A highly pathogenic GI-19 lineage infectious bronchitis virus originated from multiple recombination events with broad tissue tropism

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ABSTRACT

In the present study, an IBV strain I0305/19 was isolated from a diseased commercial broiler flock in 2019 in China with high morbidity and mortality. The isolate I0305/19 was clustered together with viruses in sublineage D of GI-19 lineage on the basis of the complete S1 sequence analysis. Isolate I0305/19 and other GI-19 viruses isolated in China have the amino acid sequence MIA at positions 110–112 in the S protein. Further analysis based on the complete genomic sequence showed that the isolate emerged through at least four recombination events between GI-19 ck/CH/LJS/120848- and GI-13 4/91-like strains, in which the S gene was found to be similar to that of the GI-19 ck/CH/LJS/120848-like strain. Pathological assessment showed the isolate was a nephropathogenic IBV strain that caused high morbidity of 100 % and mortality of 80 % in 1-day-old specific-pathogen-free (SPF) chicks. The isolate I0305/19 exhibited broader tropisms in different tissues, including tracheas, lungs, bursa of Fabricius, spleen, liver, kidneys, proventriculus, small intestines, large intestines, cecum, and cecal tonsils. Furthermore, subpopulations of the virus were found in tissues of infected chickens; this finding is important in understanding how the virulent IBV strains can potentially replicate and evolve to cause disease. This information is also valuable for understanding the mechanisms of replication and evolution of other coronaviruses such as the newly emerged SARS-CoV-2.

1. Introduction

Infectious bronchitis virus (IBV) is the etiological agent that causes infectious bronchitis (IB), which is an acute and highly contagious disease that affects chickens of all ages and leads to severe economic losses to the poultry industry, especially in terms of decrease in egg production, poor eggshell quality, reduced hatchability, increased feed conversion, and carcass condemnation at slaughter houses (Cavanagh, 2007), particularly when nephropathogenic strains or secondary infection is involved (Jackwood, 2012). Vaccines against IB are often used to reduce economic losses due to infection with field strains. However, the IB virus exists in a wide range of antigenically and genetically distinct types, and the continuous emergence of new genotypes, lineages, serotypes, and variants of IBV makes the prevention and control of this pathogen both complex and challenging.

Recently, a classification scheme based on the complete S1 sequence phylogenetic analysis categorized IBV strains into 36 lineages grouped in seven genotypes: GI-1 ~ GI-29, GII-1, GII-2, and GIII-1 ~ GVII-1 (Valastro et al., 2016; Chen et al., 2017; Jiang et al., 2017; Ma et al., 2019; Molenaar et al., 2020). GI-19 is the most widely distributed lineage worldwide. To date, the largest number of IBV strains in poultry producing countries originates from the GI-19 lineage (Valastro et al., 2016). The GI-19 strain, so-called QXIBV strain, was detected in China in 1996 when it was temporarily termed as glandular stomach-type IB strain due to the characteristic lesions in the glandular stomach of the infected chickens (Wang et al., 1998). Since then, several strains belonging to this lineage have been isolated and molecularly characterized from many cases of infection and designated as a new genotype, LX4 type; in China, these strains have been identified as nephropathogenic as they cause clinical nephritis and gross kidney lesions in infected specific-pathogen-free (SPF) chickens (Liu and Kong, 2004). According to a retrospective study, the first isolated GI-19 strain is the ck/CH/LHLJ/95I strain, which was isolated in 1995 from China (Zhao et al., 2017). However, a recently submitted sequence of an IBV strain 58HeN-93II (i.e., this strain was recently reported with accession number KC577395) shows that the lineage had originated in China in 1993. The GI-19 strain was shown to be the dominant IBV lineage in chicken flocks in China since it was detected (Liu and Kong, 2004; Zou et al., 2010; Han et al., 2011; Zhao et al., 2017; Xu et al., 2018; Fan et al., 2019).

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Since the first isolation in China, numerous reports have described the detection of GI-19 lineage in different countries and regions. In Europe, the first detection of GI-19 can be traced back to Russia (Far East and the European part) in 2001 (Bochkov et al., 2006), although some reports believed that the first detection was in the Netherlands between 2003 and 2004 (Worthington et al., 2008; Irvine et al., 2010). GI-19 viruses were also detected in France (Worthington et al., 2008; de Wit et al., 2018) and Germany in 2004 (Worthington et al., 2008); in Italy (Beato et al., 2005), the Netherlands (Worthington et al., 2008), and Slovenia in 2005 (Krapez et al., 2010); in Belgium (Worthington et al., 2008) and Poland in 2006 (Domanska-Blicharz et al., 2006); in UK in 2007 (Gough et al., 2008; Irvine et al., 2010; Valastro et al., 2010); in Sweden and Denmark in 2009 (Abro et al., 2012); in Switzerland (Sigrist et al., 2012) and Finland in 2011 (Pohjola et al., 2014); and in Hungary in 2014 (Kiss et al., 2015). Genetically related viruses were also detected in Poland (de Wit et al., 2018; Legnardi et al., 2019), Spain, Portugal (de Wit et al., 2018), and Greece (Andreopoulou et al., 2019) in recent years. Since the first detection in Europe, the incidence of infection with GI-19 has increased in many European countries, and GI-19 has become the predominant genotype (Worthington et al., 2008; Krapez et al., 2011; Ovchinnikova et al., 2011; de Wit et al., 2018).

The GI-19 lineage of IBV was also detected in other regions of Asia: North Asia such as in Japan in 1998 (JP-III) (Mase et al., 2004) and in Korea in 2003 (QX-like subgroup in K−II group) (Choi et al., 2009); South Asia such as Bangladesh (Bhuiyan et al., 2019); Southeast Asia such as Thailand (Pohuang et al., 2011), Malaysia (Khanh et al., 2017), and Indonesia (Wibowo et al., 2019); Central Asia such as Afghanistan (Sadri et al., 2019); and Middle East such as Iran (Hosseini et al., 2015; Ghalyanchilangeroudi et al., 2019) and Saudi Arabia (Alsultan et al., 2019). Epidemiological data showed that GI-19 was widespread and had become one of the dominant types in these countries. In addition, there were also reports of evidence of the circulation of GI-19-like IBV in African countries such as Zimbabwe (Toffan et al., 2011) and South Africa (Knoetze et al., 2014).

IBV GI-19 viruses were initially thought to be associated with proventriculitis outbreaks (Wang et al., 1998); however, with progress in time, it was demonstrated that these viruses are responsible for respiratory signs and nephritis (Liu and Kong, 2004; Benyeda et al., 2009; Khanh et al., 2018). Morbidity caused by IBV GI-19 viruses is 100 % and mortality is usually between 5% and 70 % (Liu and Kong et al., 2004; Benyeda et al., 2009; Choi et al., 2009; Zhong et al., 2016; Khanh et al., 2018; Xu et al., 2018; Li et al., 2019; Ren et al., 2019a,2019b; Yan et al., 2019). The present report describes the isolation and investigation of a highly pathogenic IBV strain (I0305/19) belonging to the GI-19 lineage. The genetic and pathogenic characteristics and the tissue tropism of the isolate I0305/19 were investigated.

2. Materials and methods

2.1. Virus isolation

Tissue samples of kidneys were collected from a diseased commercial broiler flock in June 2019 in Shandong province, China. The flock contained approximately 12,000 birds and had been immunized at the age of 7 days with H120 vaccine by coarse spray. Some of the broilers (∼5%) began to show very mild clinical signs at the age of 15 days. Sudden death of the broilers occurred at 16 days of age, and most of the deaths occurred at approximately 17–23 days of age, with high morbidity (up to 90 %) and mortality (∼58 %) rates. Gross examinations showed mild tracheitis and severe nephritis. Samples of kidneys from five diseased broilers were pooled together and tested negative for Newcastle disease virus (NDV) (Gohm et al., 2000) and avian influenza virus (AIV) subtypes H5 and H9 by reverse transcription-polymerase chain reaction (RT-PCR) (Chaharaein et al., 2009). However, the sample tested positive for IBV by RT-PCR (Liu et al., 2009).

