Multiple recombination events between field and vaccine strains resulted in the emergence of a novel infectious bronchitis virus with decreased pathogenicity and altered replication capacity

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ABSTRACT In this study, we isolated and identified 2 infectious bronchitis virus (**IBV**) strains from layer chickens soon after vaccination with the Massachusetts–Connecticut bivalent vaccine (**Conn**) and H120 and 4/91 booster vaccines in China in 2011. The results of cross-virus-neutralization tests and phylogenetic analysis of the S1 subunit of spike gene of these vaccine strains and other reference strains showed that strain LJL/110302 was of GI-19 lineage, whereas LLN/111169 was of the GI-1 lineage of the Conn serotype. Further comparative genomic analysis revealed that LLN/111169, an IBV strain with novel traits, originated from multiple recombination events (at least 3 recombination sites) between GI-19 and the Conn and 4/91 vaccine strains. LLN/111169 was pathogenic to specific pathogen-free (**SPF**) chickens. This is of prime importance because while IBV prevention measures worldwide are mainly dependent on modified live vaccine strains, our results showed that recombination between field and vaccine strains has produced a novel pathogenic IBV strain. In addition, LLN/111169 showed relatively broad tissue tropism (trachea, lungs, kidneys, and cecal tonsils) in infected SPF chickens. These results emphasize the importance of IBV surveillance in chicken flocks.

Key words: infectious bronchitis virus, genotype and lineage, recombination, antigenicity, pathogenicity

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INTRODUCTION

Avian infectious bronchitis virus (**IBV**) initially causes an acute, highly contagious infectious respiratory disease (infectious bronchitis, **IB**) that affects chickens and spreads directly by aerosols and direct contact and indirectly through contaminated fomites. Some IBV strains infect the kidneys and oviduct of chickens, causing a decrease in growth rate and performance in meat birds and egg quality and quantity in laying hens (Cavanagh, 2007). Mixed infection of IBV and opportunistic bacterial pathogens might result in airsacculitis and high mortality, leading to greater economic losses.

Avian infectious bronchitis virus, a gammacoronavirus belonging to the family *Coronaviridae*, is a large, enveloped, positive-strand-RNA virus, approximately 27.6 kb in size (Boursnell et al., 1987). The IBV genome encodes 4 structural proteins: the spike (**S**) and membrane (**M**) glycoproteins, nucleocapsid (**N**) phosphoprotein, and envelop (**E**) protein. It also encodes at least 4 accessory genes—3a, 3b, 5a, and 5b—which occupy one-third of the genome at the 3′ end. The S glycoprotein, an integral membrane protein, is posttranslationally cleaved into the N-terminal S1 and C-terminal S2 subunits (Cavanagh, 2007). The S1 subunit is responsible for selection of the host, induction of protective immunity, and neutralization of most neutralizing serotype-specific antibodies (Kant et al., 1992; Wickramasinghe et al., 2011). Because the S1 subunit of spike protein shows greater sequence diversity than other regions of IBV genome, it is most commonly used for molecular characterization, genetic classification, and genotyping of IBV. Such efforts have recently helped differentiate IBV strains worldwide into 7 IBV genotypes, 35 lineages, and a number of interlineage recombinants (Valastro et al., 2016; Chen et al., 2017; Jiang et al., 2017; Ma et al., 2019). However, new IBV genotypes and variants are frequently emerging in different parts of the world, and many factors are contributing to this emergence and evolution (Sjaak de Wit et al., 2011;

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Jackwood, 2012). In addition, the IBV genome contains 2 overlapping open reading frames (**ORF**), which occupy two-thirds of the genome and encode polyproteins 1a (pp1a) and 1 ab (pp1ab). These polyproteins undergo autoproteolytic cleavage to produce 15 nonstructural proteins (nsp2–nsp16).

Although point mutations are very common in coronavirus genomes, recombination between strains has also been widely reported (Kusters et al., 1990; Jia et al., 1995; Herrewegh et al., 1998; Snijder et al., 2003; Thor et al., 2011). Regions of the genome predicted to have a stable secondary structure have been reported to be recombination "hot spots" in coronaviruses (Nagy and Simon, 1997). Recombination events in the coronavirus genome are believed to have promoted the emergence of viruses with novel traits such as altered tissue tropism, antigenicity, and pathogenicity. Some of these novel viruses are even able to adapt to new hosts and ecological niches, sometimes causing zoonotic events. This has been observed in the case of a turkey coronavirus which was proved to have evolved after simultaneous infection of a host with a known IBV strain and an uncharacterized coronavirus, resulting in recombination in the S1 subunit of spike gene and a subsequent shift in host tropism from chickens to turkeys (Jackwood et al., 2010). In case of IBV, many recombinants isolated in different parts of the world have shown novel traits relative to those of their predicted parental viruses (Sjaak de Wit et al., 2011; Jackwood, 2012).

Infectious bronchitis is a major problem among chicken flocks in China. In November 2011, 2 chickens suspected to be infected with IBV were submitted to our laboratory. Infectious bronchitis virus isolates were recovered from the swollen kidneys and proventriculus of the 2 birds. In this retrospective study, we attempted to determine the genetic and antigenic characteristics of these IBV isolates to investigate their possible origin and evaluate their pathogenicity and replication capacity in the tissues of susceptible chickens.

MATERIALS AND METHODS

Ethics

This study was conducted in accordance with the animal welfare guidelines of the World Organization for Animal Health. All animal protocols were reviewed and approved by the Agricultural Animal Care and Use Committee of Heilongjiang province, China.

Chicken Embryos and Chickens

Specific pathogen-free (**SPF**) white Leghorn chicks, and 9-day-old embryonated chicken eggs were purchased from the Laboratory Animal Center, Harbin Veterinary Research Institute, Chinese Academy of Agricultural Sciences, China. All birds were maintained in isolators with negative pressure, and food and water were provided *ad libitum*.

