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Genetics, antigenicity and virulence properties of three infectious bronchitis viruses isolated from a single tracheal sample in a chicken with respiratory problems

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ABSTRACT

Three different IBV genotypes/serotypes, designated ck/CH/LDL/150434–I (LDL/150434–I), ck/CH/LDL/150434–II (LDL/150434–II) and ck/CH/LDL/150434–III (LDL/150434–III), were detected in a single tracheal sample from a chicken showing signs of respiratory disease. The viruses were isolated using a cross-neutralization test and limiting dilution in embryonated specific-pathogen-free (SPF) eggs. Isolate LDL/150434–I was a re-isolation of H120 vaccine strain that was introduced into the chicken flock by vaccination, transmitted between chickens, and later accumulated several genomic mutations. Isolate LDL/150434–II was a novel variant that originated from recombination events between H120 and ck/CH/LDT3/03-like viruses. The widespread use of H120 vaccine, which offered incomplete protection against heterotypic IBVs in the fields, may play important roles in the emergence of such a novel genetic variant. Based on the analysis of S1 and complete genomic sequence, isolate LDL/150434–III was related genetically but distinct from the established strains of nrTW I type viruses of GI-7 lineage circulating in Mainland China since 2009. The three IBV isolates were avirulent when they infected SPF chickens. Furthermore, synergistic effects on pathogenicity were not observed when the different types co-infected the SPF chickens. However, the isolates persisted in the respiratory tracts longer in combined infected birds than those in individual infected birds. The results provide insights into the evolution of the viruses and co-infection of chickens with different virus serotypes.

1. Introduction

Avian infectious bronchitis coronaviruses (IBVs) are responsible for infectious bronchitis, a highly contagious upper respiratory disease of poultry that occurs sporadically or endemically worldwide. Clinically, the disease can induce a wide spectrum of symptoms, including mild respiratory disease; drop in egg production, hatchability and quality; nephritis; enteric problems; increased feed conversion; and carcass condemnation at slaughter (Cavanagh, 2007).Therefore, IB can cause severe economic losses. Moderate to severe morbidity and mortality have been noted, which vary depending on virulence of the infecting strain; age; status of immunity, either maternal or active; and stresses such as cold or/and secondary bacterial infections (Cavanagh, 2007).

IBV belongs to the *Gammacoronavirus* genus and has a positive sense, single-stranded RNA genome approximately 27.6 kb in length (Boursnell et al., 1987) with the general organization 5′ untranslated region (UTR) –1 a, ab (or polymerase genes) –S –3a, b, c(E) –M–4b, c–5a, b–N–6b–3′ UTR (Cavanagh, 2007; Ammayappan et al., 2009). The first 20 kb of the genome are made up of the polymerase gene. During IBV infection, virions bind to the target cell receptors and release the viral genome into the cytoplasm of the host cell and the polymerase genes are then translated. The papain-like and 3C-like proteases are released from the polymerase after an autocatalytic process. The protease initiates the cleavage *in trans* of the 15 peptides (nonstructural proteins) contained in the polymerase. These peptides, probably together with some cellular proteins, form the replication–-transcription complex, and initiate transcription of a series of five nested subgenomic RNAs that are translated into the viral structural and accessory proteins (Ziebuhr et al., 2001). The genes encode the structural proteins spike (S) glycoprotein, which is a surface, rod-shaped protein that is post-translationally cleaved into two subunits, S1 and S2 (Cavanagh, 1981; Stern and Sefton, 1982); envelope (E)

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glycoprotein; membrane (M) glycoprotein and nucleocapsid (N) protein (Boursnell et al., 1987). The 3a, 3b, 4b, 4c, 5a, 5b and 6b genes encode accessory proteins and their functions are still unknown (Bentley et al., 2013).

The S1 domain of the S glycoprotein of IBV contains the receptor-binding region and is a determinant of cell tropism (Wickramasinghe et al., 2011). It also carries virus-neutralizing and serotype-specific determinants of the virus, which can induce the production of specific neutralizing antibody and the hemagglutination inhibition antibody (Kant et al., 1992).Genotyping based on the S1 gene fragment is the most commonly used system for IBV classification. The high mutation rate of the S1 gene often occurs by accumulation of point mutations, deletions and insertions. During IBV replication, mutation generates extensive antigenic variability in the progeny, with multiple serotypes and variants that the vaccine strains may not offer full cross-protection against. This makes prevention and control of IB difficult.

Evidence from epidemiological studies has revealed that co-infection with IBV and other respiratory pathogens, such as avian influenza virus (AIV) H9 subtype, is widespread in poultry in Jordan and Egypt (Roussan et al., 2008; Hassan et al., 2017). It is also reported that co-infection with different IBV serotypes is occasionally observed in the same chicken flock (Dolz et al., 2008). Recombination events occur frequently, which is another reason for the emergence of a diverse number of IBV variant strains in the field (Jackwood et al., 2012). The first prerequisite for recombination in IBV is considered to be co-infection. Consequently, the frequency of co-infection by different IBV strains/types in the same bird appears to be high in the field. However, the effects on the replicative capacity and pathogenicity by co-infection with different IBV strains/types are not clear. In this study, we isolated three IBVs belonging to different serotypes with different genetic and antigenic features from one diseased chicken. The genetic, antigenic and pathogenic features of the three isolates were investigated in this study.

2. Materials and methods

2.1. Eggs and chickens

White Leghorn SPF chickens and fertile SPF chicken eggs were obtained from the Harbin Veterinary Research Institute, the Chinese Academy of Agricultural Sciences. The birds were maintained in isolators with negative pressure, and food and water were provided *ad libitum*. All experimental procedures were approved by the Ethical and Animal Welfare Committee of Heilongjiang Province, China.

2.2. Epidemiological background, virus isolation and purification

During the course of our conventional surveillance activities for IBV in 2015, a tracheal sample was collected from a diseased layer chicken in Dalian, China. The diseased chickens showed respiratory symptoms and had suspected IBV infection. The flock in which the diseased chicken was contained about 10,000 layers. All 1-day-old chickens were vaccinated against IBV with the commercial live attenuated H120 vaccine and then boosted at age 20 and 60 days with Ma5 and H120, respectively. Some birds in this flock showed early signs of respiratory disease when they were 92 days old and the clinical signs lasted for approximately 25 days. Gross examination showed mild to severe tracheitis, and some chickens showed nephritis. The morbidity was around 3%, and the mortality was low.

The tracheal sample was used for detection of the presence of avian metapneumovirus (AMPV) using a universal RT-PCR assay that can detect the four recognized subtypes of AMPV (Chacón et al., 2011). Meanwhile, virus isolation was conducted using 9-day-old specific-pathogen-free (SPF) chicken embryos as previously described (Liu and Kong, 2004). The S1 gene of IBV was amplified from the first passage of the infectious allantoic fluid using primers S1Oligo5′ and S1Oligo3′ (Kwon et al., 1993) and cloned into a pMD 18-T vector (Takara Bio, Shiga, Japan). Ten clones were sequenced and compared with sequences published in GenBank using BLAST. The virus population of the first passage was used for further purification.

One-hundred-fold dilutions of the first passage of infectious allantoic fluid were used for purification by virus-neutralization using sera against strains H120 (Han et al., 2016), ck/CH/LDL/140520 (Gao et al., 2016) and tl/CH/LDT3/03 (Han et al., 2016). The diluted allantoic fluids were reacted with serum against two of the three genotypes (Zhang et al., 2015; Han et al., 2016) at 37 °C for 1 h and then the mixtures were used for virus purification by limiting dilution in embryonated SPF eggs. Seral 10-fold dilutions of the mixtures were inoculated into the allantoic cavity of SPF chicken embryos, which were observed for 7 days. Three days post-inoculation, the allantoic fluids were used for virus detection by RT-PCR as previously described (Liu et al., 2014). Seven days post-inoculation, the eggs were opened and examined for lesions characteristic of IBV infection. The allantoic fluids of the eggs inoculated with the highest dilution that showed lesions characteristic of IBV infection, and that were also positive for RT-PCR detection, were used for another round of purification. Virus purification was confirmed by S1 amplification, cloning and sequencing of at least 10 clones of each virus. After three rounds of virus neutralization, three viruses were isolated, purified and designated as ck/CH/LDL/150434–I (LDL/150434–I), ck/CH/LDL/150434–II (LDL/150434–II) and ck/CH/LDL/150434–III (LDL/150434–III).

