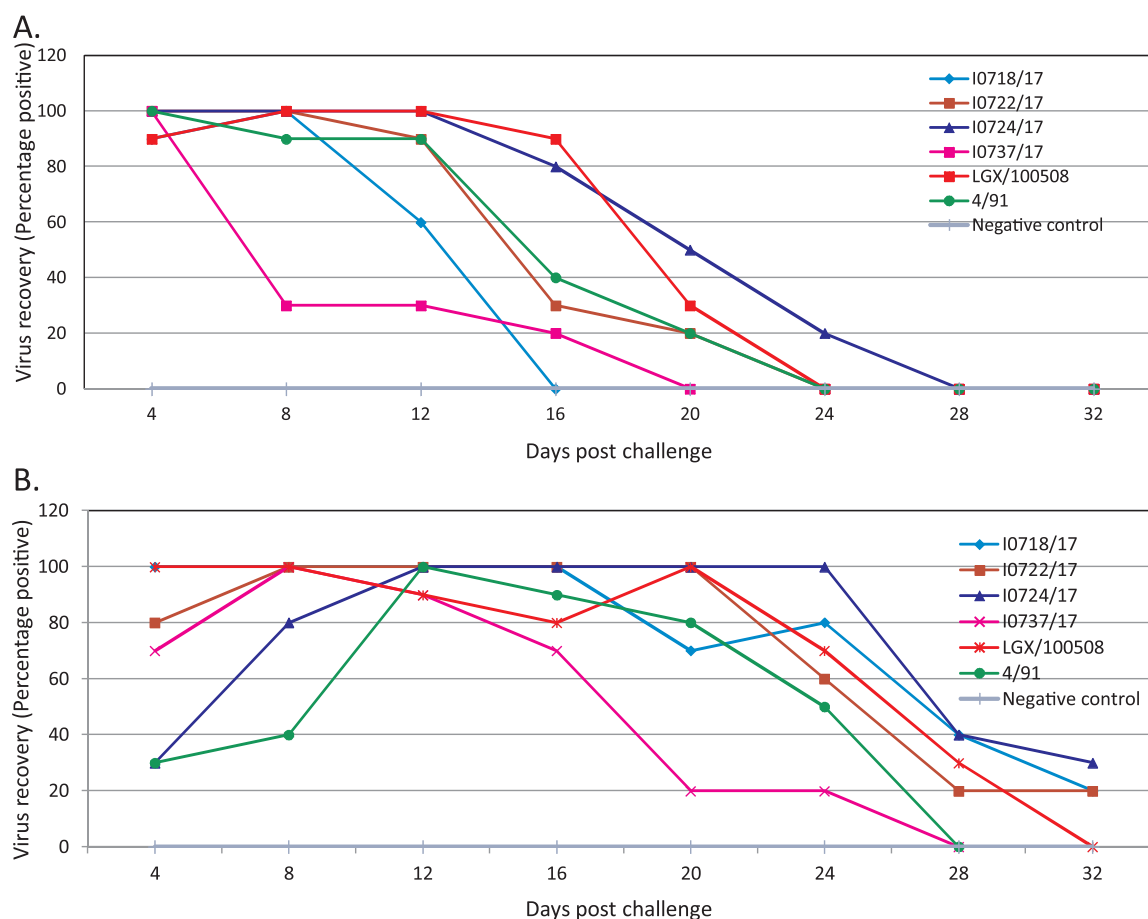




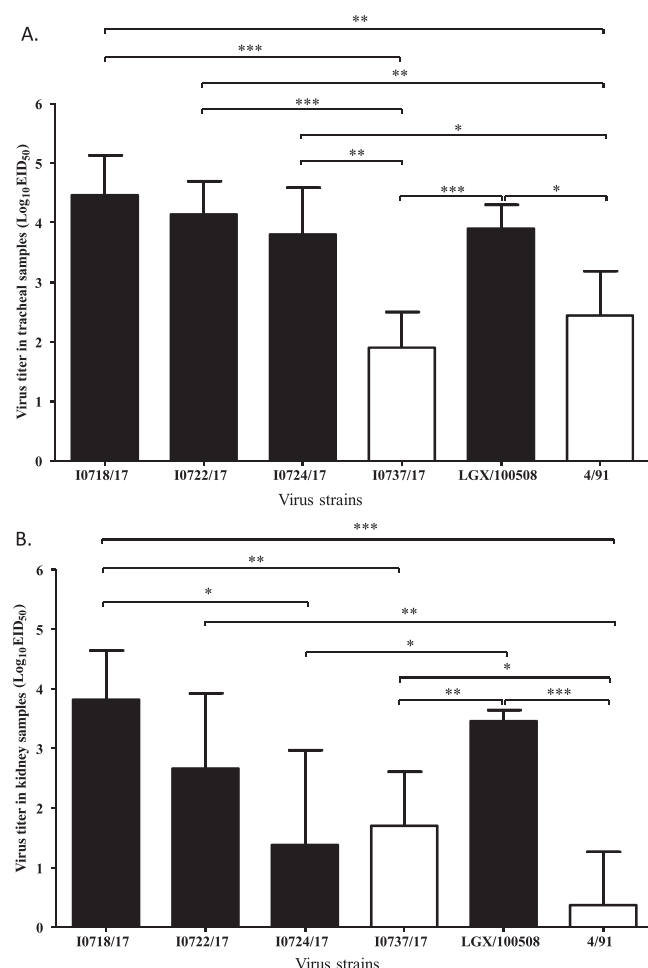
**Fig. 5.** Respiratory signs observed at 5 dpc with IBV strains LGX/100508, I0718/17, I0722/17, I0724/17, I0737/17, and 4/91. The mean score was calculated from individual scores based on the severity of respiratory signs observed in individual chickens. dpc, days post-challenge; IBV, infectious bronchitis virus.