Virus Isolation

This study retrospectively analyzed 2 IBV strains—ck/CH/LJL/110302 (**LJL/110302**) and ck/CH/LLN/111169 (**LLN/111169**)—that were isolated in 2011 from commercial layer flocks in Jilin and Liaoning provinces in China, respectively. Both commercial chicken flocks were derived from the same layer breeders and contained at least 15,000 layers each. The birds in both flocks were vaccinated under the same vaccination program: first vaccination with the Massachusetts–Connecticut (**Mass–Conn**) bivalent vaccine at 1 D of age, followed by booster vaccination with H120 and 4/91 vaccines at 14 D of age. The chickens of both flocks displayed clinical signs at approximately 20 D of age. The morbidity and mortality rates were about 65 and 10% among chickens from Jilin and 7.5 and 3% among chickens from Liaoning, respectively. Some of the dead chickens showed moderate to severe tracheitis, marked airsacculitis, and proventriculitis, and most of them showed severe nephritis, including renal swelling and accumulation of urates in renal tubules. For virus isolation, kidney and proventriculus samples from each flock were pooled and homogenized separately. The homogenates were diluted in phosphate-buffered saline (1:10 dilution), clarified by centrifugation at 300 × *g* for 5 min, and filtered through 0.22-μm membranes before inoculation into the allantoic cavity of 9-day-old SPF chicken embryos. The inoculated eggs were incubated at 37°C and candled daily. Characteristic embryonic changes (dwarfing, stunting, curling, or death) were observed between days 3 and 7, when the viruses were passaged 3 times.

Cross-Virus-Neutralization Tests

Because strains LJL/110302 and LLN/111169 were isolated from chicken flocks vaccinated with the Mass–Conn, H120, and 4/91 vaccines, we used the 3 attenuated live IBV strains to test their antigenic relationship with LJL/110302 and LLN/111169 by a cross-virus-neutralization test. Virus stocks of the 5 IBV strains—LJL/110302, LLN/111169, 4/91, H120, and Conn vaccine—were produced by inoculating 9-day-old embryonated SPF chicken eggs (Liu et al., 2013). Virus titers were calculated by the method of Reed and Muench (1938) and expressed as 50% embryo infectious dose ($\mathbf{EID_{50}}$). Antisera against the 5 IBV strains were prepared (Gao et al., 2016) and stored in 2.0-mL aliquots at −80°C until required.

Antigenicity analysis was performed by the β-cross-virus-neutralization method (with constant virus concentrations and diluted serum samples) (Gao et al., 2016). Serial 2-fold dilutions of serum were reacted with 100 EID_{50} of IBV at 37°C for 1 h. These virus–serum mixtures were inoculated into the allantoic cavity of SPF chicken embryos, which were then observed for 7 D. On the seventh day

postinoculation (**dpi**), the eggs were opened and examined for lesions characteristic of IBV infection. Antigenic relatedness values were calculated by using the formula of Archetti and Horsfall (1950).

Sequencing, Consensus-Genome Determination, and Phylogenic Analysis

The complete genomes of IBV isolates LJL/110302 and LLN/111169, in addition to those of the 4/91 and Conn vaccine strains, were sequenced as described previously (Liu et al., 2013). Viral RNA was extracted by using the RNAiso Plus reagent (Takara Bio, Shiga, Japan), and reverse transcription (**RT**)-PCR was performed by using a one-step RT-PCR kit (Takara) in accordance with the manufacturer's instructions. The 3′ and 5′ ends of the viral genomes were confirmed by rapid amplification of cDNA ends by using a 3′/5′ RACE kit (Takara) in accordance with the manufacturer's instructions. The PCR products were either sequenced directly or cloned into the pMD-18T vector (Takara) in accordance with the manufacturer's instructions. Sanger sequencing was performed (Big Dye Terminator). Each fragment of the viral genome was sequenced at least 3 times, and the consensus sequence was determined. ORF prediction was performed by comparison with the ORF of the Beaudette strain (GenBank accession number NC_001451) by using Lasergene DNAStar (version 7, Lasergene Corp, Madison, WI) and Vector NTI Advanced 10 (Invitrogen, Carlsbad, CA).

First, homologous hits were identified by an extensive search by using the Basic Local Alignment Search Tool (**BLAST**; http://www.ncbi.nlm.nih.gov) with default values. Then, 33 S1 sequences and 14 complete genome sequences of reference strains for which the sequences were available in the GenBank database were selected for phylogenetic analysis. Phylogenetic analysis was performed by using the Mega 6.0 program (http://www.megasoftware.net/). Phylogenetic trees were elaborated by the maximum-likelihood method, supplemented with statistical support by bootstrapping over 1,000 replicates.

Recombination Analysis

On the basis of the results of phylogenetic analysis, the complete genome sequences of the Conn and 4/91 vaccine strains were selected for comparison with that of LLN/111169 to analyze possible recombination events, probable parental viruses, and likely recombination breakpoints. This analysis was performed by similarity plot (SimPlot; SimPlot version 3.5.1) and bootscan (http://sray.med.som.jhmi.edu/SCRopftware/simplot/) analysis by using IBV isolate LJL/110302 as the query virus. The window width and step size were set to 1,000 bp and 50 bp, respectively. To confirm the recombination events that had occurred in the LLN111169 genome, the complete genome sequences of LJL/110302, LLN/111169, Conn, and 4/91 were analyzed by pairwise comparison, and 4 phylogenetic trees were constructed with these 4 IBV strains and 2 other IBV strains (ck/CH/LDL/091022 [GI-19] (Liu et al., 2013) and ck/CH/LGX/091109 [GenBank accession number KF411041]) on the basis of the results of the phylogenetic and SimPlot analyses.

Accession Number

The genome sequences of the LJL/110302, LLN/111169, Conn vaccine, and 4/91 vaccine strains have been submitted to the GenBank database (accession numbers KC136209, KF411040, KF696629, and KF377577, respectively).

Confirmation of Recombination in LLN/111169 Genome in Natural Conditions

To confirm that the recombinant event in the LLN/111169 genome had a natural origin and was not a result of mixture of different IBV strains or a methodological artifact that originated during egg inoculation, 2 primer pairs, LJL-U and Conn-L, and Conn-U and 4/91-L were independently used to amplify the supernatant of the kidney and proventriculus samples that had been used for isolating LLN/111169 as well as the virus stocks of LLN/111169. The 2 primer pairs were designed by aligning the sequences encoding the predicted breakpoints and flanking sequences of IBV strains LLN/111169, LJL/110302, Conn, and 4/91. One-step RT-PCR was used, and positive PCR products were subjected to direct sequencing.