The virus stocks of the three purified viruses were produced by inoculating the virus into embryonated SPF chicken eggs via the allantoic cavity and collecting the infectious allantoic fluid 72 h post-inoculation. The allantoic fluid was clarified by centrifugation at 3000 × *g* for 10 min. Before being used, the viral stocks of our three IBV isolates were detected for the presence of the AMPV (Chacón et al., 2011) as described above and tested for haemagglutination (HA) activity for detecting the viruses showing HA activity, such as avian influenza virus (AIV) and Newcastle disease virus (NDV). The viruses were used as stocks for further complete genome sequencing, virus cross-neutralization tests and virulence studies.

2.3. Sequencing and analysis of the S1 gene

The RNA of each purified virus was extracted using TRIzol reagent (Invitrogen, Carlsbad, CA, USA). The S1 gene was amplified using primers S1Oligo5′ and S1Oligo3′ (Kwon et al., 1993) with a One-Step RT-PCR kit (Takara) and cloned into a pMD 18-T vector (Takara) (Liu and Kong, 2004).Ten obtained clones were subjected to sequencing in both directions using Sanger sequencing technology. The obtained sequences from each virus were edited and aligned in a final consensus sequence using the SeqMan program (DNASTAR, Madison, WI, USA).

The BLAST program was used to search GenBank for IBV sequences that were homologous to the S1 gene of each of the three IBV isolates. The S1 genes from 40 IBV reference strains were selected for a maximum likelihood tree construction based on the JTT matrix-based model and 1000 bootstrap replicates using Mega (MEGA software version 6; available at http://www.megasoftware.net/).

2.4. Sequencing and analysis of the complete genome

To confirm the classification and further analyze the genetic features of the three IBVs, the complete genomes of the three isolates were amplified and sequenced as previously described (Liu et al., 2013). Some of the primers did not work due to sequence differences; hence, new primers were designed based on the newly determined sequences flanking those genome regions. The 3′ and 5′ ends of each virus genome were determined using 3′ and 5′ RACE protocols, respectively, as previously described (Liu et al., 2013). Positive bands of the expected size that had strong signals and without additional bands were used for sequencing. All regions were sequenced at least three times. The

A

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ck/CH/LDL/150434-I     AAGTCCACTACGAAGGAAACCCCATTTTCTAAAAAGGTTGTTGTAGGTTGTGGTCCCATTATAAGAAGGATTAAATGGATTAAACCAC  25467
ck/CH/LDL/150434-II    AAGTCAGCTACGAAGGAACCCCCATCTTTAAAAAAGGTTGTTGCAGATTGTGGTCGAGTTATAAAAAAGATTAGA---------CCAC  25461
ck/CH/LDL/150434-III   AAGTCCACTACCAAGGAACCCCTATCTTTCAAAAAGGTTGTTGTAGGCTCTGGTCCCATTATAAAAAGGATTAGAATTATTAAGCCGC  25515

                                                              Start codon of 5a ***
ck/CH/LDL/150434-I     CTACACTACTTACTTGTAATAAGGGCGTTTGGACTTACAAGCGCTTAA---CAAATACAGACGATGAAATGGCTGACTAGTTTTGGAA  25536
ck/CH/LDL/150434-II    CTACAACACTAGTTTCTAATAAAGGCGTTTGGACTTACAAGCGCTTAAAAACAAATACAGACGATGAAATGGCTGACTAGTTTTGGAA  25533
ck/CH/LDL/150434-III   CAACTACACTTATTTTTATAAGAGGCGTTTTATCTTACAAACGCTTAA---CAAATACGGACGATGAAATGGCTGACTAGTTTTGGAA  25584

ck/CH/LDL/150434-I     GAGCAGTTATTTCTTGTTATAAAGCCCTACTATTAACTCAGTTAAGAGTATTAGATAGGTTAATTTTAGATCACGGACCAAAACGCGT  25624
ck/CH/LDL/150434-II    GGGCATTCATCTCTTGTTATAAATCCCTATTATTAACTCAGCTTAGAGTTTTAGATAGGTTAATTTTAGAGCACGGACCAAAAAGTAC  25621
ck/CH/LDL/150434-III   GAGCAGTTATTTCATGTTATAAAGCCTTACTATTAAATCAATTAAGATTGTTAGATAGGTTAATTCTAGATCACGGACCGAGGCGTAC  25672

                                                                                Start codon of 5b ***
ck/CH/LDL/150434-I     CTTAACGTGTGGTAGGCGAGTGCTTTTATCTCAATTAGATTTAGTTTATAGGTTGGCATATACGCCCACCCAATCGCTGGTATGAATA  25712
ck/CH/LDL/150434-II    ATTAACGTGTGCTAGGCGAGTGCTTTTAGTTCAATTAGATTTAGTTTATAGGTTGGCATATACTTCCAACCATTCGCTGGTATGAATA  25709
ck/CH/LDL/150434-III   TTTAAGTTGTGCCAGGCGCGTGCTTTTAGTTCAATTAGATTTAGTTTATAGGTTGGCTTATACGCCCGACCAGTCGCTGGTATGAATA  25760
                                                                                 Stop codon of 5a ***

ck/CH/LDL/150434-I     ATAGTAAAGATAATCCTTTTCGCGGAGCAATAGCAAGAAAAGCGCGAATTTATCTGAGAGAAGGATTAGAGTGTGTTTACTTTCTTAA  25800
ck/CH/LDL/150434-II    ATAGTAAAGATAATCCTTTTCGCGGAGCAATAGCAAGAAAAGCGCGAATTTATCTGAGAGAAGGATTAGATTGTGTTTACTTTCTTAA  25797
ck/CH/LDL/150434-III   ATAGTAAAGATAACCCTTTTCGCGGAGCAATAGCAAGAAAAGCGCGAATTTATCTGAGAGGAGGATTAGATTGTGTTTACTAACTTAA  25848

ck/CH/LDL/150434-I     CAAAGCAGGACAAGCAGAGCCTTGTCCCGCGTGTACCTCCCTAGTATTCCAAGGGAAAACTTGTGAGGAACACACAGATAATAATAAT  25888
ck/CH/LDL/150434-II    CAAAGCAGGACAAGCAGAGCCTTGTCCCGCGTGCACATCACTAGTATTCCAAGGGAAAACTTGTGAGGAGCACATAAATAATAACAAC  25885
ck/CH/LDL/150434-III   CAAAGCAGGACAAGCAGAGCCTTGTCCCGCGTGCACATCACTAGTATTCCAAGGGAAAACTTGTGAGGAGCACATACATAATAACGAC  25936
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B