The pooled kidneys were also processed for attempted virus isolation by inoculating into the allantoic cavity of 9-day-old SPF eggs (Liu and Kong, 2004). Five eggs were used for each passage. The allantoic fluid was harvested on day 3 post inoculation from three of the inoculated eggs, pooled, and subjected to examination by negative-contrast electron microscopy and detection of NDV and AIV by hemagglutination (HA) activity and of IBV by RT-PCR (Liu et al., 2009). The remaining two eggs were continuously cultured for another 4 days to detect typical lesions such as stunting and/or curling with feather dystrophy. The allantoic fluids were passaged three times until typical lesions of eggs were observed.

2.2. RNA extraction, RT-PCR, and genome sequencing

The allantoic fluid of IBV-infected eggs at passage level 3 was used for viral RNA extraction and genome sequencing. Briefly, viral RNA was extracted from 200 μL of infectious allantoic fluid by using the RNAiso Plus (Takara Bio Inc, Shiga, Japan) according to the manufacturer's instructions. Conventional RT-PCRs were performed to amplify the complete genomic sequence of the IBV isolate by using 14 sets of primers (Liu et al., 2013). The RT-PCR was performed using a commercially PrimeScript™ One Step RT-PCR kit (Takara) according to the manufacturer's instructions. The 3′/5′-UTRs of the viral genomes were sequenced using a SMARTer® RACE 3′/5′ kit (Takara) according to the manufacturer's instructions. The RT-PCR products were cloned into the pMD-18 T vector (Takara), and each fragment of the viral genome was sequenced at least three times.

2.3. Genotyping and comparison of S1 subunit

The complete S1 nucleotide sequence of the isolate I0305/19 was phylogenetically analyzed against 50 reference sequences (Supplemental Table 1), including three sequences of GI-1/Massachusetts (Mass) lineage (H120 vaccine, M41 and Beaudette strains), two sequences of GI-13 lineage (4/91 vaccine and ck/CH/LBJ/140402 strains), and 45 sequences of GI-19 lineage strains downloaded from the National Center for Biotechnology Information (NCBI) GenBank database (https://www.ncbi.nlm.nih.gov/genbank/). The 45 S1 sequences of the GI-19 lineage strains included 30 from strains isolated in China, six in Sweden, three in Thailand, three in Malaysia, two in France, and one in the Netherlands. The 30 S1 sequences of China GI-19 lineage strains included two to six sequences of viruses belonging to seven different sublineages (A–G) in GI-19 lineage (Xu et al., 2018). The S1 nucleotide sequence of the isolate I0305/19 was aligned, and a phylogenetic tree was constructed using the maximum likelihood method and the Jukes-Cantor model in Mega 6.0 program (Tamura et al., 2007) with 1000 bootstrap replicates for branch support. In addition, the S1 amino acid sequences of the isolate I0305/19 were pairwise compared with those of the 45 reference S1 sequences. The amino acid sequences of S1 fragment containing the critical receptor binding domain (RBD) of GI-19 virus that enable binding to the trachea and kidney of infected chickens (Bouwman et al., 2020) were also pairwise compared between the isolate I0305/19 and M41 and five GI-19 strains showing different pathogenicity to SPF chickens (Casais et al., 2003; Promkuntod et al., 2014; Leyson et al., 2016; Parsons et al., 2019).

2.4. Complete genomic sequence determination and analysis

The obtained sequences were assembled using BioEdit software version 4.0.6 (http://www.mbio.ncsu.edu/bioedit/bioedit.htmL); the consensus sequence was determined, and ORF predictions were performed using the ORF finder program (https:/www.ncbi.nlm.nih.gov/orffinder) by comparing with that of the GI-19 representative strain LX4 (AY338732) (Liu and Kong, 2004). The complete genomic sequence of the isolate I0305/19 was then phylogenetically compared using the maximum likelihood method in Mega 4.0 program with 87 reference

sequences, including three sequences of GI-1/Mass H120, M41, and Beaudette strains; two sequences of GI-13 (4/91) and ck/CH/LBJ/140202 strains; and 82 sequences of GI-19 lineage strains downloaded from the NCBI GenBank database (https://www.ncbi.nlm.nih.gov/genbank/). The 82 reference sequences of the GI-19 lineage were genetically closely related to that of I0305/19 (> 93.0 % identity) selected by BLAST using the complete genomic sequence of the isolate I0305/19.

2.5. Recombination analysis

The complete genomic sequence of the isolate I0305/19 was first compared and aligned with those of 19 IBV strains, including 4/91 and H120 vaccine strains, and 17 GI-19 lineage strains selected on the basis of the results of complete genomic sequence phylogenetic trees by using multiple alignment with fast Fourier transformation (MAFFT) algorithm (Katoh et al., 2002) available at the EMBL-EBI website (http://www.ebi.ac.uk/Tools/msa/mafft/). After clustering, the complete genomic sequence of the isolate I0305/19 was aligned with that of the IBV strains ck/CH/LJS/120848 (LJS/120848) and 4/91 vaccine by using the MAFFT algorithm, and the sequence alignment was then introduced into similarity plot (SimPlot) (http://sray.med.som.jhmi.edu/SCRopftware/simplot/) to confirm the recombination events in the genome of the isolate I0305/19, with a window size and step size of 1000 and 50 nucleotides, respectively. The complete genomic sequence of I0305/19 was used as a query. Lastly, pairwise comparison of the complete genomic sequence of the isolate I0305/19 was performed with those of the IBV LJS/120848 and 4/91 strains to identify the recombination breakpoints.

2.6. GenBank accession number

The complete genomic sequence of the isolate I0305/19 was deposited into GenBank with accession number MN794188.

2.7. Assessment of pathogenicity

Experiments involving chickens were conducted at the Harbin Veterinary Research Institute under the approval of the Ethical and Animal Welfare Committee of the Heilongjiang province, China (no. HSY-IACUC-2019−129). Sixty 1-day-old SPF chicks were divided equally into two groups, each of which was housed in a separate positive-pressure isolator. At 1 day of age, 30 birds in group 1 were inoculated intranasally and intraocularly with 0.1 mL of the isolate I0305/19 containing 10^5 50 % egg infectious dose (EID_{50}). Birds in group 2 were mock inoculated with PBS through the same routes as birds in group 1. Ten birds from each group were randomly marked and humanely killed on day 4 post-inoculation (pi), and tissues of tracheas, lungs, bursa of Fabricius, spleen, liver, kidneys, proventriculus, small intestines (duodenum, jejunum, and ileum), large intestines (cecum, large intestinal segment connecting the ileocecal junction with the cloaca), cecum, and cecal tonsils were carefully removed from the surrounding tissues and used for viral titration in 9-day-old SPF embryonated eggs (Han et al., 2018). Viral titers were expressed as an EID_{50}. At least five eggs were used for each dilution, and the EID_{50} values were calculated using the method of Reed and Muench (1938). Blood samples were collected on 4, 8, 12, 16, and 20 days post-inoculation (dpi) from the remaining birds in the two groups and used for antibody detection (ELISA; IDEXX Corporation) according to the manufacturer's instructions. The clinical signs and mortality were investigated daily. The birds were examined daily for signs of infection for a period of 30 days.