In Vivo Infection Experimental Design

Three groups of 60 one-day-old SPF white Leghorn layer chicks were housed in separate isolators. The birds in groups 1 and 2 received $10^{5.0}$ EID_{50}/0.1 mL doses of IBV strains LJL/110302 and LLN/111169, respectively, through the ocular-nasal route at 1 D of age. The birds in group 3 received only sterile allantoic fluid and served as the negative control. At 4 and 8 dpi, 5 chickens from each group were humanely killed and necropsied. Trachea, lung, kidney, and cecal tonsil samples were collected and used for virus titration by real-time RT-PCR. The remaining birds in all 3 groups were observed until 25 dpi, and their morbidity and mortality were recorded. Dead chickens were autopsied, and gross lesions were recorded. Blood samples were collected from the remaining chickens in each group at 4, 8, 12, 16, 20, and 24 dpi. The IBV-specific IgG in serum was detected by using a commercial IBV enzyme-linked immunosorbent assay kit (IDEXX Lab. Inc., Westbrook, Maine) in accordance with the manufacturer's protocol.

Immunohistochemistry

Trachea and kidney samples collected at 4 dpi were used for detection of IBV antigens by immunohistochemistry. Immunohistochemistry was performed as described previously (Benyeda et al., 2010) with slight modification. Briefly, the tissue samples were fixed in 10% neutral

buffered formalin for 24 h, processed routinely, and embedded in paraffin wax. Sections were dewaxed and incubated in citrate buffer (pH 6.0) in a microwave oven at 700 to 800 W for 10 min and then at 200 to 300 W for 30 min. Endogenous peroxidase was blocked using 3% H_2O_2 at room temperature for 10 min. The sections were covered with monoclonal antibody 6D10, which is directed against the nucleoprotein (Han et al., 2013). Then, dextran coupled with peroxidase and goat secondary antibody (Santa Cruz Biotechnology Inc., Santa Cruz, CA) was applied for the secondary reaction, incubated for 30 min, and washed with 0.01 M phosphate buffered saline. Thereafter, substrate working solution (BOSTER, Wuhan, China) was applied to the sections and incubated for 5 to 10 min. Normal bovine serum albumin was used as a control substitute for the monoclonal antibody.

Real-Time RT-PCR

Same amount of tissues (0.2 mg) were used to extract the viral genomic RNA. Homogenized tissue samples were analyzed by one-step real-time RT-PCR in a LightCycler1 480 real-time PCR system (Roche, Basel, Switzerland) by using the One-Step PrimeScript1 RT-PCR kit (TaKaRa) as described previously (Liu et al., 2013). Then, the concentrations of the viral RNA were calculated to express as "viral genomic RNA copy number/μg of tissue" from the each tissue. The data were analyzed by using the LightCycler1 480 software, version 1.5, and the PCR products were confirmed by electrophoresis on a 2.0% agarose gel and visualized by staining with ethidium bromide.

Statistical Analysis

Data are expressed as mean ± standard deviation. Viral titers were analyzed by the Student *t*-test by using GraphPad Prism for Windows, version 5 (GraphPad Software, La Jolla, CA). Differences were considered significant if the P value was <0.05 ($^{*}P < 0.05$; $^{**}P < 0.01$; and $^{***}P < 0.001$).

RESULTS

Genotype and Serotype of the Two IBV Isolates

Two IBV strains, designated as LJL/110302 and LLN/111169, were isolated from diseased chickens from 2 farms in the northeast region of China. The results of genotyping revealed that isolate LJL/110302 was of GI-19 lineage (Figure 1), whereas isolate LLN/111169 belonged to the GI-1 lineage and was genetically closely related to the Conn strain (Figure 1). Cross-virus-neutralization findings revealed that LLN/111169 was antigenically related to the Conn vaccine strain but was different from the Mass H120 and 4/91 vaccine strains (Figure 2); LLN/111169 was isolated from diseased chickens that were vaccinated with these 3 types of vaccines. LLN/111169 was also genetically and antigenically different from LJL/110302.

Genomic Characteristics of the Two IBV Isolates

The sequences of isolates LJL/110302 and LLN/111169 and vaccine strains Conn and 4/91 were assembled into one contiguous sequence to represent each of the complete viral genomes. The sequences of 27,673, 27,651, 27,630, and 27,606 nucleotides were derived from the LJL/110302, LLN/111169, Conn, and 4/91 strains, respectively, excluding the poly-A tail at the 3′ end. The genome organization of these strains are typical of IBVs, with the genes arranged in the following order (5′ to 3′): replicase ORF1ab, S, E, M, and N (Supplementary Figure 1). The 5′ and 3′ ends both contain short untranslated regions. The replicase ORF1ab encodes a number of putative proteins, including nsp3 (which encodes a putative papain-like protease), nsp5 (which encodes a putative chymotrypsin-like protease), nsp12 (which encodes a putative RNA-dependent RNA polymerase), nsp13 (which encodes a putative helicase), and other proteins of unknown functions.

Phylogenetic trees based on the complete genomes showed LJL/110302 clustered into the same group as strains of GI-19 lineage, including strains ck/CH/LDL/091022 (Liu et al., 2013) and LLN/111169. In line with these results, isolate LJL/110302 shared higher genomic sequence identity with the GI-19 lineage (97.8% with strain ck/CH/LDL/091022 and 93.3% with LLN/111169). In contrast, isolate LLN/111169 was clustered into a separate group, different from those of the Conn and 4/91 vaccine strains (Figure 3). LLN/111169 shared only 86.3 and 85.8% genetic identity with Conn and 4/91, respectively.