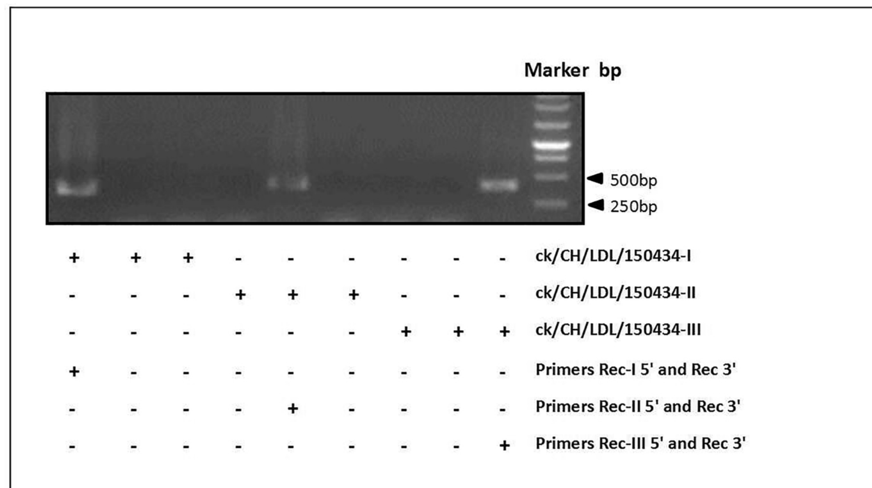

Fig. 1. Differentiation of IBV isolates LDL/150434-I, LDL/150434-II and LDL/150434-III by RT-PCR. Multiple sequence alignment of ORF5 and flanking sequences, and the positions and sequences of the primers (in black) using for differentiate the three IBV isolates (A). The numbers on the right of each alignment show the nucleotide positions in the genome of each virus. The three IBV isolates were identified directly by RT-PCR individually using primers Rec-I 5′ and Rec 3′, Rec-II 5′ and Rec 3′, and Rec-III 5′ and Rec 3′, respectively (B). Each of the viruses was shown to be positive only using its specific primers.

obtained nucleotide sequences were manually edited by removing any residual PCR primer sequences and then assembled, and analyzed using the Clustal W method available in the BioEdit software package (version 7.0.3.0; http://www.mbio.ncsu.edu/bioEdit/bioedit) to produce the final sequences of the viral genomes. They were compared using BLAST with sequences published in GenBank. Phylogenetic trees were inferred with the complete genomic sequences of the strains corresponding to those of the S1 genes that were available in GenBank, using the maximum likelihood method as described above.

The complete genomic sequence of isolate LDL/150434–II was further compared with that of reference strains ck/CH/LGX/130530, tl/CH/LDT3/03, LDT3-A, H120 and M41 using the Multiple Alignment with Fast Fourier Transformation (MAFFT) version v6 (http://mafft.cbrc.jp/alignment/software/). Meanwhile, the nucleotide identities were calculated to determine distances between each of our isolates and reference strains.

To confirm the possible recombination, SimPlot analysis was performed using the nucleotide alignment of the genome sequences of isolate LDL/150434–II, strains tl/CH/LDT3/03 and H120, and SimPlot version 3.5.1, as described previously (Lole et al., 1999). The analysis was conducted using a sliding window of 1000 nucleotides moving in 100-nucleotide steps with genome sequences of strain 4/91 as the query. Possible recombination sites suggested by the MAFFT and SimPlot analysis were confirmed through multiple sequence alignments that were separated and pairwise compared based on the potential breakpoints into non-recombined regions, to identify the potential recombination switch sites and clarify the genetic relationship of each fragment of isolate LDL/150434–II with those of H120, M41, tl/CH/LDT3/03 and LDT3-A.

2.5. GenBank accession numbers

The complete genomic sequences of three IBV isolates LDL/150434–I, LDL/150434–II and LDL/150434–III were deposited into GenBank (accession numbers KT736031, KT736032 and KX077987, respectively).

2.6. Virus cross-neutralization tests

The titers of the three IBV isolates were determined using 10-day-old embryonated chicken eggs. The 50% embryo infectious dose (EID_{50}) was calculated by the method of Reed and Muench (1938). Sera against the three IBV strains were prepared as previously described (Gao et al., 2016). All sera were inactivated at 56 °C for 30 min and stored in 2.0 ml aliquots at −80 °C until required. The β VN method with constant virus and diluted serum was used in SPF chicken embryos for serotyping (Liu et al., 2013).

2.7. Virulence studies in 7-day-old chickens

Animal experiments were performed to gain insight into whether an increase in virulence occurred when different strains/types of IBV co-infected SPF chickens. Seven groups of 10 1-day-old SPF chickens were placed in isolators and challenged by oculonasal administration at 7 days old. Birds in groups 1–3 were challenged with 10^5 EID_{50} per chicken of /LDL/150434–I, LDL/150434–II or LDL/150434–III, respectively, in 0.1-ml volumes. Birds in group 4 were challenged with 10^5 EID_{50} of LDL/150434–II and 10^5 EID_{50} of LDL/150434–III (LDL/150434–II + III). Birds in group 5 were challenged with 10^5 EID_{50} of LDL/150434–I, 10^5 EID_{50} of LDL/150434–II and 10^5 EID_{50} of /LDL/150434–III (LDL/150434–I + II + III). Birds in group 6 were challenged with 10^5 EID_{50} of M41 (as a positive control). Birds in group 7 were mock-inoculated with sterile allantoic fluid and served as the negative control. Nasopharyngeal swabs and blood samples were collected from all birds at 4, 8, 12, 16, 20 and 24 days after challenge. 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). The nasopharyngeal swabs were placed into separate tubes containing 0.6 ml PBS containing antibiotics (2000 U/ml penicillin G, 200μg/ml gentamicin sulfate, and 4μg/ml amphotericin B; Sigma–Aldrich, St Louis, MO, USA) and stored at −70 °C until virus recovery. The serum collected in this study was stored at −70 °C until ELISA testing. Chickens were examined daily for signs of infection for 30 days after inoculation.

2.8. Virus recovery and detection

To identify and differentiate the three IBV isolates, the complete genomic sequences of our three purified viruses were aligned and the OFR5 and flanking sequences were selected for genome-sense and anti-genome-sense primers design, respectively, because of the abundance of this fragment in the virions (Spencer and Hiscox, 2006).Three genome-sense primers (Rec-I 5′, Rec-II 5′ and Rec-III 5′), which specifically identified each of the three viruses, and one consensus anti-genome-sense primer (Rec 3′) were designed. The positions and sequences of the primers are illustrated in Fig. 1A. The specificity and validity of the primers were confirmed by amplifying the three viruses using each of the primers (Fig. 1B).

To investigate virus shedding, the viruses were recovered from the oropharyngeal swab samples of the challenged chickens as described previously (Zhang et al., 2015). Each nasopharyngeal swab tube was centrifuged at 1500 × g for 10 min and the supernatant samples were inoculated into SPF embryonated eggs via the allantoic cavity. The eggs were candled daily to examine for characteristic IBV lesions in the embryos. Allantoic fluid from the inoculated embryos was collected at 72 h post-inoculation and subjected to RT-PCR amplification for differentiating the three viruses using the aforementioned primer pairs. A positive sample was recorded if specific lesions were observed and the RT-PCR amplification was positive.

To confirm that the three purified viruses were from a tracheal sample and had a natural origin, and were not a methodological artifact that originated during inoculation of the eggs, the supernatant of the tracheal swab had been used for virus isolation was used for direct amplification with the primers Rec 3′ and Rec-I 5′, Rec 3′ and Rec-II 5′ and, and Rec 3′ and Rec-III 5′respectively, using a One-Step RT-PCR kit (Takara).

3. Results

3.1. Three genotypes of IBV were isolated from a single trachea

The tracheal sample was negative for AMPV and other viruses showing HA activity such as AIV and NDV. However, obvious lesions, such as dwarfing, stunting, or curling of embryos, were observed at the first passage. The S1 gene of IBV was detectable using RT-PCR from the first passage when the infectious allantoic fluid was collected 72 h post inoculation using primers S1Oligo5′ and S1Oligo3′ (Kwon et al., 1993).

BLAST searching revealed three distinct types of S1 gene sequences (H120-, Taiwan I [TW I]- and tl/CH/LDT3/03-like) in the amplification products, implicating that at least three different IBV types co-existed in the tracheal sample. Then, three IB viruses, designated as LDL/150434–I, LDL/150434–II and LDL/150434–III, were isolated and purified from a single tracheal sample from a diseased chicken suspected to be infected with IBV, using virus-neutralization and three rounds of limiting dilution analysis. This result was confirmed by RT-PCR that was used to amplify the tracheal sample in which the three viruses were detected and isolated (data not shown), using three sets of specific primers targeting the sequences corresponding to ORF5 and flanking sequences of the three IBV isolates.