2.8. Immunohistochemistry

Tissue samples of tracheas, lungs, bursa of Fabricius, spleen, liver, kidneys, proventriculus, small intestines, large intestines, cecum and cecal tonsils of the dead chickens were fixed in 10 % neutral buffered formalin, embedded in paraffin wax, and sectioned. Immunohistochemistry (IHC) was performed on serial sections prepared from the tissue samples (Ren et al., 2019a,2019b). The tissue sections were dewaxed and incubated in citrate buffer (pH 6.0) in a microwave oven at 700–800 W for 10 min and then at 200–300 W for 30 min. Endogenous peroxidase activity was blocked with 3 % H_2O_2 solution. IBV was detected with the monoclonal antibody 6D10, which is directed against the N protein (Han et al., 2013). Antibody binding was detected using a peroxidase-labeled goat secondary antibody (Santa Cruz Biotechnology Inc., Santa Cruz, CA). The sections were washed with 0.01 M PBS. A substrate working solution (Boster, Wuhan, China) was subsequently applied to the sections. Normal bovine serum albumin was used as a control substitute for the monoclonal antibody.

2.9. Sequencing of the S1 gene from tissues of I0305/19-infected chickens

Eleven tissue samples (tracheas, lungs, bursa of Fabricius, spleen, liver, kidneys, proventriculus, small intestines, large intestines, cecum and cecal tonsils) were collected from each of the 10 I0305/19-infected chickens and total RNA was individually extracted by using the RNAiso Plus (Takara). The S1 gene was amplified by conventional RT-PCRs with primers S1Oligo5′ (Kwon et al., 1993) and S1Oligo3′N (5′-CATA ACTAACATAAGAGCAAC-3′). Sanger sequencing was directly performed (Big Dye Terminator) on the RT-PCR product using the primers S1Oligo5′ and S1Oligo3′N.
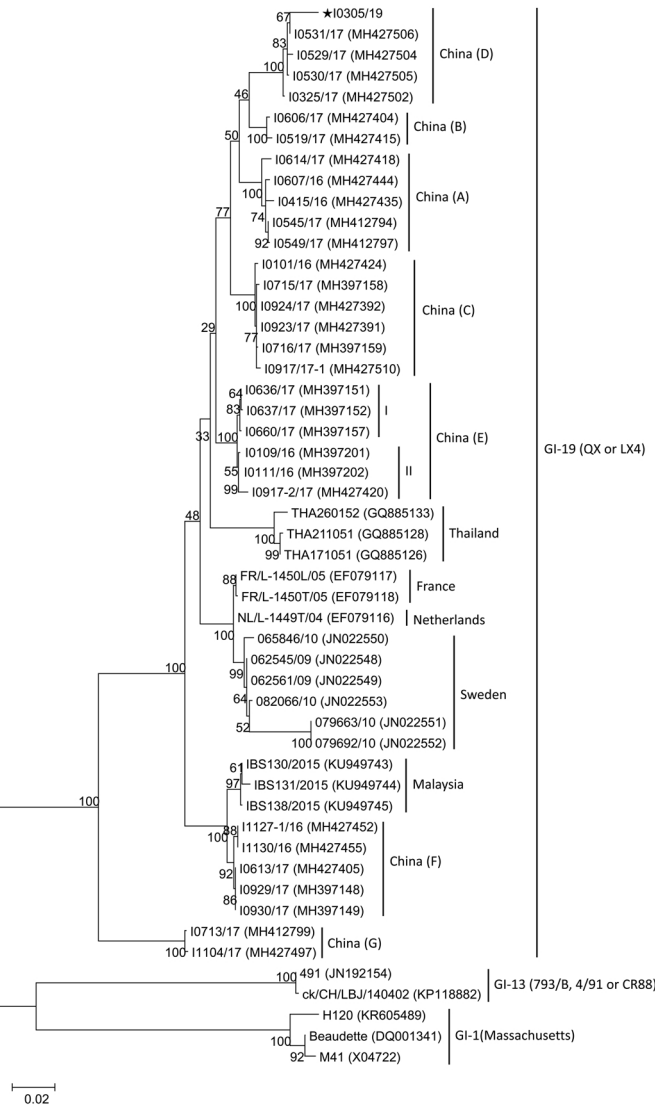
3. Results

3.1. IBV I0305/19 was isolated from the diseased broilers and grouped to the GI-19 lineage

Both the pooled sample of kidneys and the collected allantoic fluids from each passage were found to be negative for NDV and AIVs according to the results of RT-PCR, HA, or morphology of the virus particles. However, the pooled kidney was positive for IBV in RT-PCR analysis, and an IBV, designated as I0305/19, was isolated from the sample using 9-day-old SPF embryonated chicken eggs.
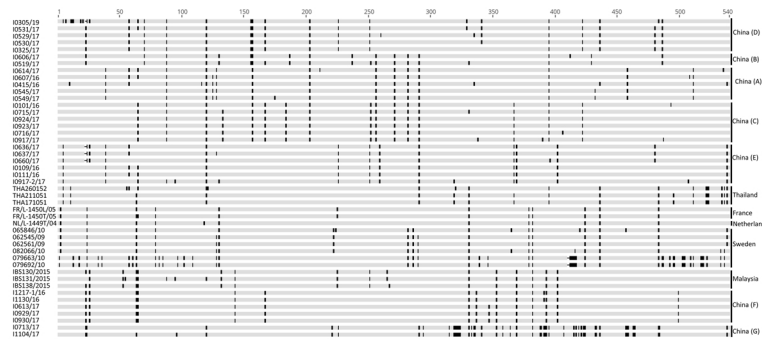
The topologies of phylogenetic trees constructed using the maximum likelihood method and the Jukes-Cantor model with the complete S1 nucleotide sequences of the isolate I0305/19 and 50 reference strains were very similar. The results of the maximum likelihood method are shown in Fig. 1A. The isolate I0305/19 was clustered within the GI-19 lineage and showed a closer genetic relationship with viruses in sublineage D than with those in other sublineages (Xu et al., 2018). Moreover, the isolate I0305/19 shared the same unique amino acid substitutions in S1 subunit with those of IBV strains in sublineage D, which were different from those of other sublineages (Fig. 1B). The isolate I0305/19 showed a high degree of nucleotide and amino acid identities of 98.1 %–98.4 % and 97.1%–97.8 %, respectively, with viruses of sublineage D, in contrast to 89.5 %–95.7 % and 87.5 %–94.3 %, respectively, with viruses of other sublineages. The results also demonstrated that GI-19 strains isolated in European countries clustered together, although viruses isolated in one European country showed closer relationship than those in other countries. In contrast, GI-19 viruses isolated in other Asian countries were closely genetically related to viruses isolated in China; furthermore, viruses isolated in Malaysia were closely related to viruses in sublineage F, and viruses isolated in Thailand showed a close relationship with viruses in sublineages A–E. In addition, amino acid sequence alignment showed that the isolate I0305/19 exhibited intensive substitutions in the 30-amino acid region at the N terminal of S1 subunit.

Isolate I0305/19 and other GI-19 viruses isolated in China have the amino acid sequence MIA at positions 110–112 in the S protein. However, the GI-19 CK/SWE/0658946/10 strain has the KIP and GI-19

A.

B.

C.