Isolate LLN/111169 was Derived From Multiple Recombination Events

As shown in Figure 4A, the genome of isolate LLN/111169 showed greater similarity to that of LJL/110302 than to those of the Conn and 4/91 vaccine strains from 5′ UTR to the 5′ end of nsp14 in gene 1 (nucleotides 1–17,108) as well as in most parts of the N gene (nucleotides 26,118–26,637). However, in the region extending from the 5′ end of nsp14 in gene 1 to the 5′ end of the N gene—including the complete S gene, gene 3, M gene, and gene 5 (nucleotides 17,135–26,115)—isolate LLN/111169 showed greater nucleotide sequence identity to the Conn vaccine strain. Of significant interest is the finding that in the genomic region containing nucleotides 26,643–27,632, LLN/111169 appeared to be closely related to the 4/91 vaccine strain. These results were confirmed by the phylogenetic trees and nucleotide identity percentages calculated by using the 4 fragments (Figure 4B). These data strongly suggest that LLN/111169 arose from multiple recombination events (at least 3 breakpoints) between LJL/110302-like, Conn-like, and 4/91-like viruses. The

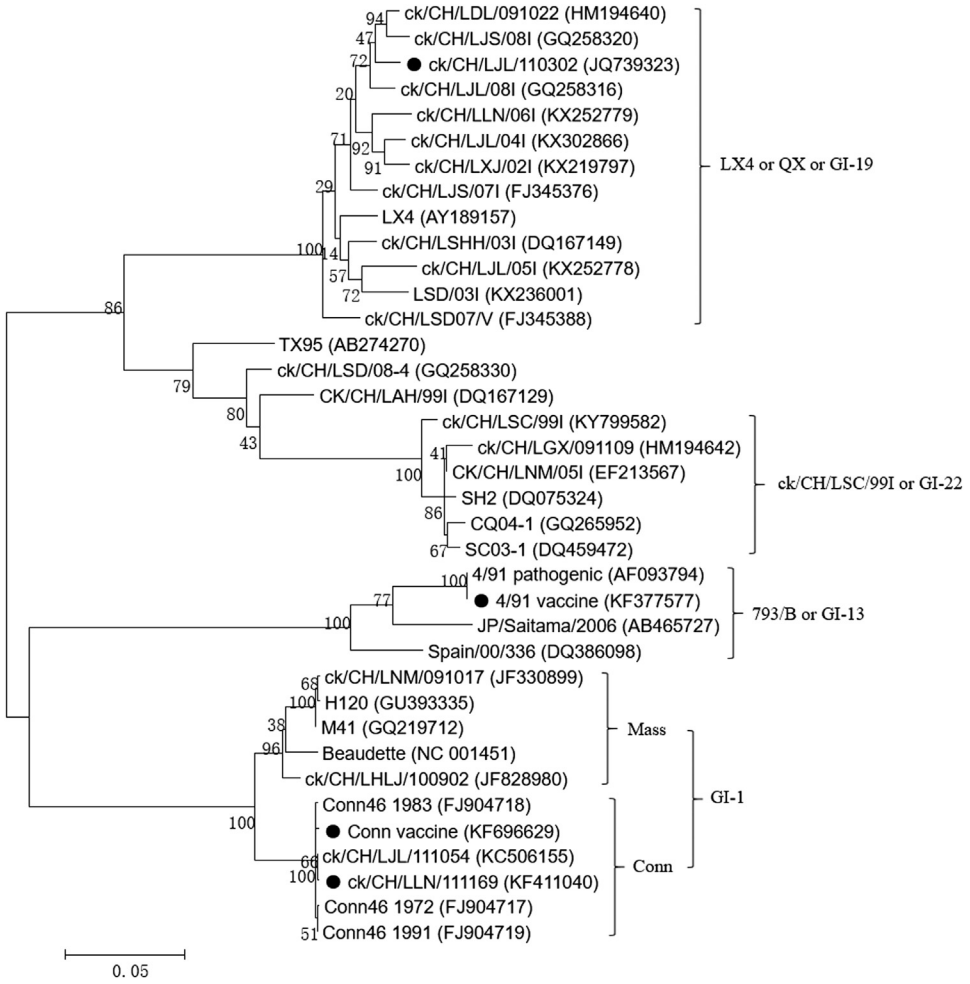

Figure 1. Maximum-likelihood phylogenetic analysis based on the complete S1 subunit of spike gene sequences of the 2 isolates and 2 vaccine strains sequenced in this study and 34 reference strains. The IBV strains sequenced in this study are indicated with black circles. The scale bars indicate the number of nucleotide mutations per site. IBV, infectious bronchitis virus.

presence of 3 breakpoints (nucleotides 17,109–17,134 in nsp14 in ORF1b and nucleotides 26,116–26,117 and 26,638–26,642 in the N gene) was also observed in the genome of LLN/111169 (Supplementary Figure 2), implying that template switches occurred within the nsp14 and N genes.

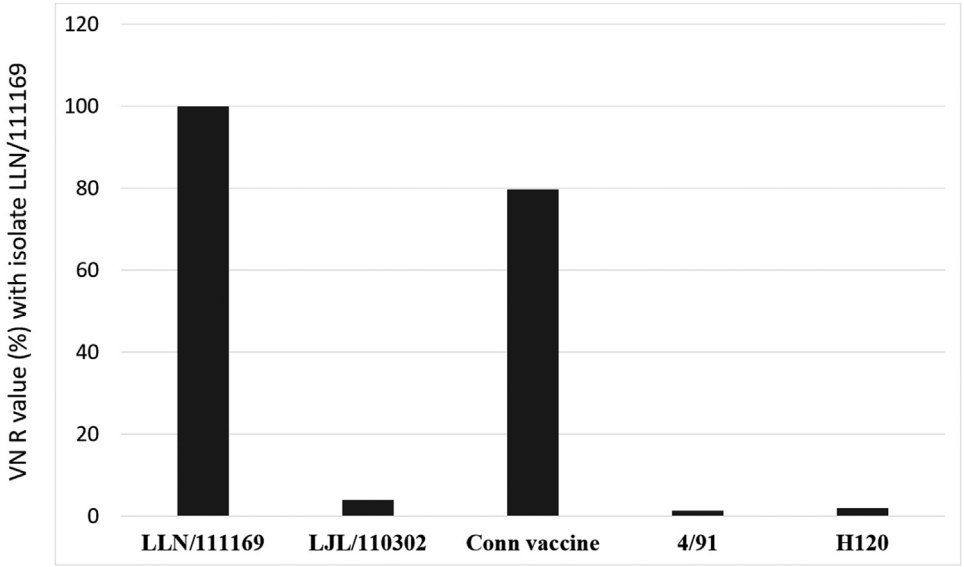

Figure 2. Calculated antigenic relatedness values, R, of isolate LLN/111169 against homologous and heterologous strains (LJL/110302, Conn vaccine, 4/91, and H120).