According to the above results, H120, M41, tl/CH/LDT3/03 and LDT3-A, and four TW I genotype IBV strains, TW1171/92, TW2575/98, T03/01 and ck/CH/LHLJ/141105, which were isolated in Taiwan and Mainland China in 1992, 1998, 2001 and 2014 (Wang and Tsai, 1996; Xu et al., 2016), respectively, were selected for comparison of S1 gene sequences. Phylogenetic analysis of the complete S1 genes showed that isolate LDL/150434–I belonged to Massachusetts (Mass) genotype (Fig. 2A) and was more closely related to H120 vaccine strain than other Mass strains. This hypothesis was supported by the analysis of nucleotide and amino acid identities shared between isolate LDL/150434–Iand H120 (99.8% and 99.6%, respectively), and between isolate LDL/150434–Iand M41 strain (97.6% and 96.5%, respectively). Isolate LDL/150434–II formed a separate group with the tl/CH/LDT3/03-like (Liu et al., 2005) viruses. The nucleotide and amino acid sequence identities reached 99.8% and 99.1% between isolate LDL/150434–IIand the vaccine strain LDT3-A, and 99.6% and 99.0% between isolate LDL/150434–II and strain tl/CH/LDT3/03, respectively. Isolate LDL/150434–III showed a close relationship with TW I-like viruses (Xu et al., 2016), however, it was grouped to a sub-lineage within this group (Fig. 2A). The nucleotide and deduced amino acid sequence identities ranged from 90.2% (between LDL/150434–III and ck/CH/LHLJ/141105) to 96.4% (between LDL/150434–III and T03/01), and from 87.1% (between LDL/150434–III and ck/CH/LHLJ/141105) to 95.4% (between LDL/150434–III and TW2575/98), respectively (Table 1).

Comparison of deduced amino acid sequences of S1 subunit of S proteins, especially in the hypervariable regions (HVRs) I, II and III, between our three isolates and the reference viruses showed that LDL/150434–Iand H120 shared nearly identical sequences. LDL/150434–II was similar to both tl/CH/LDT3/03 and vaccine strain LDT3-A, and LDL/150434–III was closely related to TW I-like viruses (Fig. 3). The differences between the three IBV isolates were mainly at the regions corresponding to known HVRs: HVR1, residues 50–69; HVR2, residues 117–131; and HVR3, residues 269–289 (corresponding to the S1 subunit of S protein of H120 vaccine strain). These results were in agreement with phylogenetic analysis based on the S1 nucleotide sequences.

3.2. Characteristics of the complete genomes of the three IBV isolates

The complete genome sequences of isolates LDL/150434–I, –II and

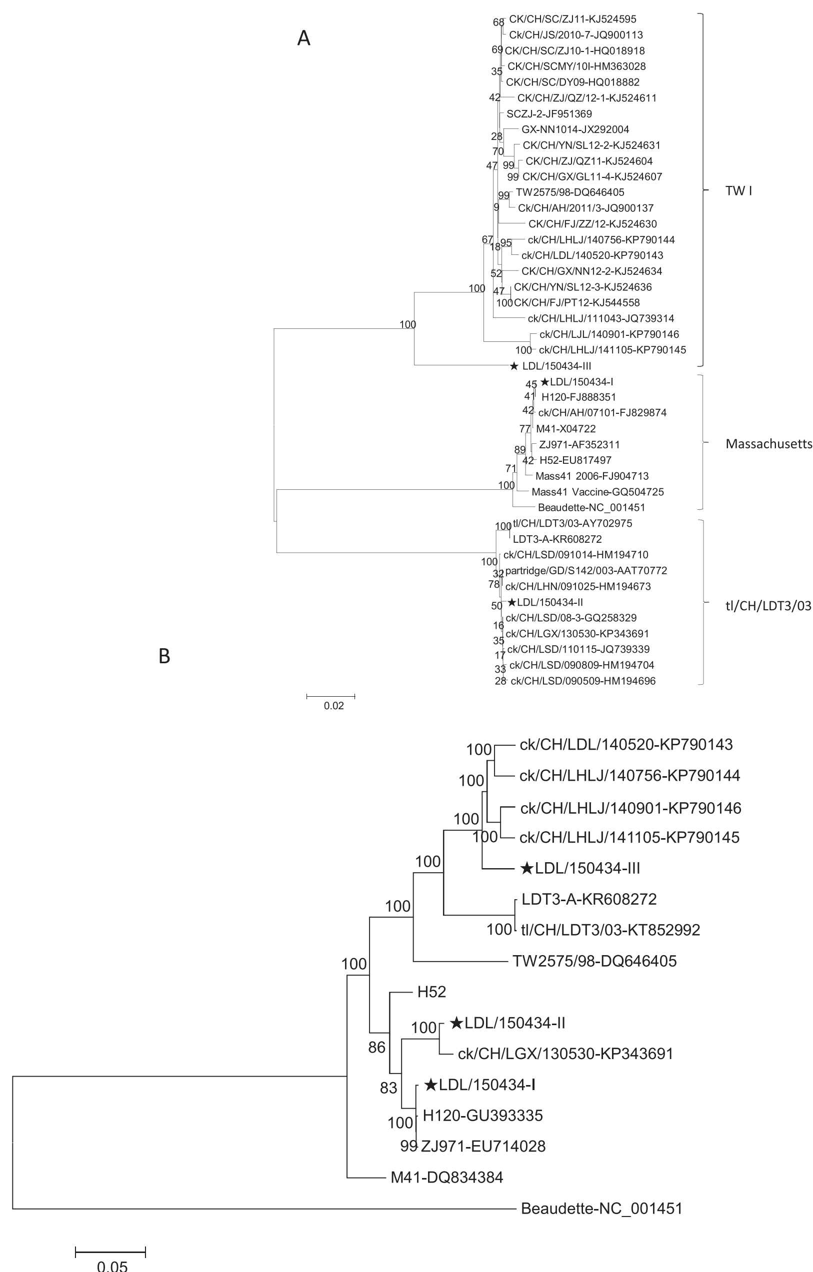

Fig. 2. Maximum-likelihood phylogenetic trees constructed based on the complete S1 genes of the 35 reference IBV strains and our three isolates (the first 1592 nucleotides, starting at the AUG translation initiation codon, of the S gene) (A) and complete genomic sequences of 13 reference strains and our three isolates (B). Bootstrap values are shown next to the branches. The scale bar indicates the number of nucleotide substitutions per site. Our three IBV isolates are indicates by black stars. The GenBank accession numbers for the reference IBV strains are showed after the names of each virus in the trees.

–III were 27 630, 27 603 and 27 654 nucleotides in length, respectively, excluding the 3′ poly A tails. Putative genes and regions of isolates LDL/150434–I, -II and –III are shown in Table 2, compared with those of IBV reference strains H120, M41, tl/CH/LDT3/03, LDT3-A, TW2575/98 and ck/CH/LHLJ/141105. In parallel with the phylogenetic results from the S1 gene, isolate LDL/150434–I formed a similar group with the H120 vaccine strain based on the complete genomic sequences (Fig. 2B), and was genetically similar to H120 (99.7%) compared to the M41 strain (91.2%). Unlike the S1 gene, LDL/150434–II belonged to a new sub-group showing a close phylogenetic relationship with a natural recombinant virus, tl/CH/LGX/130530, which originated from recombination between parental IBV H120 strain and pathogenic tl/CH/LDT3/03-like virus (Han et al., 2016). LDL/150434–II shared 98.7% and 95.7% nucleotide identity with tl/CH/LGX/130530 and H120, respectively, compared to approximately 89.6% with tl/CH/LDT3/03 and LDT3-A. Isolate LDL/150434–III was found to be a unique member of a cluster which closely related natural recombinatant TW I (nr TW I) strains (95.5% identity with ck/CH/LHLJ/141105) circulating in Mainland China in recent years (Xu et al., 2016). Comparatively, LDL/150434–III was distinct from strain TW2575/98 (87.4% identity).