Fig. 1. Comparison of the S1 gene of the isolate I0305/19 with those of other reference strains. A phylogenetic tree was constructed with the complete S1 nucleotide sequences of the isolate I0305/19 (★) and 50 reference strains using the maximum likelihood method in Mega 6.0 program with 1000 bootstrap replicates for branch support (A). The GenBank accession number of each strain is indicated in parentheses. The complete S1 amino acid sequence of the isolate I0305/19 was compared with those of the 45 GI-19 lineage reference strains using MAFFT version v6 (B). The GenBank accession number of each strain is the same as that shown in Fig. 1A. Alignment of the amino acid sequences of the S1 fragment among M41, CK/SWE/0658946/10, I0305/19, and 4 other GI-19 viruses were shown (C). This fragment contained the critical amino acids (*) that were previously identified in the GI-19 CK/SWE/0658946/10 strain that enable binding of the virus to the kidney of infected chickens. The numbers on the right of each alignment show the amino acid positions in the S1 subunit of the spike protein of each virus. The sequences of M41 are listed, and only amino acids differing from those of M41 are depicted.
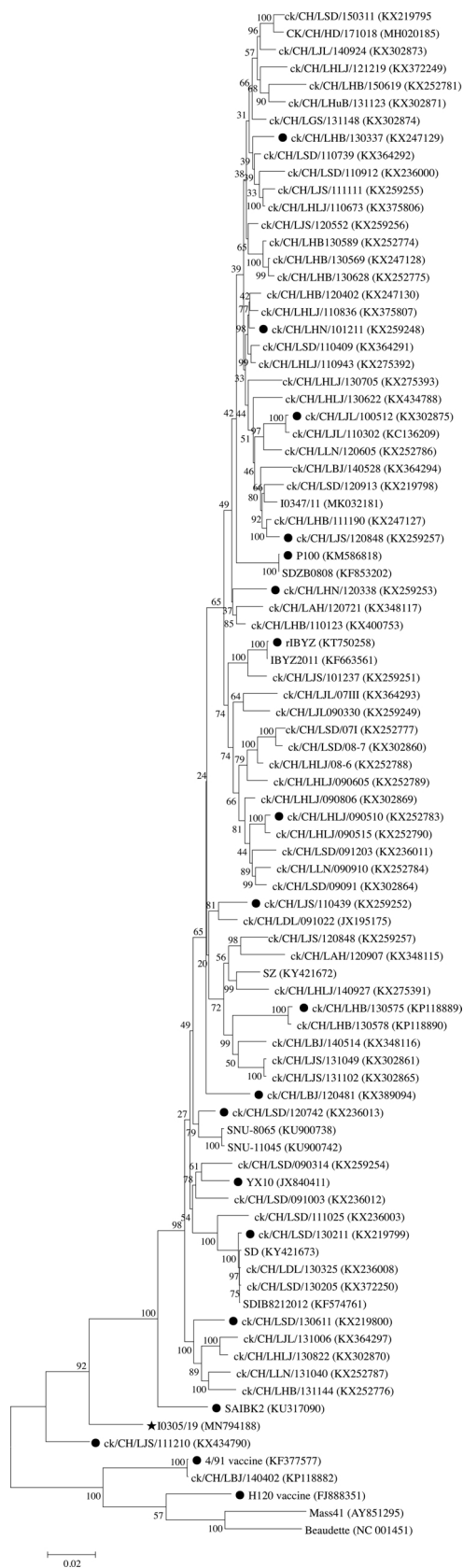
Fig. 2. Phylogenetic analyses based on the complete genomic sequence of the isolate I0305/19 with those of 87 reference IBV strains. The maximum likelihood method in Mega 4.0 program was used with 1000 bootstrap replicates. The complete genomic sequences used for further comparison with multiple alignment with fast Fourier transformation (MAFFT) algorithm are indicated by black circles.

strains isolated in South Korea have MIE at positions 110–112 in the S protein (Fig. 1C).

3.2. Isolate I0305/19 was derived from multiple recombination events

The complete genomic sequence of the IBV isolate I0305/19 was 27,607 nucleotides in length, excluding the poly A tail. At least 10 ORFs (5′ UTR-1a-1ab- S-3a-3b-E-M-5a-5b-N-3′-UTR) were predicted in the genome. A phylogenetic analysis using the complete genomes of 87 IBV strains was performed to investigate the genetic relationship with the sequence of isolate I0305/19 (Fig. 2). The isolate I0305/19 showed a close relationship with IBV GI-19 lineage strains, especially the strains LJS/111210 (94.0 %) and SAIBK2 (92.9 %). The complete genomic sequence-based MAFFT alignment of the isolate I0305/19 and those of the 4/91, H120 and 17 GI-19 linage strains selected based on the phylogenetic trees provided five fragments of I0305/19 that were changing identity with the reference strains 4/91 and LJS/120848 (Fig. 3A). This result was confirmed by the SimPlot analysis, which showed that the isolate I0305/19 was possibly derived from recombination events of the potential parental LJS/120848- and 4/91-like strains (Fig. 3B). Four crossover points were found: the first was located at the 3′ end of nsp2 (between 2374 and 2391 nucleotide positions), the second at the 3′ end of nsp4 (between 8562 and 8565), the third at the 3′ end of nsp15 (between 19,205 and 19,207), and the last at the 3′ end of the S gene (between 20,391 and 20,418) (Fig. 3C).

3.3. Isolate I0305/19 was highly pathogenic to the 1-day-old SPF chickens

When the IBV isolate I0305/19 was inoculated intraocularly and intranasally into the 1-day-old SPF chickens, approximately one-third of the animals developed very mild clinical signs at 2 dpi; the birds showed clinical signs with occasional mouth breathing, head shaking, and head twitches. All birds showed clinical signs at 3 dpi (100 % morbidity), although the signs were still very mild. Some birds began to die at 4 dpi, and the mortality was up to 80 % (Fig. 4A). Gross lesions were detected in the kidneys of the dead birds, which showed swollen and pale kidneys with urate deposition in the organ and ureters (Supplemental Fig. 1). Mild catarrhal exudate in the nasal cavity were detected in two-thirds of the dead birds. The mild clinical signs lasted approximately 8 days. No clinical signs were found in the negative control group. No birds were positive for antibody against IBV at 4 dpi, and nearly half of the birds and all the birds were positive for antibody against IBV at 8 and 12 dpi onward, respectively. All the birds were negative for antibody against IBV in the negative control group.