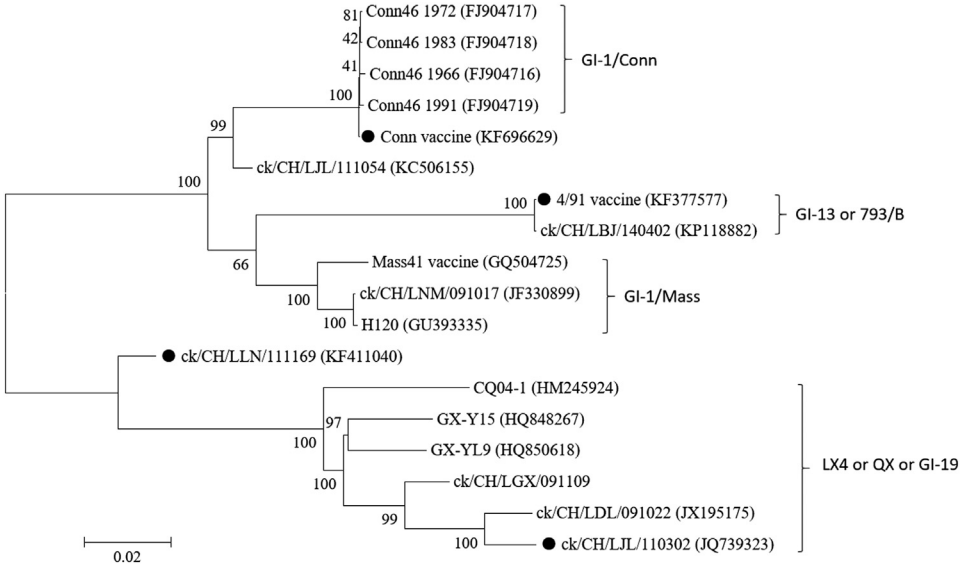

Figure 3. Maximum-likelihood phylogenetic analysis based on the complete genome sequences of the 2 isolates and 2 vaccine strains sequenced in this study and 13 reference strains. The IBV strains sequenced in this study are indicated with black circles. The scale bars indicate the number of nucleotide mutations per site. The GenBank accession number of each virus is indicated in parentheses. IBV, infectious bronchitis virus.

This possibility was confirmed by the results of RT-PCR (Figure 5, A and B) with 2 sets of specific primers targeting the sequences containing the predicted breakpoints and flanking sequences of isolate LLN/111169 as well as the results of subsequent sequencing (data not shown).

The Two IBV Isolates Are Pathogenic to SPF Chickens

While chicks in the control group showed no overt signs of disease, some of the LJL/110302-infected and LLN/111169-infected chickens showed mild clinical signs at 2 and 3 dpi, respectively. All LJL/110302-infected chickens showed severe clinical signs from 3 to 10 dpi. However, LLN/111169-infected chickens showed mild to severe clinical signs. The clinical signs were more severe in chickens infected with LJL/110302 than in those infected with LLN/111169. In both groups of chickens, the signs disappeared at 13 dpi. The clinical signs included listlessness, huddling, ruffled feathers, dark and shrunken combs, and/or watery diarrhea (flushing). Five of the 10 chicks in the LJL/110302-infected group died—2 on day 5 and 3 on day 6—during the experiment. The dead chicks showed gross lesions confined mainly to the kidneys as well as mild to severe tracheitis. None of the chicks in the LLN/111169-infected group died during the experiment.

All chickens infected with the IBV isolates were negative for IBV-specific antibodies at 4 D of age. All LJL/110302-infected chickens and fewer than half of the LLN/111169-infected chickens were positive for IBV-specific antibodies from 8 dpi. No antibodies were detected in the control group.

The Two IBV Isolates Possess Different Replication Capacities in SPF Chickens

As illustrated in Figure 6, relative to LLN/111169-infected chickens, LJL/110302-infected chickens showed higher concentrations of viral RNA in all investigated tissues at 4 dpi, except in the cecal tonsils, where the viral RNA concentrations in both groups were comparable. Similarly, antigen-positive cells were more intensely labeled in the trachea and kidneys of LJL/110302-infected chickens than in those of LLN/111169-infected chickens (Figure 7). In contrast, the replication efficiency of isolate LLN/111169 in chicken tissues was higher than that of LJL/110302 at 8 dpi. However, both isolates showed a somewhat high affinity for the trachea at this timepoint, as reflected in the high numbers of viral genome copies detected by real-time RT-PCR. In particular, both isolates showed a strong affinity to the kidney at 8 dpi. No IBV-derived RNA was detected in the tissues of control chickens (data not shown).

DISCUSSION

During our longitudinal surveillance of IBV in China in 2011, 2 commercial layer farms in the northeast region of China (Jilin and Liaoning provinces) reported disease outbreaks consistent with IB. The chicken flocks in these 2 farms were from the same layer breeders and had been vaccinated under similar IB vaccination programs. Chicken in both farms showed signs of the disease at approximately 20 D of age. It was, therefore, speculated that these 2 disease outbreaks were caused by similar agents and that IB vaccine strains were likely involved in the disease, although morbidity and mortality rates varied between the 2 farms. In this study, IBVs were