In addition, isolate LDL/150434–I shared 95.7% and 86.5% nucleotide identities with isolates LDL/150434–II and LDL/150434–III, respectively. Isolate LDL/150434–II shared 87.8%nucleotide identity with isolate /LDL/150434–III.

3.3. Recombination analysis

Phylogenetic analysis showed inconsistent positions for isolate LDL/150434–II in S1 and complete genome trees (Fig. 1A and B); therefore, we performed MAFFT and SimPlot analysis on this virus using the

Table 1
Amino acid and nucleotide similarities of the S1 gene[a] among the three IBV isolates and other IBV strains[b].

Strain	1	2	3	4	5	6	7	8	9	10	11
							Amino acid identity (%)				
1. LDL/150434–I		99.6	96.5	80.0	80.0	79.3	82.4	81.5	80.0	82.3	83.0
2. H120	99.8		96.3	80.0	80.0	79.3	82.4	81.5	80.0	82.3	83.0
3. M41	97.6	97.6		79.5	79.5	78.7	82.1	80.6	78.9	81.3	82.1
4. LDL/150434–II	82.6	82.7	83.0		99.6	99.0	81.5	82.4	81.0	83.7	83.0
5. LDT3-A	80.8	81.0	81.0	99.8		97.8	81.5	82.4	81.0	83.7	83.0
6. tl/CH/LDT3/03	82.5	82.5	82.9	99.1	81.4		80.6	81.7	80.2	83.2	82.3
7. LDL/150434–III	81.6	81.5	82.0	82.5	80.8	82.3		89.3	87.1	88.5	93.0
8. TW2575/98	82.2	82.3	82.3	82.9	81.5	82.7	92.6		95.4	90.2	91.9
9. ck/CH/LHLJ/141105	80.6	80.7	80.9	82.5	81.1	82.5	90.2	96.1		88.4	89.1
10. T03/01	82.6	82.5	82.7	83.0	81.4	83.0	96.4	95.0	92.1		91.1
11. TW1171/92	82.5	82.6	82.7	84.7	81.0	84.6	93.0	94.5	92.3	94.7	
				Nucleotide identity (%)							

[a] First 1623 nucleotides, starting at the AUG translation start codon, of the S1 gene were compared.
[b] IBV reference strains were selected for comparison based on the results of phylogenetic trees using the complete sequences of S1 genes.

complete genomic sequence. Results from MAFFT (Fig. 4) and SimPlot (Fig. 5A) analysis clustered between the isolate LDL/150434–II and strain H120 from 5′ UTR to the middle part of non-structural protein 16, however, clustered between the isolate LDL/150434–II and strain tl/CH/LDT3/03 from the remaining genomes (Figs. 4 and 5A). A multiple sequence alignment using isolate LDL/150434–II and strains H120 and tl/CH/LDT3/03 further indicated that upstream of position 19932, isolate LDL/150434–II possessed nucleotides identical to strain H120; however, isolate LDL/150434–II possessed nucleotides identical to strain tl/CH/LDT3/03 downstream of 19934 (Fig. 5B).

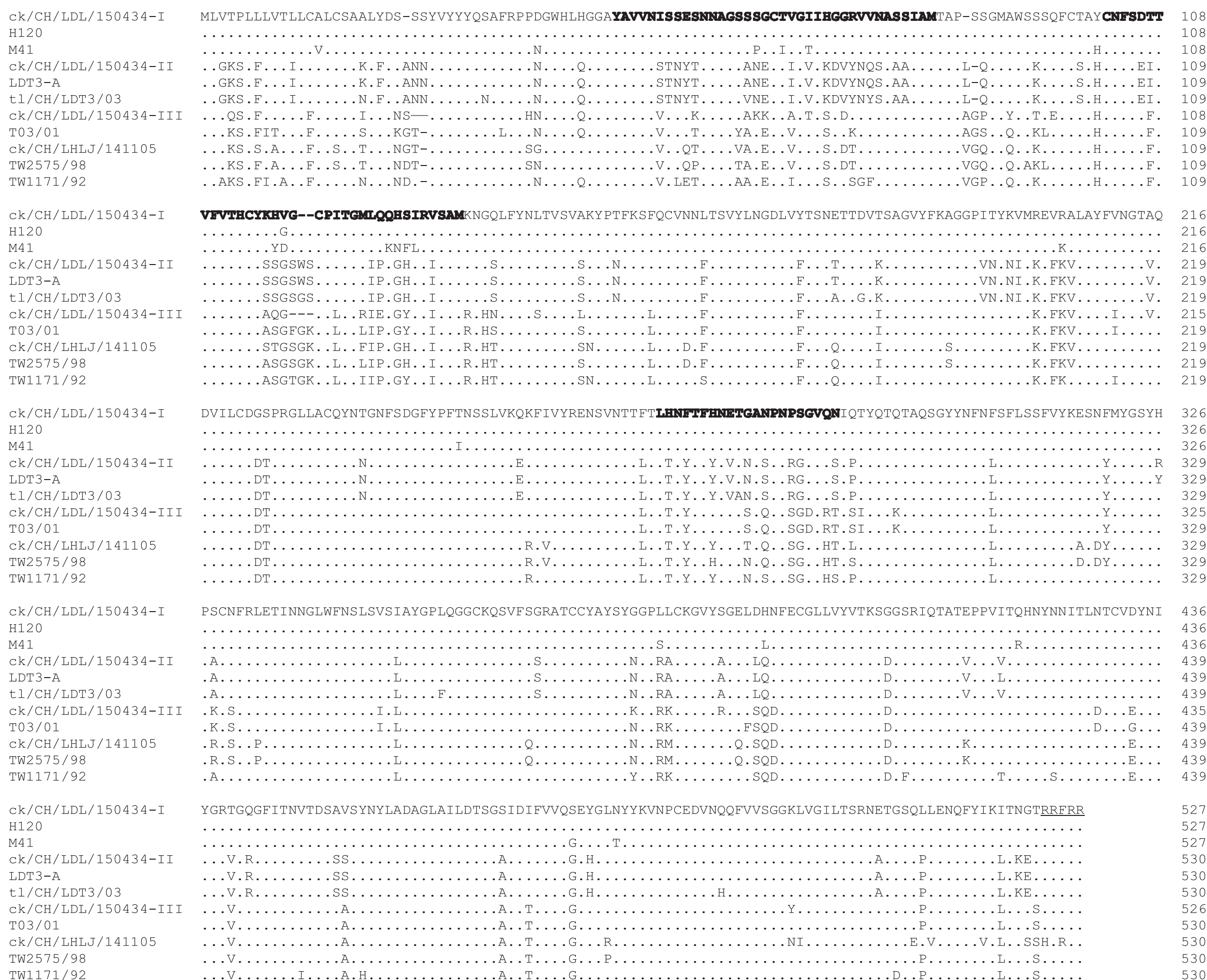

Fig. 3. Multiple sequence alignment of S1 amino acid sequences from eight IBVs with those of our three isolates LDL/150434-I, LDL/150434-II and LDL/150434-III. The numbers on the right of each alignment show the nucleotide positions in S1 subunit of S protein of each virus. The sequences of LDL/150434-I are listed and only amino acids differing from those of LDL/150434-I are depicted. The three HVRs are bold. The deleted nucleotides are represented as –.The cleavage sites of S1 subunit of S protein are underlined. The GenBank accession numbers are: T03/01(AY606315) and TW1171/92(DQ646406). The GenBank accession numbers of other IBV strains are listed in Fig. 2.

Table 2
Comparison of putative genes/regions in our three IBV isolates LDL/150434–I, –II and –III with those in six reference strains.