3.4. Isolate I0305/19 showed broader tissue tropism in the infected SPF chickens

Two criteria were used for evaluating tissue tropism of the isolate I0305/19 in the infected chickens. First, the viral titers were tested in 11 tissues, including tracheas, lungs, bursa of Fabricius, spleen, liver, kidneys, proventriculus, small intestines, large intestines, cecum, and cecal tonsils, by using 9-day-old SPF chickens, and the total amount of infectious viruses recovered from the entire organ were calculated. As shown in Fig. 4B, the $\log_{10}$ titers of infectious virus were below 2.0 only in the spleens and livers of the infected chickens. In contrast, the $\log_{10}$ titers of the challenge virus in the remaining nine organs were 3.0 or greater. No virus was recovered in the tissues of negative control chickens. Second, the 11 tissues of the dead chickens were subjected to IHC for detecting the presence of IBV antigen using a monoclonal antibody directed against the N protein. As shown in Fig. 5, the IBV antigen was positive in seven of the 11 selected tissues, including trachea, the secondary bronchus in the lung, bursa of Fabricius, spleen, kidneys, small intestines, large intestines, and cecal tonsils. No IHC-positive staining for IBV antigen was observed in the tissues of birds in the negative control group.
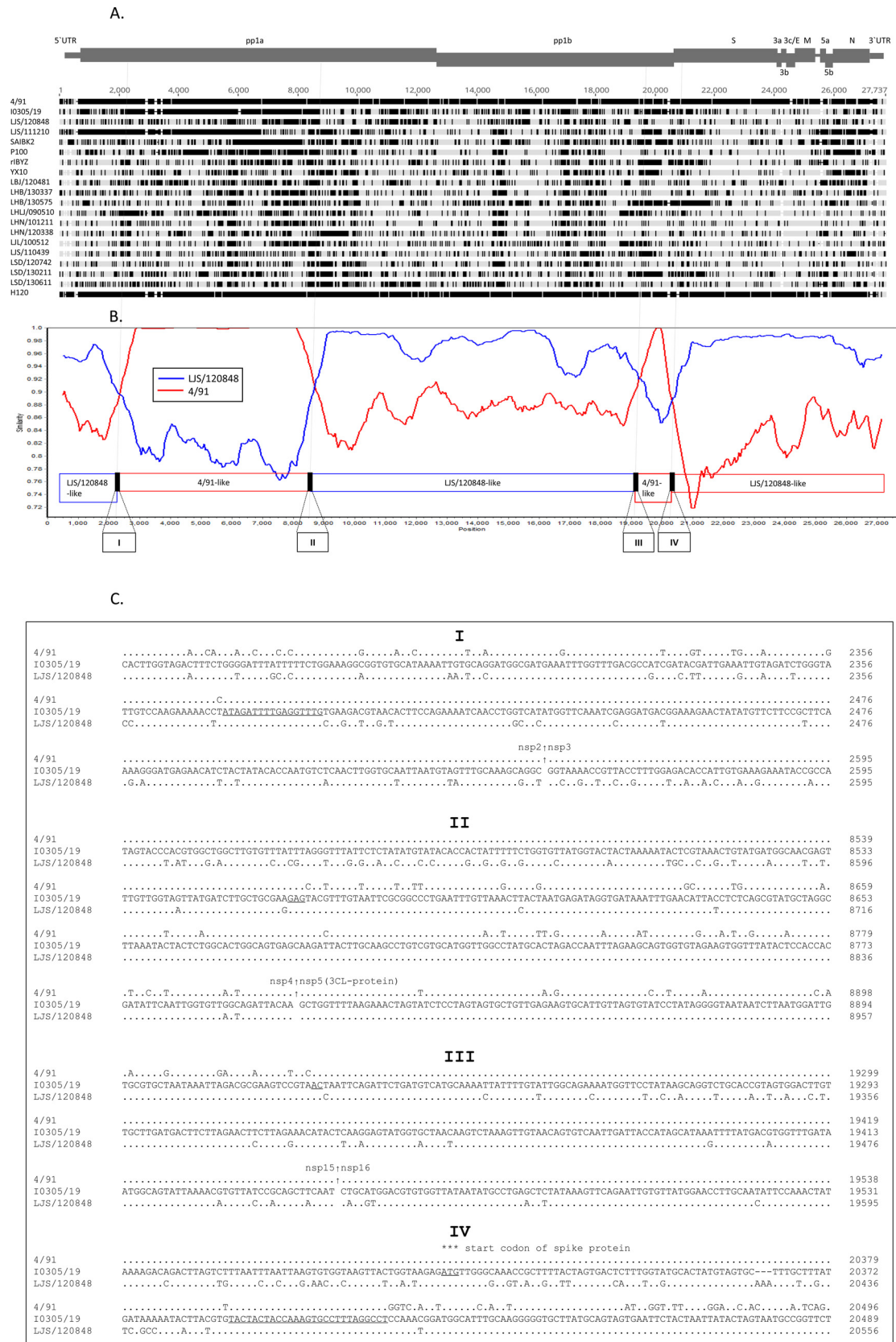
Fig. 3. Recombination event analysis of the isolate I0305/19. The complete genomic sequence of the isolate I0305/19 was compared with those of 19 IBV strains on the basis of the results of complete genomic sequence phylogenetic trees by using multiple alignment with fast Fourier transformation (MAFFT) algorithm (A). The black lines indicate differences. The genomic scale and the genes/fragments are shown at the top of the plot. Sequence alignment was introduced into similarity plot (SimPlot) to confirm the recombination events in the genome of the isolate I0305/19 (B), with a window size and step size of 1000 and 50 nucleotides, respectively. Pairwise comparison of the complete genomic sequence of the isolate I0305/19 was performed with those of the IBV ck/CH/LJS/120848 strain and 4/91 vaccine strain (C). Regions I, II, III, and IV correspond to those indicated in Fig. 3B.
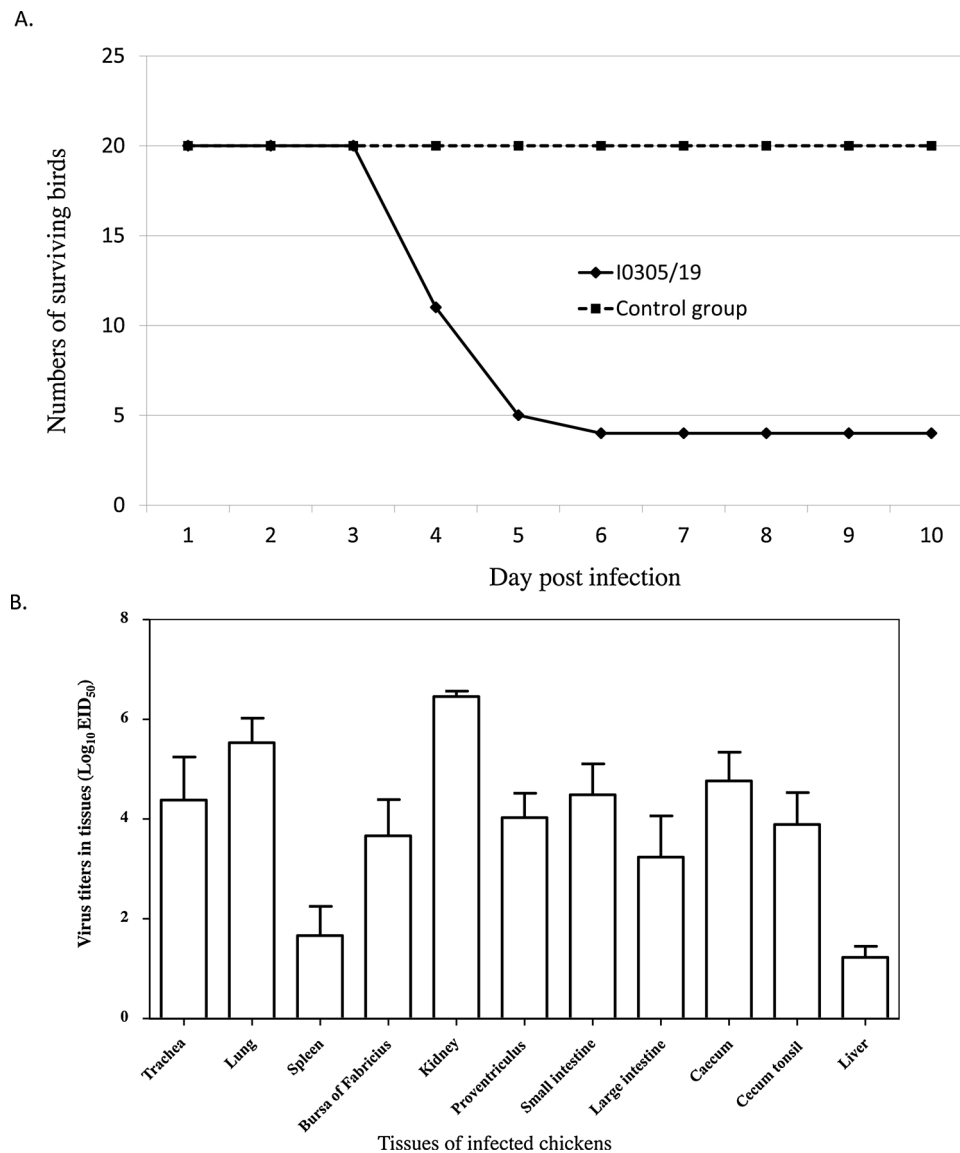
A.

B.

Fig. 4. Pathogenicity of the isolate I0305/19 in chickens. One-day-old chicks were intranasally and intraocularly inoculated with 10^5 EID$_{50}$ virus, and the number of survived birds was recorded daily (A). Replication of the isolate I0305/19 in chickens (B). One-day-old chickens were challenged with the isolate I0305/19, and the tracheas, lungs, bursa of Fabricius, spleen, liver, kidneys, proventriculus, small intestines, large intestines, cecum, and cecal tonsils were collected at 4 dpi for virus titration in embryonated eggs.