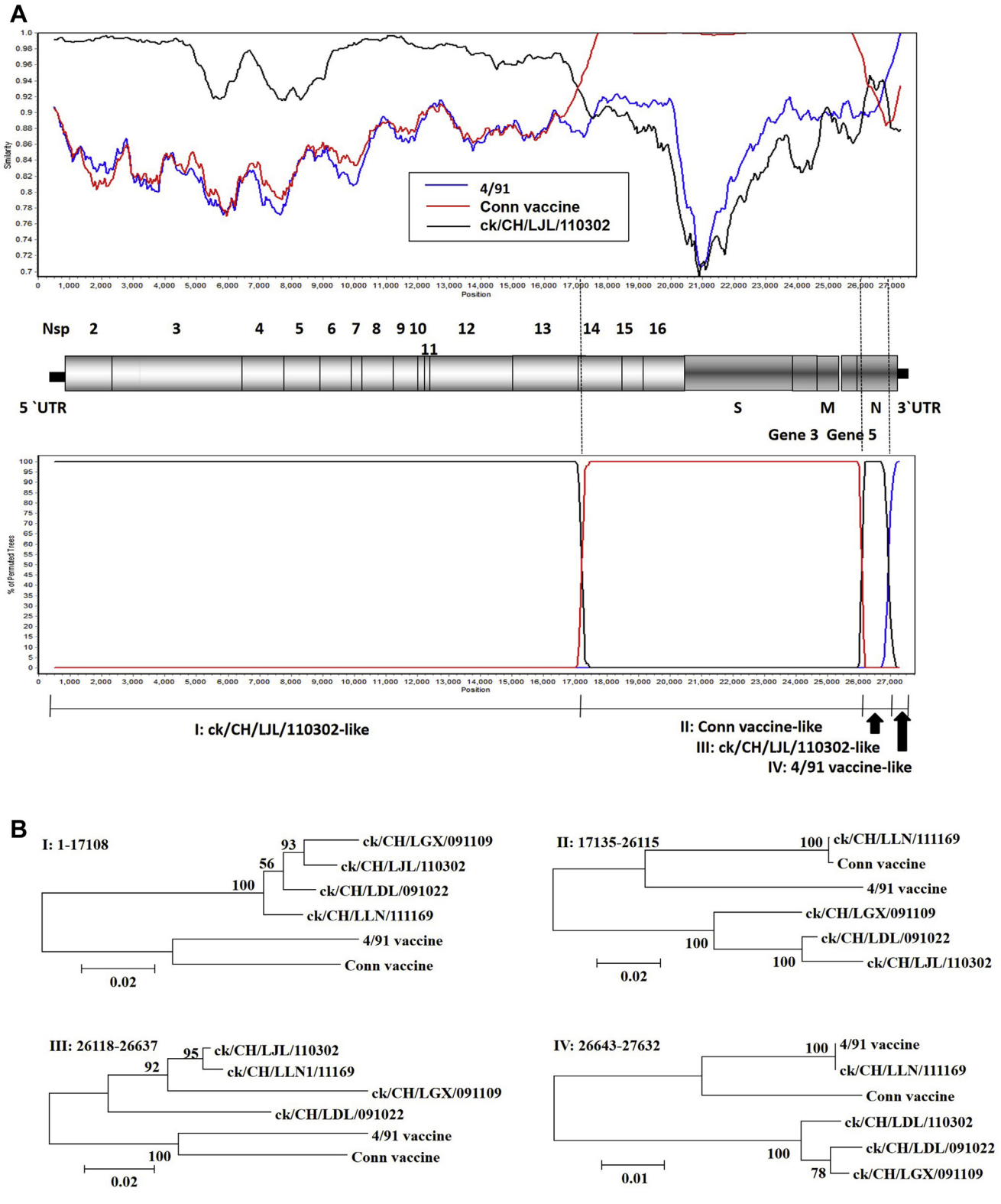

Figure 4. Detection of possible recombination events in IBV isolate LLN/111169. SimPlot and bootscan analyses of the full-length genome sequence of LLN/111169 were performed by using SimPlot, version 3.5.1 (http://sray.med.som.jhmi.edu/SCRopftware/simplot/) (A). IBV isolate LJL/110302 was used as the query virus. The full-length genome sequences of the vaccine strains investigated in this study (4/91 and Conn) were used as reference sequences. The analysis was performed by employing the Kimura model, with a window size of 1,000 bp and step size of 50 bp. Phylogenetic analysis based on different gene fragments (B). The trees were constructed by the maximum-likelihood method by using the MEGA 6.0 program (http://www.megasoftware.net/). The scale bars indicate the number of nucleotide mutations per site. The GenBank accession numbers for the viruses are the same as those in Figure. 2. IBV, infectious bronchitis virus.