Strain	5' UTR	Gene 1		S	Gene 3			M	Gene 5		N	3' UTR
		ORF1a	ORF1ab		3a	3b	3c		5a	5b		
LDL/150434–I	1-528 (528)[a]	529-12330 (11,802)	529-20363 (19,835)	20314-23802 (3489)	23802-23975 (174)	23975-24169 (195)	24150-24479 (330)	24451-25128 (678)	25488-25685 (198)	25682-25930 (249)	25873-27102 (1230)	27103-27630 (528)
H120	1-528 (528)	529-12330 (11,802)	529-20363 (19,835)	20314-23802 (3489)	23802-23975 (174)	23975-24169 (195)	24150-24479 (330)	24451-25128 (678)	25488-25685 (198)	25682-25930 (249)	25873-27102 (1230)	27103-27630 (528)
M41	1-529 (529)	530-12391 (11,862)	530-20424 (19,895)	20375-23863 (3489)	23863-24036 (174)	24036-24230 (195)	24211-24540 (330)	24512-25189 (678)	25540-25737 (198)	25734-25982 (249)	25925-27154 (1230)	27155-27475 (321)
LDL/150434–II	1-528 (528)	529-12330 (11,802)	529-20363 (19,835)	20314-23811 (3498)	23811-23984 (174)	23984-24172 (189)	24156-24482 (327)	24454-25131 (678)	25485-25682 (198)	25679-25951 (273)	25870-27099 (1230)	27100-27603 (504)
tl/CH/LDT3/03	1-529 (529)	530-12,421 (11,892)	530-20454 (19,925)	20405-23902 (3498)	23902-24075 (174)	24075-24263 (189)	24247-24573 (327)	24545-25222 (678)	25569-25766 (198)	25763-26035 (273)	25954-27183 (1230)	27184-27699 (516)
LDT3-A	1-529 (529)	530-12,421 (11,892)	530-20430 (19,901)	20381-23878 (3498)	23878-24051 (174)	24051-24239 (189)	24223-24549 (327)	24521-25198 (678)	25552-25749 (198)	25746-26018 (273)	25937-27166 (1230)	27167-27681 (515)
/LDL/150434–III	1-525 (525)	526-21420 (11,895)	526-20417 (19,892)	20368-23853 (3486)	23853-24026 (174)	24026-24214 (189)	24201-24527 (327)	24499-25176 (678)	25536-25733 (198)	25730-25978 (249)	25921-27150 (1230)	27151-27654 (504)
TW2575/98	1-526 (526)	527-12454 (11,928)	527-20487 (19,961)	20438-23935 (3498)	23935-24108 (174)	24108-24302 (195)	24283-24612 (330)	24584-25261 (678)	25621-25818 (198)	25815-26063 (249)	26006-27235 (1230)	27236-27710 (475)
ck/CH/LHLJ/141105	1-525 (525)	526-12420 (11,895)	526-20417 (19,892)	20368-23865 (3498)	23865-24038 (174)	24038-24226 (189)	24213-24539 (327)	24511-25188 (678)	25552-25749 (198)	25746-25994 (249)	25937-27166 (1230)	27167-20417 (251)

[a] Sizes of the putative genes/regions in parentheses.

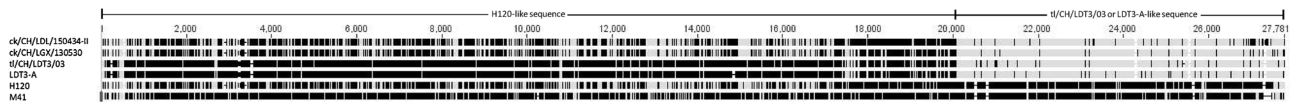

Fig. 4. Multiple alignments of the complete genomes of isolate LDL/150434-I and five reference strains performed using MAFFT version 6 (http://mafft.cbrc.jp/alignment/software/). The numbers at the top the alignment show the nucleotide positions in the genome of isolate LDL/150434-I. The genome sequence of the IBV LDL/150434-I was set as the reference sequence. The nucleotide sequences of other viruses that disagree with the reference sequence at the indicated positions are represented in black, while the nucleotide sequences of other viruses that agree with the reference sequence at the indicated positions are represented by gray. The GenBank accession numbers of other IBV strains are listed in Fig. 2.

We compared the nucleotide sequences of the 3′ 7.0-kb region of IBV isolate LDL/150434-II with those of pathogenic tl/CH/LDT3/03 and LDT3-A vaccine strains, and 35 mutations were found between isolate LDL/150434-II and pathogenic tl/CH/LDT3/03 and LDT3-A. Of the 35 mutations, 19 were the same in the isolate LDL/150434-II and vaccine LDT3-A strain, but in contrast, 16 mutations were the same in the isolate LDL/150434-II and pathogenic tl/CH/LDT3/03. A 7-bp deletion at positions 25,266–25,272 in the genome was found in pathogenic tl/CH/LDT3/03, compared to that of isolate LDL/150434-II and vaccine LDT3-A strain (Table 3).

3.4. Antigenic properties of the three IBVs by virus cross-neutralization tests

As illustrated in Table 4, virtually no cross-neutralization was observed between the three isolates, which confirmed the absence of an antigenic relationship between the three isolates.

3.5. Pathogenic properties of the three IBVs

In some chickens, respiratory signs started at 2 days post-challenge with M41 strain. At 5 days post-challenge, symptoms were observed in nearly all birds in this group, with respiratory signs including mild to severe respiratory depression and distress, nasal discharge, sneezing, coughing, rales, and watery eyes. The respiratory signs disappeared within 10 days after the appearance of the disease. For the chickens challenged with isolates LDL/150434-II, LDL/150434-III, LDL/150434-II + III and LDL/150434-I + II + III, only two or three showed mild signs such as shaking head, and most did not show obvious clinical signs. The isolate LDL/150434-I and mock-inoculated control chickens were free of respiratory signs. The mean scores of the chickens in each group were illustrated in Fig. 6.

Virus was detectable from oropharyngeal swabs of all the chickens challenged with LDL/150434–I at days 4 and 8 post-challenge (Table 5). The numbers of chickens shedding virus started to decrease progressively and none shed virus after 20 days post-challenge. Some of the chickens were still shedding viruses from the respiratory tract at 20 and 24 days post-challenge in the combined infection group. No viruses were detected in the negative group.

Most of the chickens in both the individual and combined infected groups seroconverted by 8 days. All of the chickens challenged with viruses had seroconverted by 12 days post-challenge (data not shown). We did not observe seroconversion in any chickens at 4 days post-challenge or in any chickens in the negative control group.

4. Discussion

In this study, three different IBV serotypes were detected and isolated from a single tracheal sample from a chicken with respiratory symptoms suggestive of IBV infection. To our knowledge, although co-infection with different single-stranded RNA viral families in the same