3.5. Subpopulations of virus were found in tissues of infected chickens

The sequence of S1 genes of the virus from nine tissues, including tracheas, lungs, bursa of Fabricius, kidneys, proventriculus, small intestines, large intestines, cecum, and cecal tonsils of infected chickens, was amplified (the S1 gene had not been amplified from the liver and spleen by using the primers S1Oligo5′ and S1Oligo3′N), and the amplified PCR products were directly sequenced and compared with those of the isolate I0305/19. The results showed that the sequence of the S1 gene of the challenge I0305/19 virus had not changed in the kidneys and cecal tonsils of all the 10 infected chickens after replication of virus. However, there were five differences between the S1 nucleotide sequences of the challenge I0305/19 strain and the virus isolated from tissues of some infected chickens (Fig. 6). Of these five changes, one major nucleotide peak was found at position 278 in the S1 gene of the virus from the bursa of Fabricius and small and large intestines of two infected birds (the first and sixth birds), which resulted in a change of Phe93 to Ser93. Another major nucleotide peak was observed at position 353 in the virus from proventriculi and lungs of two infected birds

(the fourth and eighth birds), which led to a change of Gly118 to Asp118. The sequences of the S1 gene had not changed in the remaining nine tissues of most of the infected chickens. These results suggested mixed subpopulations after replication of the challenge I0305/19 virus in some of the infected chickens.

4. Discussion

On the basis of the sequence of the S1 gene, the isolate I0305/19 was most closely related to strains of sublineage D of genotype I lineage 19 (Valastro et al., 2016; Xu et al., 2018) and showed ≥98.1 % nucleotide and ≥97.1 % amino acid sequence identities. The GI-19 lineage is believed to be originated from China (Franzo et al., 2017), extensively spread to different regions of the country, and rapidly became the predominant IBV genotype in China since its first report (Wang et al., 1998; Liu and Kong, 2004; Zou et al., 2010; Han et al., 2011; Zhao et al., 2017; Xu et al., 2018; Fan et al., 2019), possibly because of the unavailability of antigenically matched vaccine that could likely prevent the occurrence of outbreaks. In the present study,
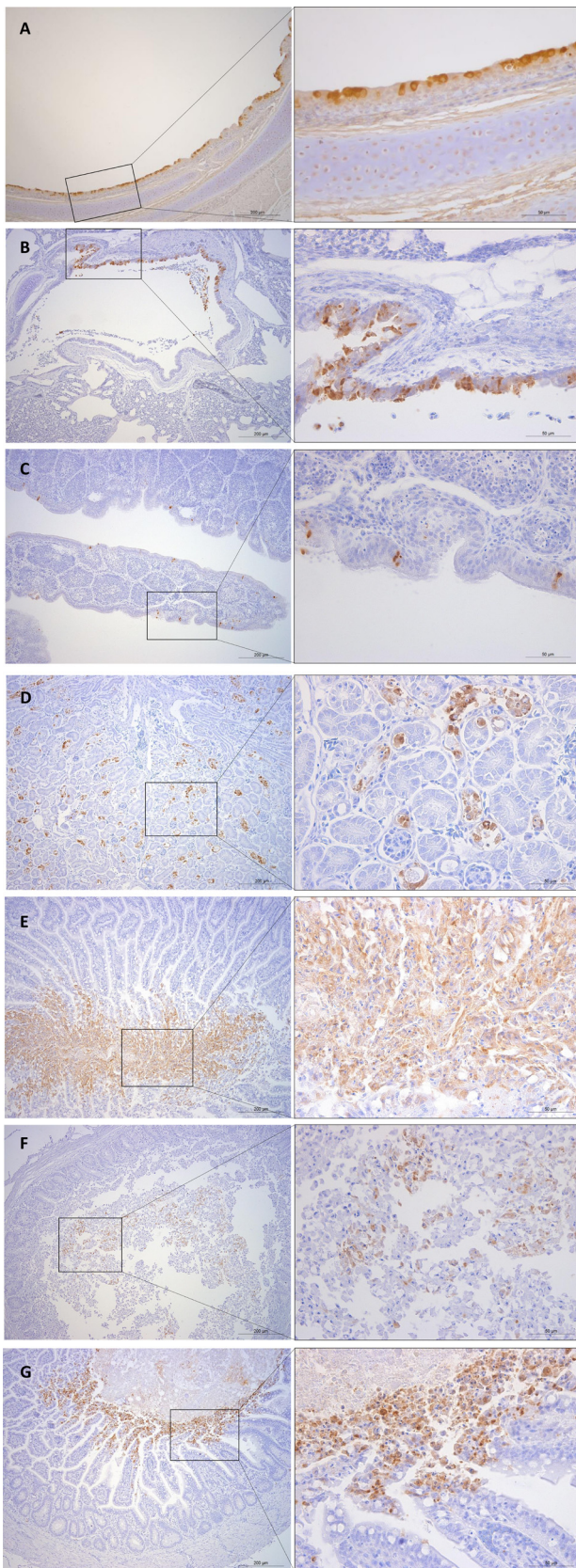
Fig. 5. Immunohistochemical staining of the IBV isolate I0305/19-infected chickens. Tissues of dead chicks were collected at 4 days post infection. Immunohistochemical staining detected virus antigens (brown color signals) in the trachea (A), lung (B), bursa of Fabricius (C), kidneys (D), small intestine (E), large intestine (F), and cecal tonsils (G) by IBV N protein-specific monoclonal antibody.

we found multiple substitutions at the N terminal of the S1 subunit in the isolate I0305/19 compared to those of other GI-19 viruses. It remains to be investigated whether these substitutions have resulted in antigenic differences and even pathogenic changes.

IBV causes mainly a contagious upper respiratory tract disease, with morbidity of typically 100 % and mortality reaching 50 % when infected with some strains that cause nephritis or opportunistic infection (Jackwood, 2012). GI-19 field strains showed different levels of virulence to SPF chickens in experimental infections. Some strains isolated in China were nonpathogenic to SPF chicken (Yan et al., 2017), while others showed low to high pathogenicity to SPF chickens, with morbidity ranging from 40 % to 100 % and mortality ranging between 10 % and 80 % (Table 1) (Liu and Kong, 2004; Zhong et al., 2016; Yan et al., 2017; Xu et al., 2018; Li et al., 2019; Ren et al., 2019a,2019b). The GI-19 virus strain isolated in Malaysia was also highly virulent to SPF chickens with 100 % morbidity and 41.3 % mortality (Khanh et al., 2018). Comparatively, GI-19 viruses isolated in South Korea, France, Slovakia, Greece, and Hungary induced only clinical signs in some or all the infected birds with 70 %–100 % morbidity (Benyeda et al., 2009; Choi et al., 2009). The isolate I0305/19 caused relevant pathological alterations in commercial broilers that resulted in a high mortality of these birds in the field. The experimental infection of SPF chickens confirmed this results and suggested that the isolate I0305/19 was a highly pathogenic IBV strain that was more virulent than other GI-19 strains isolated thus far. However, these results should be taken with caution because birds with different ages and numbers, and challenge viruses with different doses and routes were used in different studies.