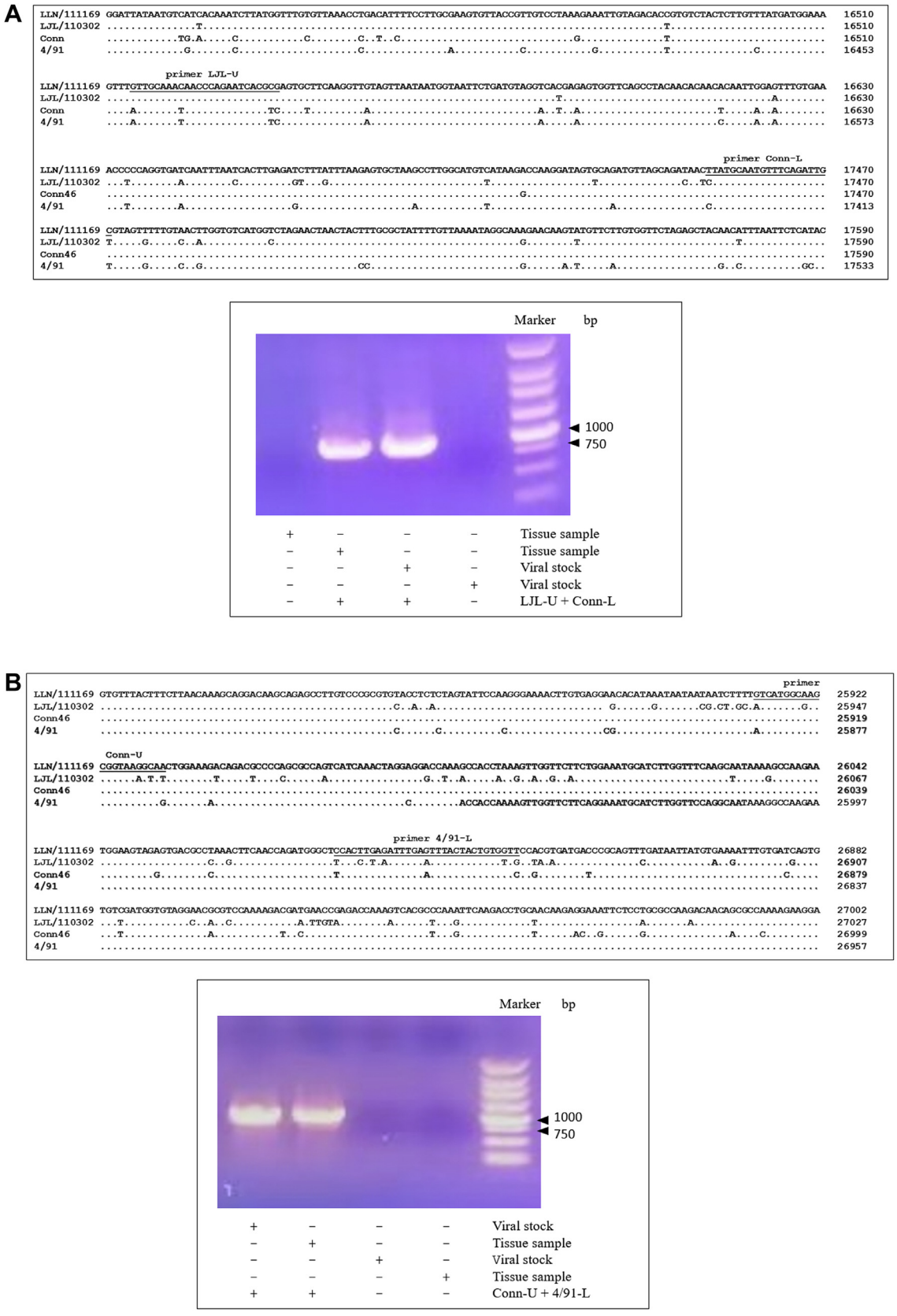

Figure 5. Primer design and identification of the recombination events. The first pair of primers (LJL-U and Conn-L) were designed to identify the first recombination event in the genome of LLN/111169 (A). Sequences encoding the regions of first predicted breakpoints and flanking sequences of IBV strains LLN/111169, LJL/110302, Conn, and 4/91 were aligned. The sequences of LLN/111169 are listed, and the only amino acids differing from those of LLN/111169 are depicted. The numbers on the right side of each alignment indicate the nucleotide positions in the genome of each virus. Both the tissue sample and viral stock were positive for RT-PCR with primers LJL-U and Conn-L (about 960 bp). The second pair of primers (Conn-U and 4/91-L) were designed to identify the second and third recombination events in the genome of LLN/111169 (B). Both the tissue sample and viral stock were positive for RT-PCR (about 910 bp) with primers Conn-U and 4/91-L. RT-PCR, reverse transcription-PCR.

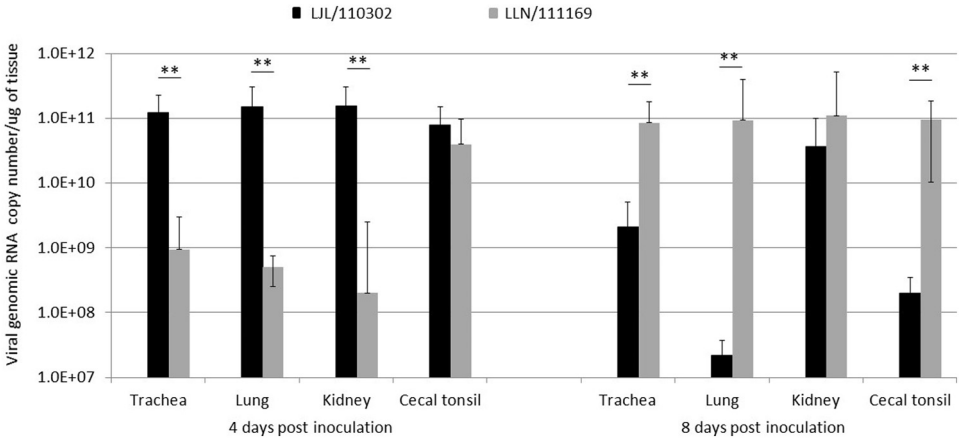

Figure 6. Viral RNA copy numbers in the tissues of chickens inoculated with isolates LJL/110302 and LLN/111169. Viral RNA copy numbers in the same amount of tissues (0.2 mg) of 5 chickens at 4 and 8 dpi were measured by real-time RT-PCR. Then, the concentrations of the viral RNA were calculated to express as "viral genomic RNA copy number/μg of tissue" from the each tissue. The results represent the mean of 3 independent experiments with 3 replicates per experiment. Each bar indicates the mean ± SD. The data were analyzed by using SAS; $^{**}P < 0.01$. RT-PCR, reverse transcription-PCR.

isolated from both chicken flocks. However, isolate LLN/111169 was found to be genetically and antigenically identical to the Conn vaccine-like strain, and isolate LJL/110302 was genetically and antigenically different from isolate LLN/111169, and the 3 vaccine strains H120, Conn, and 4/91, which implied that different IBV lineages/serotypes were involved in the 2 farms.

The GI-19 lineage generally has a higher prevalence in China (Xu et al., 2018), and previous epidemiologic investigations had established that LJL/110302 (GI-19)-associated infections had previously occurred on the same farm and in the vicinity. This virus lineage was involved again in the latest disease outbreak. In the present report, it was demonstrated that isolate LLN/111169 is a chimeric virus possessing an LJL/110302-like (GI-19) genome sequence, but with a unique S gene sequence which showed high similarity to that of the Conn vaccine strain and a 4/91-like (GI-13) fragment containing about a half-length of the N gene sequence at the 3′ end and a complete 3′ UTR. This chimeric virus was probably generated by multiple recombination events between the Conn and 4/91 vaccine strains and the circulating LJL/110302-like field strain. It is believed that recombination events can occur and can sometimes be observed in cases where birds have been