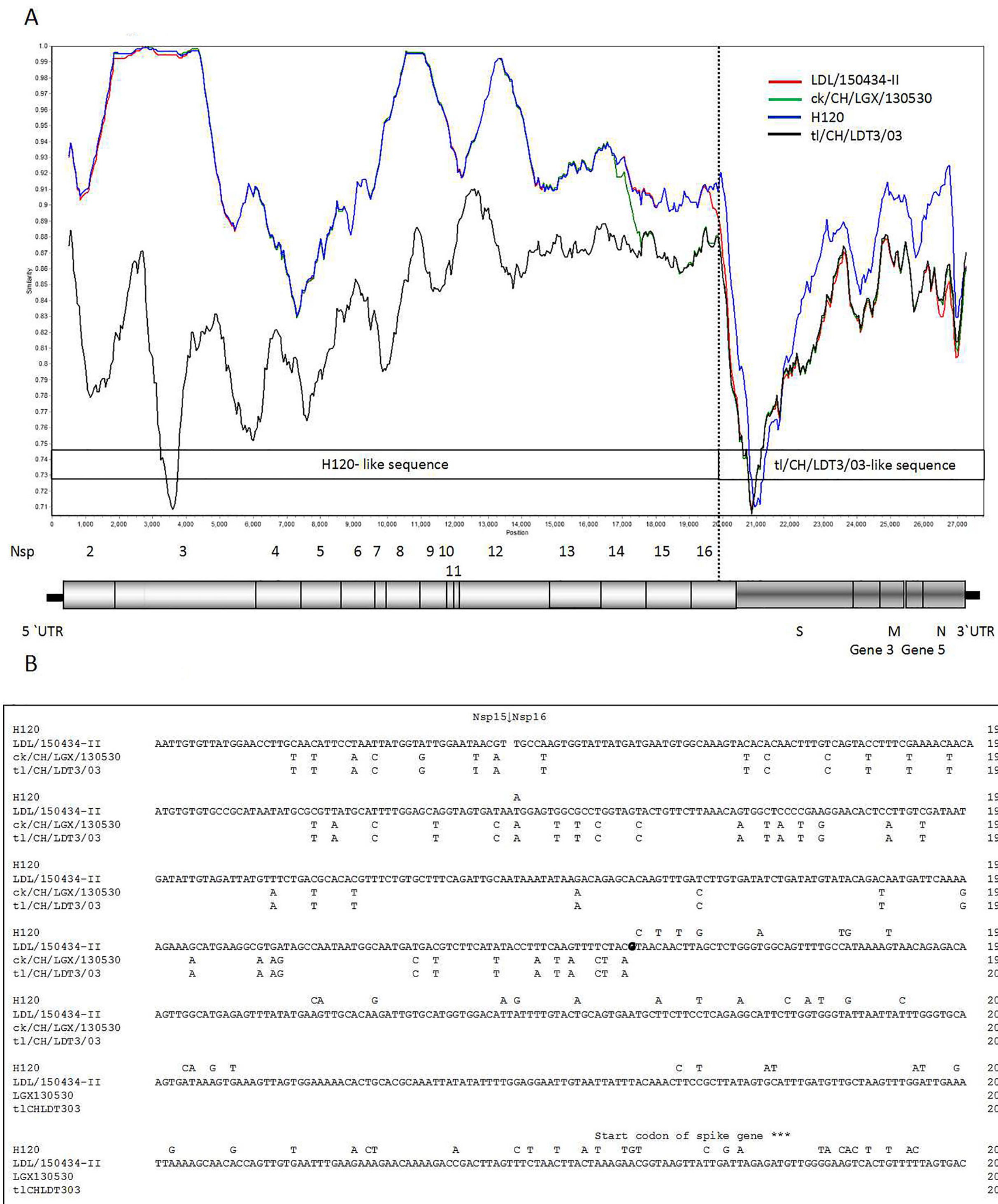

Fig. 5. Recombination analysis of the IBV isolate LDL/150434-I. Similarity plot using M41 as the query sequence (A). The dotted lines show the deduced recombination breakpoints. The hollow arrows show the different fragments. The numbers show the nucleotide positions of the corresponding fragments in the genome of LDL/150434-II. Multiple sequence alignment of the predicted breakpoint and flanking sequences among IBV ck/CH/LDL/150434-II, H120, tl/CH/LDT3/03 and ck/CH/LGX/130530-A strains (B). The numbers on the right of each alignment show the nucleotide positions in the genome of each virus. The sequences of LDL/150434-II are listed, and only the nucleotides differing from those of ck/CH/LDL/150434-II are depicted. The region where the template switches (breakpoint) have taken place is bold. The GenBank accession numbers are listed in Fig. 2A.

animal has only been reported in two studies (Kemenesi et al., 2014; Rizzo et al., 2017), infection with multiple coronaviruses in the same species/bat/colony is well known and has been previously reported in China and Europe (Ge et al., 2013; Lau et al., 2010; Yuan et al., 2010). Increased surveillance of IBV has resulted in a large number of variants being discovered in the vaccinated and non-vaccinated flocks. However, co-infection with multiple genotypes of IBV in the same bird is scare and was only found in a single epidemiological study in 2013 in European broilers (de Wit et al., 2017), which detected two different IBV genotypes in the same tracheal sample. To control IBV infection, vaccination remains the most effective means. However, IBV infection is difficult to control because the virus has extensive genetic diversity

Table 3
Pairwise comparisons of nucleotide sequences of the 3′ 7.0-kb region of IBV isolate LDL/150434-II with those of pathogenic tl/CH/LDT3/03 and LDT3-A vaccine strains.

Strain	Genome position																	
	20470	20411	20501	20542	20544	20674	20857	20867	20895	21154	21298	21382	21574	21810	21894	22886	22967	23061
	S																	
	57	98	188	229	231	361	544	554	575	841	985	1069	1268	1497	1581	2573	2654	2748
1	A	G	C	C	A	T	A	A	A	A	T	C	T	G	C	T	T	C
2	A	G	C	C	A	T	A	A	A	A	C	C	G	G	C	C	T	T
3	T	A	T	T	T	G	G	G	G	G	C	T	G	C	T	C	C	T
Strain	Genome position																	
	23441	23676	24279	24369	24520	24927	24972	25132	25241	25266-25272	25534	25660	26043	26658	27675	27104	27464	27582
	S		3c		M			M-5a			N					3'UTR		
	3128	3363	124	214	67	474	519	1	110	135-141	50	176	176	509	997	1	361	478
1	T	C	T	A	T	A	T	A	T	TAAGCAA	T	T	T	T	T	T	T	G
2	C	T	G	C	C	A	C	A	C	TAAGCAA	T	C	C	C	T	C	T	A
3	C	T	G	C	C	T	C	G	C	-------	C	C	C	C	C	C	A	A

We compared the nucleotide sequences of the 3′ 7.0-kb region of IBV isolate LDL/150434-II with those of pathogenic tl/CH/LDT3/03 and LDT3-A vaccine strains. Of the 35 mutations, 19 were the same in the isolate LDL/150434-II and vaccine LDT3-A strain (in gray); in contrast, 16 mutations were the same in the pathogenic tl/CH/LDT3/03 and isolate LDL/150434-II. In addition, a 7-bp deletion at position 25,266–25,272 in the genome was found in pathogenic tl/CH/LDT3/03, compared to those of isolate LDL/150434-II and vaccine strain LDT3-A. Nucleotide positions correspond to those in the sequence of the IBV isolate LDL/150434-II genome. GenBank accession numbers are the same as those in Fig. 2B.
1: vaccine LDT3-A strain; 2:isolate ck/CH/LDL/150434-II; 3: pathogenic tl/CH/LDT3/03.

Table 4
Titers were obtained in reciprocal β virus neutralization tests (diluted serum, constant virus)[a].

Virus	Serum		
	LDL/150434–I	LDL/150434–II	LDL/150434–III
LDL/150434–I	366	< 2	< 2
LDL/150434–II	< 2	338	< 2
LDL/150434–III	< 2	< 2	294

[a] End-point values of the viruses were calculated.

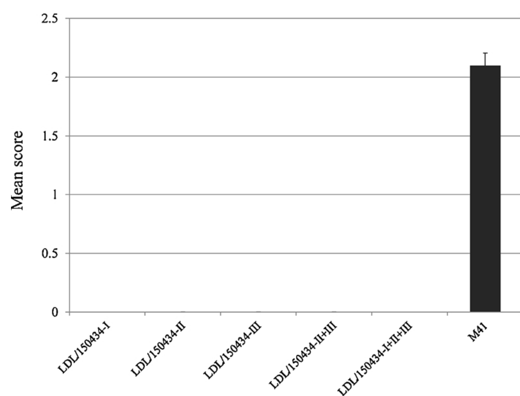

Fig. 6. Respiratory signs detected at 5 days post-challenge in chickens with isolates LDL/150434-I, LDL/150434-II, LDL/150434-III, LDL/150434-II + III, LDL/150434-I + II + III and strain M41. The mean score was calculated from individual scores that were based on severity of respiratory signs detected in individual chickens. Bars indicate mean with standard error of the mean.

and is constantly changing and evolving to avoid the host immune response (Jackwood et al., 2009). Co-infection with different IBV genotypes in the same bird makes control of infection even more difficult.