An initial report showed that GI-19 viruses were associated with proventriculitis outbreak (Wang et al., 1998); however, with progress in time, it was demonstrated that the viruses cause respiratory signs, nephritis, and reproductive disorders. Coincidentally, no obvious gross lesions were observed and viral antigens were not detected by IHC in the proventriculi of chickens infected with the isolate I0305/19, although the isolate I0305/19 can replicate efficiently in the proventriculi by viral titration using 9-day-old SPF chicken eggs. In addition, only very mild respiratory signs were observed in both the commercial broilers and the SPF chickens infected with isolate I0305/19; however, other reports demonstrated that the GI-19 viruses could induce obvious respiratory signs (Liu and Kong et al., 2004; Benyeda et al., 2009; Choi et al., 2009; Zhong et al., 2016; Khanh et al., 2018; Xu et al., 2018; Li et al., 2019; Ren et al., 2019a; Yan et al., 2019). The isolate I0305/19 can replicate efficiently in both the trachea and lungs in the infected SPF chickens, which was demonstrated by viral titration in SPF chicken eggs and viral antigen detection by IHC. A previous study also showed that some of the nephropathogenic IBV strains are less pathogenic for tracheal tissues (Fernando et al., 2013, 2017) despite the epithelium of the trachea being the initial and principal site of viral replication (Ignjatović and Sapats, 2000). Indeed, the isolate I0305/19 was a nephropathogenic strain, as shown by nephritis of the infected chickens, viral titration in kidneys using chicken eggs, and results of IHC of the kidneys. Pathogenicity of IBV strains for the oviduct of layers varies greatly. Several IBV GI-19 strains can replicate in the epithelial cells of the oviduct, thereby causing pathological changes (Crinion and Hofstad, 1972; Benyeda et al., 2009; de Wit et al., 2011; Khanh et al., 2018). In the present study, only four birds survived in the challenge test and two of them were layers. Both the layers showed no gross oviduct lesions at 30 dpi when compared with the control birds. Because of the small number of layers in the present study, it remains unclear whether the isolate I0305/19 can induce pathogenic changes in the oviducts. Further investigation using a defined group of female chickens is needed to confirm this observation. In addition, virus was also detected at high titration in the bursa of Fabricius, cecal tonsils, and gastrointestinal tissues, and at low titration in the spleen and liver at 4 dpi; the rapid replication and broader tissue tropism of the virus during the early stage of infection are important factors to consider that this isolate is highly pathogenic to chickens.
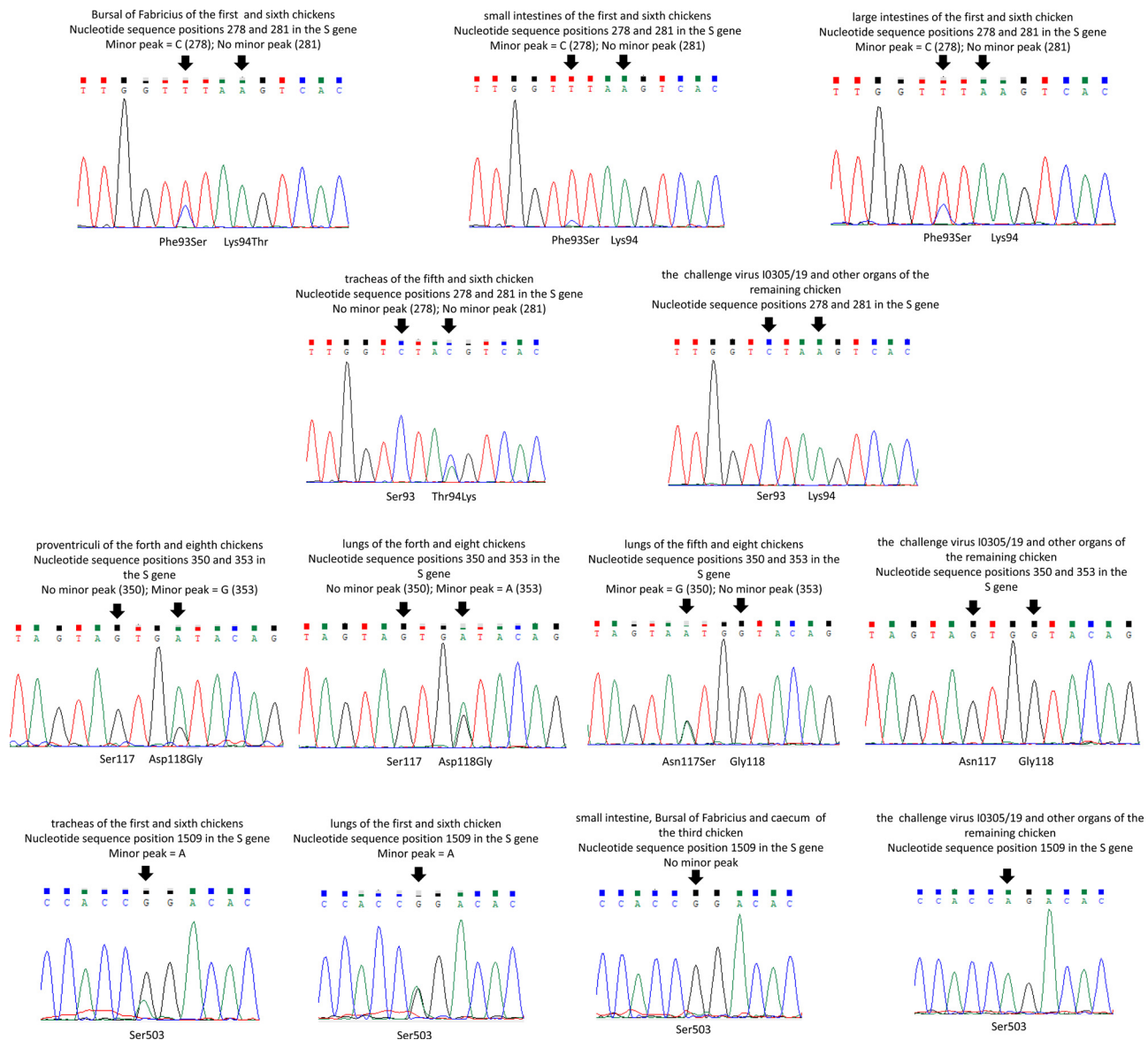
Fig. 6. Chromatograms of I0305/19 virus showing major and minor peaks indicating the existence of mixed subpopulations in tissue samples of some of the infected chickens after replication of the challenge I0305/19 virus.

In the present study, the isolate I0305/19 was proven to originate from at least four recombination events between LJS/120848- and 4/91-like strains in which the S gene sequences were found to be similar to that of the LJS/120848-like strain clustered in the GI-19 lineage (Valastro et al., 2010). Binding to host tissues is the first step in the viral life cycle of IBV and is therefore critical in determining tissue tropism and pathogenicity. Tissue tropism differs depending on the amino acid composition of the S1 protein (Casais et al., 2003; Promkuntod et al., 2014; Leyson et al., 2016; Parsons et al., 2019); therefore, S1 is considered to be responsible for host receptor binding (Wickramasinghe et al., 2011). It was demonstrated that the RBD of GI-19 virus binding to the trachea and kidney is dependent on a different sialylated glycan ligand compared to M41-RBD (Promkuntod et al., 2014); furthermore, three amino acids (KIP at positions 110–112) have been identified in an GI-19 lineage strain CK/SWE/0658946/10 that enable binding to the kidney of infected chickens (Bouwman et al., 2020). Interestingly, we found that isolate I0305/19 and other GI-19 viruses isolated in China have the amino acid sequence MIA at positions 110–112 in the S protein. It is unknown whether these amino acid changes affected the RBD of the S1 protein of these IBV isolates, in particular for interaction with

alpha-2,3-sialic acid or other putative IBV receptors on the surface of the host cells. However, we found that Chinese GI-19 viruses with different tissue tropism and pathogenicity shared the same RBD-MIA. Thus, mutations in this region, even in the S1 protein, of these IBV isolates did not seem to be the only factor responsible for viral tissue tropism and pathogenicity. Coincidentally, a previous study also indicated that genetic alterations in the S1 gene of IBV strains could not change their tissue tropism and pathogenicity (Fernando et al., 2017). One of the potential parental viruses of the recombinant I0305/19, a 4/91-like strain, is a vaccine strain that attenuated the pathogenicity. Hence, it remains unknown whether or to what extent the swapped genes influenced the viral pathogenicity. More studies based on the reverse genetic techniques are needed to elucidate the mechanisms contributing to the altered tissue tropism and pathogenicity of the isolate I0305/19.

The introduction and establishment of low virulent virus, such as vaccination with the vaccine strain, could provide, at least in part, protection against and competition with other invading viruses in a flock. This introduction could hamper the occurrence of disease outbreaks caused by other invading viruses; however, it could also

Table 1
Comparison of the pathogenicity of the isolate I0305/19 with those of other GI-19 viruses.