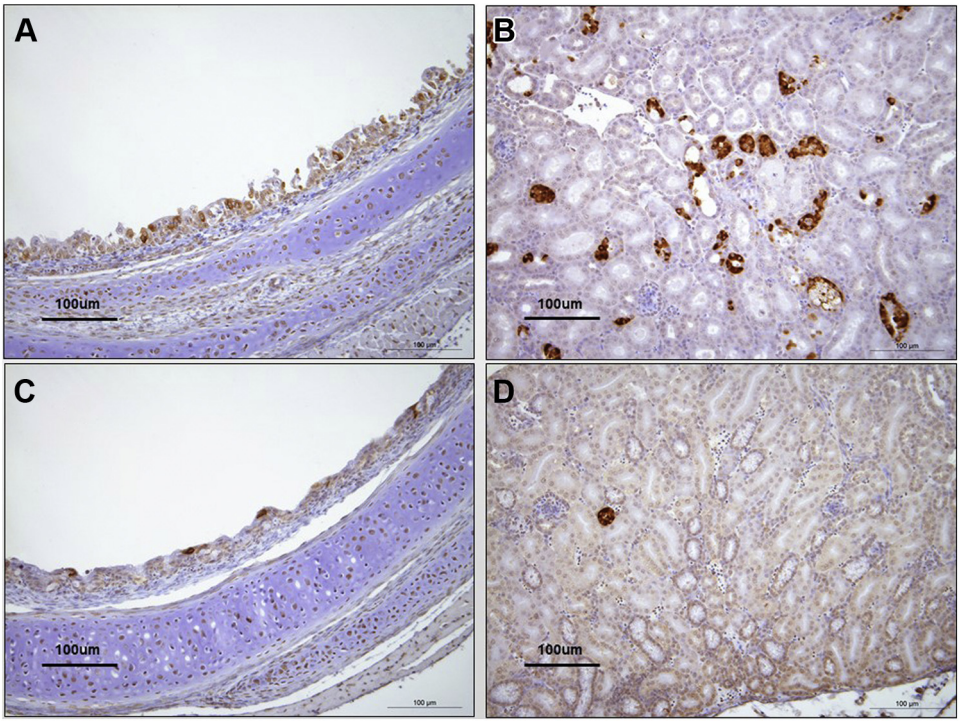

Figure 7. Immunohistochemical staining of IBV in the trachea (A and C) and kidneys (B and D) of chickens infected with LJL/110302 (A and B) and LLN/111169 (C and D). The images were acquired at 100× magnification. IBV, infectious bronchitis virus.

vaccinated or infected with a mixture of IBV serotypes/lineages. In the present case, infection of GI-19-lineage IBVs in Conn-vaccinated and/or 4/91-vaccinated birds was possible because vaccination with the 2 vaccine strains could not provide complete protection against the GI-19 lineage (Liu et al., 2014; Han et al., 2017). Thus, mixed infection of vaccine and virulent strains might occur under such conditions, which is the premise for the occurrence of recombination events. On the basis of this phenomenon—as observed in other IBV strains previously isolated in China—the Conn-like and 4/91-like sequences found in the genome of isolate LLN/111169 can be assumed to have been derived from the Conn and 4/91 vaccine strains (Liu et al., 2014; Han et al., 2017).

Recombination is a common phenomenon in coronaviruses. It has been found to be responsible for the emergence of SARS-CoV (Ge et al., 2013; Hu et al., 2015; Lau et al., 2015) as well as new strains of other coronaviruses, including human CoV HKU1, human CoV OC43, feline CoV type II strains (Herrewegh et al., 1998; Woo et al., 2006; Lau et al., 2011), and deltacoronaviruses (Lau et al., 2018). Recombination involving the S protein is believed to be a common phenomenon that might generate new genotypes and facilitate interspecies transmission and adaptation to new animal hosts. In avian coronaviruses, recombination events involving the S protein are believed to have promoted the emergence of turkey (Jackwood et al., 2010; Mardani et al., 2010) and guineafowl (Brown et al., 2016) coronaviruses; in this case, the turkey coronavirus was proved to have evolved after simultaneous infection of a host with a known IBV strain and an uncharacterized coronavirus, resulting in recombination in the S1 subunit of spike gene and a subsequent shift in host tropism from chickens to turkeys (Jackwood et al., 2010). Infectious bronchitis virus is among the most important coronaviruses responsible for heavy economic losses worldwide. Recombination events in the S protein of IBV can not only result in the emergence of novel genotypes/serotypes and variants but also potentially result in the loss of vaccine efficacy, because the S protein plays an important role in antigenicity, tissue tropism, and virulence (Wickramasinghe et al., 2014). Vaccine efficacy could also be reduced by antigenic differences among vaccine and field strains due to mutations in the S protein and incorrect vaccine choice. In the present study, although the recombinant LLN/111169 strain had the backbone of a GI-19-like virus, it was highly similar in its S protein sequence to the Conn vaccine strain and was antigenically related to the Conn vaccine strain. However, the LLN/111169 was isolated from a commercial layer flock that were vaccinated the Mass–Conn bivalent vaccine. It was possibly that other factors such as the improper use of vaccine were involved in the loss of vaccine efficacy although it was required to be confirmed by vaccination-challenge test in the further investigation. However, isolate LLN/111169 was less pathogenic to SPF chickens than isolate LJL/110302, although both isolates showed broader tissue tropism. Interestingly, LJL/110302 and LLN/111169 showed different replication patterns in the tissues of SPF chickens, with the more virulent LJL/110302 isolate showing a greater replication capacity than the LLN/111169 isolate in the earlier stages of infection and lower replication capacity at 8 dpi. This difference in replication patterns might be associated with the recombination events in the N gene and 3′ UTR of isolate LLN/111169 (Tsai et al., 2018; Lo et al., 2019).

In the present study, we were unable to establish whether the diseased chicken flock was a "mixing vessel" for producing the recombinant LLN/111169 strain, although the fact that the chickens were vaccinated with 2 of the predicted parental viruses, Conn and 4/91, before the disease outbreak forms a very interesting link. However, strain LLN/111169 have emerged from multiple recombination events of the vaccines used, including Conn and 4/91 and another G1-19 field strain. It seems unlikely that all those recombination events have occurred simultaneously during a production cycle. It is more possible that those multiple recombination events have occurred over multiple flocks, and strain LLN/111169 emerged after the recombination of previous intermedial recombinants; the exact recombination schemes between the predicted parental viruses that produced the recombinant LLN/111169 strain remain unknown. Therefore, the present results should be interpreted with caution, and further investigations are necessary to advance our understanding of the mechanisms involved in recombination events in IBVs. Currently, IBV prevention is mainly dependent on vaccination with modified live vaccines. The identification of recombination events between field and vaccines strains, resulting in novel IBV strains with altered features, raises significant concerns over the use of IB vaccines in chicken flocks. In addition, the findings of this study highlight the significance of constant epidemiologic and molecular surveillance for IBV.

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SUPPLEMENTARY DATA

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