By investigating the HVRs in S1 gene, the complete S1 gene and complete genomic sequences using pairwise comparison and phylogenetic analysis, we found that isolate LDL/150434–I was most likely re-isolation of H120 vaccine strain. This result was confirmed by our virulent study in which SPF chickens were inoculated with isolate LDL/150434–I and showed no clinical signs. This was expected considering that the diseased flock had been vaccinated with H120 strain at age 60 days, although the respiratory signs appeared at approximately 30 days later. It has been demonstrated that IBV vaccine strains, such as H120, may persist in chickens with maternal antibodies and that periodically shed virus from the trachea and cloaca for 77 days post-inoculation (Naqi et al., 2003). Moreover, H120 live vaccine is able to spread extensively within groups of chickens vaccinated at different ages (Matthijs et al., 2008). Hence, the vaccine strains may be transmitted between chickens after introduction into the chicken flocks by vaccination, and accumulations of genetic mutations in the viral genome occur later. The persistence and transmission of vaccine strains might provide protection against homologous strains. However, incomplete protection against heterologous strains might result from co-existence of vaccine and heterologous strains in the same bird, as found in our study, and give rise to recombination events between these different genotypes (Chen et al., 2015). Therefore, infection with multiple types of virus in the same chicken might be a result of multiple independent introductions of different IBV genotypes/variants; some of which might be related to vaccination or later transmission of vaccine strains between chickens, and later accumulation of mutations and recombination events between these genotypes.

Both phylogenetic and amino acid substitution analysis based on the S1 genes showed that the isolate LDL/150434–III was a TW I-like virus of GI-7 lineage (Valastro et al., 2016). Although isolate LDL/150434–III was similar to those of known TW I strains, it consisted of a new sub-lineage that was markedly distinct from established TW I virus of GI-7 lineage. The amino acid substitutions and deletions were found in the S1 subunit of S protein of isolate LDL/150434–III, compared to those of the known TW-I viruses. This suggests that TW I-like viruses have undergone extensive evolution, with diverse strains circulating in chicken flocks since they emerged in Mainland China 10 years ago (Xu et al., 2016), and have become endemic in Mainland China (Xu et al., 2016, 2018). It is unclear whether the substitutions and deletions in S1 domain changed the antigenic relatedness between LDL/150434–III and other TW I virus, and a neutralization test was needed. Comparison of complete genome sequences of isolate LDL/150434–III with those of TW I-like virus (TW2575/98) (Wang and Tsai, 1996) and nrTW I-like virus (ck/CH/LHLJ/141105) (Xu et al., 2016) indicated that isolate LDL/150434–III was an nrTW I-like virus. TW I virus was first isolated and identified in Taiwan in 1992, and caused clinical signs of depression, sudden decrease in feed consumption, diarrhea and increased

Table 5
Virus recovery from oropharyngeal swabs of chickens challenged with isolates LDL/150434–I, LDL/150434–II, LDL/150434–III, LDL/150434–II + III and LDL/150434–I + II + III.

Group	Virus recovery																				
	4[a]			8			12			16			20			24			28		
	-I	-II	-III	-I	-II	-III	-I	-II	-III	-I	-II	-III	-I	-II	-III	-I	-II	-III	-I	-II	-III
LDL/150434-I (-I)	10/10	0/10	0/10	10/10	0/10	0/10	5/10	0/10	0/10	2/10	0/10	0/10	0/10	0/10	0/10	–	–	–			
LDL/150434-II (-II)	0/10	9/10	0/10	0/10	10/10	0/10	0/10	8/10	0/10	0/10	6/10	0/10	0/10	0/10	0/10	–	–	–	–	–	–
LDL/150434-III (-III)	0/10	0/10	6/10	0/10	0/10	10/10	0/10	0/10	2/10	0/10	0/10	0/10	–	–	–	–	–	–	–	–	–
LDL/150434-II + III	0/10	10/10	6/10	0/10	10/10	9/10	0/10	6/10	0/10	0/10	4/10	0/10	0/10	2/10	0/10	0/10	1/10	0/10	0/10	0/10	0/10
LDL/150434-I + II + III	9/10	8/10	2/10	10/10	10/10	10/10	9/10	3/10	1/10	4/10	3/10	0/10	1/10	1/10	0/10	0/10	0/10	0/10	–	–	–
Control	0/10	0/10	0/10	0/10	0/10	0/10	0/10	0/10	0/10	0/10	0/10	0/10	0/10	0/10	0/10	0/10	0/10	0/10	0/10	0/10	0/10

[a] Days after inoculation.

mortality of infected birds (Wang and Tsai, 1996). Infection of SPF chickens with TW I strains caused respiratory distress and depression that commenced at 24 h after challenge (Wang et al., 1996). However, respiratory system disease, kidney damage, decreased egg production, cystic oviducts and mortality were observed in commercial layers and SPF chickens infected with nrTW I viruses (Xu et al., 2016; Gao et al., 2016). Although the chickens from which LDL/150434–III was isolated showed mild to severe tracheitis and some showed nephritis, this virus did not induce obvious clinical signs in SPF chickens. This is not surprising because no relationship exists between pathotype and genotype (Wang and Huang, 2000). IBV strains have variable pathogenicity in chickens and some strains commonly persist in chickens in the absence of clinical disease (Jones and Ambali, 1987).

Recombination among human or animal coronaviruses has been described previously (Herrewegh et al., 1998; Lau et al., 2010, 2011; Sabir et al., 2016).Recombination is also a well-recognized mechanism by which IBVs generate diversity. In this study, inconsistent phylogenetic clustering between the S1 gene and complete genomic sequence was found in isolate LDL/150434–II, implicating the occurrence of recombination events in the genome of LDL/150434–II. However, we should be cautious when we interpret the possible recombination events solely on the basis of inconsistent phylogenetic clustering in trees based on different viral genes, to avoid drawing premature conclusions. Hence, SimPlot and MAFFT analyses were used to investigate the recombination and the results revealed evidence of recombination for IBV isolate LDL/150434–II. In line with previous evidence in other coronaviruses (Woo et al., 2005; Licitra et al., 2014), the junction was found to be located between ORF1b and S of isolate LDL/150434–II, which was the most frequently identified location for the recombination breakpoints. The high occurrence of recombination between IBVs could be mostly likely due to the presence of co-infection or re-infection with IBVs with multiple genotypes/serotypes in the same bird. For recombination as a consequence of mixed infections, we believe that widespread use of vaccines that offer incomplete protection against heterotypic IBVs may play an important role in the emergence of such novel genetic variants.

Previous studies have indicated the synergistic mechanism between IBV and AIV-H9N2, possibly by trypsin-like proteases encoded by coronaviruses that enhance the AIV-H9N2 hemagglutinin cleavage (Haghighat-Jahromi et al., 2008; Hassan et al., 2017), which increases replication and pathogenicity of AIV-H9N2. In this study, we did not find any synergistic effects on pathogenicity when different types of IBV co-infected SPF chickens. Replication and infection of IBV are primarily initiated in the respiratory tract regardless of the tissue tropism of the strains. From the respiratory tract, the virus spreads through the host via viremia (Jones and Jordan, 1972) to the ciliated epithelium of the oviduct, causing necrosis and malformation (Chousalkar and Roberts, 2007; Crinion et al., 1971; Jones and Jordan, 1971), and to the renal tubular epithelial cells, causing renal failure with urate obstruction due to tubular necrosis with mononuclear inflammation (Chen and Itakura, 1996, 1997; Condron and Marshall, 1986; Jones, 1974; Purcell et al., 1976). The severity of the disease in various organs depends on the IBV subtype and ultimately determines the mortality in chickens. Single and combined infections with our three isolates did not cause obvious clinical signs and we did not evaluate the histopathological lesions. However, we found that viruses persisted in the respiratory tract longer in some chickens incombined infected group than those in individually infected group. Further study is needed to confirm the results on pathogenicity of different IBV types using more virulent strains, especially with different tropism, to evaluate whether damage in different organs caused by strains with different tropism has synergistic effects on pathogenicity when the birds are co-infected. The results from the experimental infection in our study might not hold true under field conditions because it is not true that same amounts of different genotypes co-infected a bird in the field conditions.

Acknowledgements

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