Strain	Country[A]	Year[B]	Day[C]	Number[D]	Dosage	Route	Morbidity (%)
I0305/19	China	2019	1	20	$10^{5.0}EID_{50}$	intranasal and intraocular	100
SD	China	2013	21	22	$10^{5.0}EID_{50}$	intranasal and intraocular	100
SZ	China	2012	21	22	$10^{5.0}EID_{50}$	intranasal and intraocular	0
GH/15	China	2015	10	10	$10^{5.0}EID_{50}$	intranasal and intraocular	100
DYW/16	China	2016	10	10	$10^{5.0}EID_{50}$	intranasal and intraocular	100
MS/17	China	2017	10	10	$10^{5.0}EID_{50}$	intranasal and intraocular	100
DYYJ/17	China	2017	10	10	$10^{5.0}EID_{50}$	intranasal and intraocular	100
PZ/17	China	2017	10	10	$10^{5.0}EID_{50}$	intranasal and intraocular	70
NA[E]	China	2005	1	50	$10^{5.0}EID_{50}$	intranasal and intraocular	100
NA[E]	France	NA[E]	1	50	$10^{5.0}EID_{50}$	intranasal and intraocular	100
NA[E]	Slovakia	NA[E]	1	50	$10^{5.0}EID_{50}$	intranasal and intraocular	100
NA[E]	Greece	NA[E]	1	50	$10^{5.0}EID_{50}$	intranasal and intraocular	100
NA[E]	Hungary	NA[E]	1	100	$10^{5.0}EID_{50}$	intranasal and intraocular	100
IBS130/2015	Malaysia	NA[E]	1	46	$10^{5.0}EID_{50}$	intraocular	100
IBS130/2015	Malaysia	2015	30	46	$10^{5.0}EID_{50}$	intraocular	100
GDJ	China	2017 or 2018	14	10	$10^{6.0}EID_{50}$	ocularly	90
GDS	China	2017 or 2018	14	10	$10^{6.0}EID_{50}$	ocularly	90
LN1	China	2017 or 2018	14	10	$10^{6.0}EID_{50}$	ocularly	60
LN2	China	2017 or 2018	14	10	$10^{6.0}EID_{50}$	ocularly	90
TJ	China	2017 or 2018	14	10	$10^{6.0}EID_{50}$	ocularly	40
Kr/Q43/06	South Korea	2006	7	10	$10^{5.7}EID_{50}$	Eye drop	100
CK/CH/JS/TAHY	China	2017	42	30	$10^{5.5}EID_{50}$	intranasally and via the eye drop	100
YN[F]	China	2005	21	74	$10^{5.5}EID_{50}$	intranasal and intraocular	100

Strain	Mortality (%)	Respiratory Sign	Lesions in the organ				Reference
			trachea	proventrculus	kidney	oviduct	
I0305/19	80	mild	yes	no	yes	no	In this study
SD	67	yes	yes	no	yes	NA[E]	Yan et al., 2017
SZ	0	no	no	no	no	NA[E]	Yan et al., 2017
GH/15	20	yes	yes	no	yes	NA[E]	Li et al., 2019
DYW/16	50	yes	yes	no	yes	NA[E]	Li et al., 2019
MS/17	30	yes	yes	no	yes	NA[E]	Li et al., 2019
DYYJ/17	0	yes	yes	no	yes	NA[E]	Li et al., 2019
PZ/17	0	yes	yes	no	yes	NA[E]	Li et al., 2019
NA[E]	0	yes	yes	no	yes	yes	Benyeda et al., 2010
NA[E]	0	yes	yes	no	yes	yes	Benyeda et al., 2010
NA[E]	0	yes	yes	no	yes	yes	Benyeda et al., 2010
NA[E]	0	yes	yes	no	yes	yes	Benyeda et al., 2010
NA[E]	0	yes	yes	no	yes	yes	Benyeda et al., 2010
IBS130/2015	19.57	yes	yes	no	yes	yes	Khanh et al., 2018
IBS130/2015	41.3	yes	yes	no	yes	yes	Khanh et al., 2018
GDJ	10	yes	yes	no	yes	NA[E]	Yan et al., 2019
GDS	0	yes	yes	no	yes	NA[E]	Yan et al., 2019
LN1	0	yes	yes	no	yes	NA[E]	Yan et al., 2019
LN2	0	yes	yes	no	yes	NA[E]	Yan et al., 2019
TJ	0	yes	yes	no	yes	NA[E]	Yan et al., 2019
Kr/Q43/06	0	yes	yes	no	yes	NA[E]	Choi et al., 2009
CK/CH/JS/TAHY	80	yes	yes	no	yes	NA[E]	Ren et al., 2019a
YN	40.5	yes	yes	no	yes	NA[E]	Zhong et al., 2016

[A] Country where that viruses were isolated.
[B] Year when the viruses were isolated.
[C] Age when the chickens were challenged.
[D] Numbers of challenged chickens.
[E] NA = not avaliable.
[F] Commercial birds were used in this report.

facilitate the occurrence of infection by highly virulent strains that were not well matched with the established viruses. This may be the case for the introduction of the isolate I0305/19 in the diseased chicken flock; the isolate caused high mortality in the vaccinated chickens. The high poultry farm density and high chicken concentrations in a farm, as commonly observed in Chinese poultry, surely create favorable conditions for viral replication, maintenance, and circulation of such viral infection, thus resulting in the emergence of novel viruses and genesis of viral population. Therefore, in addition to the regular vaccination, improved management and biosecurity practices may help to reduce viral emergence and control the disease outbreaks. Presently, little information is available on the population dynamics of the isolate I0305/19-like viruses in China. Surveillance based on properly planned sampling in different regions may be needed for better understanding the epidemiology and evolution of the isolate.

Coronaviruses including IBV have been shown to exist in biological samples as a group of closely related subpopulations (quasi-species) that are generated through mutations and recombination events that occur when the viral RNA-dependent RNA polymerase, which lacks proofreading capabilities, replicates the viral genome (McKinley et al., 2011). The genetic changes allow rapid adaption to the host by selection of more fit viral subpopulation(s); this, however, can possibly lead to changes in pathogenesis and emergence of new pathogens. In the present study, we sequenced the S1 gene from nine tissues of isolate I0305/19-infected chickens by the Sanger method, because it was reported that most genetic changes occur in the S1 gene during adaption and infection in the host (Cavanagh et al., 2005; Huang and Wang, 2007; Liu et al., 2007). We sequenced the RT-PCR products because that data represented all the possible genetic diversities within the population of viruses in a given tissue sample. Our data showed

differences in S1 sequences between the challenge I0305/19 virus and the virus from tissues of some infected chickens; this was most likely due to the induction of mutations through replication of the genome. It needs to be further evaluated whether these mutations can lead to phenotypic changes in the virus by reverse genetic techniques and animal challenge tests. However, recognizing the presence of subpopulations in tissues of infected chickens is important for our understanding of how the virulent IBV strains can potentially replicate and evolve to cause disease; this information will also contribute to our further understanding of the replication and evolution of virus in the host and pathogenesis with regard to other coronaviruses such as the newly emerged SARS-CoV-2 (Ji et al., 2020).

All authors of this paper have read and approved the final version submitted

2: Author contributions: Y. H., L. Z., M. R., Z. H. and S. L. performed the experiment. J. S., Y. Z. and S. L. analyzed the data. S. L. wrote the paper.

CRediT authorship contribution statement

Yutong Hou: Methodology, Validation, Formal analysis, Investigation. **Lili Zhang:** Validation, Methodology, Investigation, Formal analysis. **Mengting Ren:** Methodology, Validation, Formal analysis. **Zongxi Han:** Methodology, Resources, Formal analysis, Investigation. **Junfeng Sun:** Software, Formal analysis. **Yan Zhao:** Methodology, Formal analysis. **Shengwang Liu:** Conceptualization, Methodology, Supervision, Project administration, Writing - review & editing, Funding acquisition.

Declaration of Competing Interest

The authors declare that they have no conflict of interest.

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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.2020.198002.

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