coronaviruses, the S1 subunit of the S protein of IBV contains an RBD (Koch et al., 1990; Schultze et al., 1992; Spaan et al., 1988; Sturman et al., 1985). The location of the RBD in IBV genome has been mapped to the most N-terminal 19–272 amino acid residues of the S1 subunit (Promkuntod et al., 2014). Moreover, amino acids N38, H43, P63, and T69 appear to be critical for the binding of the S protein to host cells in the chicken respiratory tract (Promkuntod et al., 2014). In the present

study, although strains I0737/17 and 4/91 showed a difference of ten amino acid substitutions, only four were found to be located in the RBD domain. Moreover, when tested by means of experimental infection in SPF birds, these two viruses showed no drastic differences in their affinity for replication in the trachea or their pathogenicity. Although the C-terminal sequences of the RBD of strains I0718/17, I0722/17, and I0724/17 differed greatly from that of strain LGX/100508, all these viruses showed a similar affinity for replication in the trachea of infected chickens. These results suggest that these sequence exchanges might have no effect on the affinity of the viruses for the trachea, particularly for interaction with  $\alpha$ -2,3-sialic acid or other potential IBV receptors on the surface of cells in the respiratory tract of chickens (Shahwan et al., 2013; Winter et al., 2006). However, strain LGX/100508 was more pathogenic to SPF chickens than the other strains analyzed in the present study. Therefore, the point mutations observed in the RBD might have an effect on the affinity of the viruses for different tissues, rendering them highly pathogenic to SPF chickens. Interestingly, although strain I0718/17 differed from strains I0722/17 and I0724/17 by only six amino acid substitutions in the S1 protein, it showed a higher affinity for kidneys and was more pathogenic to SPF chickens. This indicates that these amino acid changes might affect receptor binding. Otherwise, the S1 protein of these IBV strains might not be the only factor responsible for their expression of pathogenicity. The binding of susceptible host cells is the first step in the viral life cycle, and host innate immune responses could also be a major contributing factor to the pathological outcome of IBV infection. In addition, we cannot exclude that the differences in pathogenicity are not due to other changes between the parental 4/91 strain and the



**Fig. 6.** Virus recovery from oropharyngeal (A) and cloacal (B) swabs from chickens challenged with IBV strains LGX/100508, I0718/17, I0722/17, I0724/17, I0737/17, and 4/91. Virus recovery was performed by inoculating 9-day-old embryonated, specific pathogen-free eggs through the allantoic route with supernatant from the swabs. IBV, infectious bronchitis virus.



**Fig. 7.** Replication of IBV strains LGX/100508, 10718/17, 10722/17, 10724/17, 10737/17, and 4/91 in chickens. Chickens were challenged with each of the IBV strains, and the trachea (A) and kidneys (B) from each bird were collected at 5 dpc for virus titration in eggs. Each bar represents the virus titer from an individual bird. dpc, days post-challenge; IBV, infectious bronchitis virus. Differences were considered significant if the p value was < 0.05 (\*p < 0.05, \*\*p < 0.01, \*\*\*p < 0.001).

recombinant viruses. By using reverse genetics, a previous report showed that the replicase gene of IBV is a determinant of pathogenicity (Armesto et al., 2009) and recent studies have shown that IBV pathogenicity is also due to the accessory proteins (Laconi et al., 2018; van Beurden et al., 2018). In order to establish the mechanisms, further study by reverse genetics and animal studies is needed to verify the association between changes in the genome sequence and the pathogenicity of IBV strains.

While all IBV strains appear to initially infect chickens through the respiratory tract, viremia enables the viruses to spread to secondary sites for further replication and persistence. It has been considered that the IBV strain replicates mainly in the respiratory tract, although it can also spread and replicate in a range of other tissues, such as the digestive and urogenital tracts. In this study, we found that IBV-infected birds shed the virus for a longer duration from the cloaca than from the respiratory tract; moreover, the birds shed the virus from the respiratory tract even after the clinical signs had disappeared. It is important to consider that, during an active IBV infection, even in the absence of clinical disease, and during the course of chronic and persistent infection, the gastrointestinal tract contains a significant amount of virus, and such persistently infected tissues are a likely source of re-infection in chickens, as reported previously (Alexander and Gough, 1978, 1977; Chong and Apostolov, 1982; Cook, 1968; Jones and

Ambali, 1987). In addition, our results from analysis of oropharyngeal and cloacal swabs showed that most of the challenged birds shed their challenge viruses from the digestive tract at 20 dpc, by which time the birds were positive for anti-IBV antibodies. This suggests that these IBVs had reached a somewhat persistent status despite the presence of systemic anti-IBV immunoglobulin G. Thus, these anti-IBV antibodies present in the serum seem to exert a non-complete protective role against IBV infection in SPF chickens, as reported previously (Santos Fernando et al., 2017).

Taken together, the results of our genetic and biological analyses clearly demonstrate that our four IBV strains, which emerged from recombination events between GI-13- and GI-19-like viruses, are antigenically different from their deduced parental viruses. They also show variations in their pathogenicity and affinity for the trachea and kidneys of chickens after infection.

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## Appendix A. Supplementary data

Supplementary material related to this article can be found, in the online version, at doi:<https://doi.org/10.1016/j.virusres.2019.01.007>.

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