



# Molecular and antigenic characteristics of Massachusetts genotype infectious bronchitis coronavirus in China



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## ABSTRACT

In this study, 418 IBVs were isolated in samples from 1717 chicken flocks. Twenty-nine of the isolates were classified as the Massachusetts genotype. These 29 isolates, as well as two previously isolated Massachusetts genotype IBV strains, were studied further. Of the 31 strains, 24 were H120-like and two were M41-like isolates as determined by complete genomic sequence analysis, indicating that most of the IBV isolates were likely the reisolated vaccine virus. The remaining five IBV isolates, ck/CH/LHB/111172, ck/CH/LSD/111219, ck/CH/LHB/130598, ck/CH/LDL/110931, and ck/CH/LHB/130573, were shown to have originated from natural recombination events between an H120-like vaccine strain and other types of viruses. The virus cross-neutralization test found that the antigenicity of ck/CH/LHB/111172, ck/CH/LSD/111219, and ck/CH/LHB/130598 was similar to that of H120. Vaccination with the H120 vaccine offered complete protection against challenge with these isolates. However, isolates ck/CH/LDL/110931 and ck/CH/LHB/130573 were serotypically different from their parental viruses and from other serotypes in this study. Furthermore, vaccination with the H120 vaccine did not provide protection against challenge with these two isolates. The results of this study demonstrated that recombination is the mechanism that is responsible for the emergence of new serotype strains, and it has the ability to alter virus serotypes. Therefore, IBV surveillance of chicken flocks vaccinated with IBV live vaccines, as well as the consideration of new strategies to effectively control IBV infection using inactivated or/and genetically engineered vaccines, is of great importance.

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## 1. Introduction

Avian infectious bronchitis virus (IBV) is a ubiquitous, highly contagious respiratory pathogen of chickens that inflicts serious economic losses to the commercial poultry industry worldwide. IBV belongs to the genus *Gammacoronavirus* in the subfamily *Coronavirinae*, family *Coronaviridae*, order *Nidovirales* (de Groot et al., 2012). It has a single-stranded, positive-sense RNA genome of approximately 27.6 kilobases in length that encodes four structural proteins: the nucleocapsid (N), membrane (M), envelope (E), and spike (S). Serotype and genotype classifications, which are usually based on features of the S1 part of the S protein gene, are used to

classify IBV strains (de Wit, 2000). Furthermore, recombination may be involved in the evolution of IBV, and it probably occurs at many positions within a given genome during mixed infections (Lai and Cavanagh, 1997).

Historically, the Massachusetts (Mass)-type viruses were believed to be the first and only serotype found in the USA and other regions of the world. However, many IBV serotypes have arisen and disappeared in the poultry industry since then (Cook et al., 2012). The control of IB is mostly accomplished through the use of live attenuated vaccines. The use of commercially produced Mass-type modified live virus vaccines began in the 1950s and has continued to this day. This type of vaccines, such as the Mass strains M41 and H120, are the most commonly used around the world because such vaccines have proven to be capable of protecting against a wide range of IBV strains (Cavanagh and Gelb, 2008). However, the Mass-type of IBVs is constantly isolated from chicken flocks with respiratory clinical signs.

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In this study, the surveillance of IBVs was conducted on 1717 chicken flocks suspected to be infected with IBV between 2009 and 2013 in China. Twenty-nine Mass genotype IBVs were isolated and, together with our previously isolated two strains, the genomic and antigenic characteristics of these IBV isolates were investigated to provide a better understanding of the emergence, circulation, evolution, and antigenicity of Mass-type IBVs in chicken flocks in China.

## 2. Material and methods

### 2.1. Eggs and chicks

Fertile White Leghorn specific pathogen-free (SPF) chicks were obtained from the Harbin Veterinary Research Institute, as were White Leghorn SPF chicken eggs. The birds were maintained in isolators with negative pressure, and food and water were provided *ad libitum*.

### 2.2. Samples, testing, and virus isolation

Samples of tissues and organs were obtained from 1717 chicken flocks with suspected IB clinical signs in China between 2011 and 2013 that were vaccinated with the H120 vaccine (Table 1). These samples were first screened for IBV by reverse transcription-polymerase chain reaction (RT-PCR) as previously described (Liu et al., 2009). Then the IBV-positive samples were used for virus isolation using 10-day-old embryonated SPF chicken eggs as previously described (Liu et al., 2009). Subsequently, a Mass genotype-specific RT-PCR assay targeting the IBV S1 gene (Cavanagh et al., 1999) was conducted on the infected allantoic fluids, and only those viruses of the Mass genotype were used in this study.

### 2.3. Total RNA isolation and complete genome sequencing and analysis

Total viral RNA was isolated from 200 µl of infected allantoic fluids using TRIzol reagent (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's instructions. The complete genomes of 31 Mass genotype of IBV strains were amplified and sequenced as previously described (Liu et al., 2013). The obtained nucleotide sequences were manually edited, assembled, and analyzed using the Clustal W method available in the BioEdit software package (version 7.0.3.0., available at: <http://www.mbio.ncsu.edu/bioEdit/bioedit>) to produce the final sequences of the viral genomes. The S1 gene and genomes of our 31 IBV strains were compared to those of the M41 and H120 strains and other 19 reference strains available in GenBank. Genetic distances were calculated using a maximum composite likelihood model with 1000 bootstrap replicates as implemented in the MEGA 5.0 program (Tamura et al., 2011).

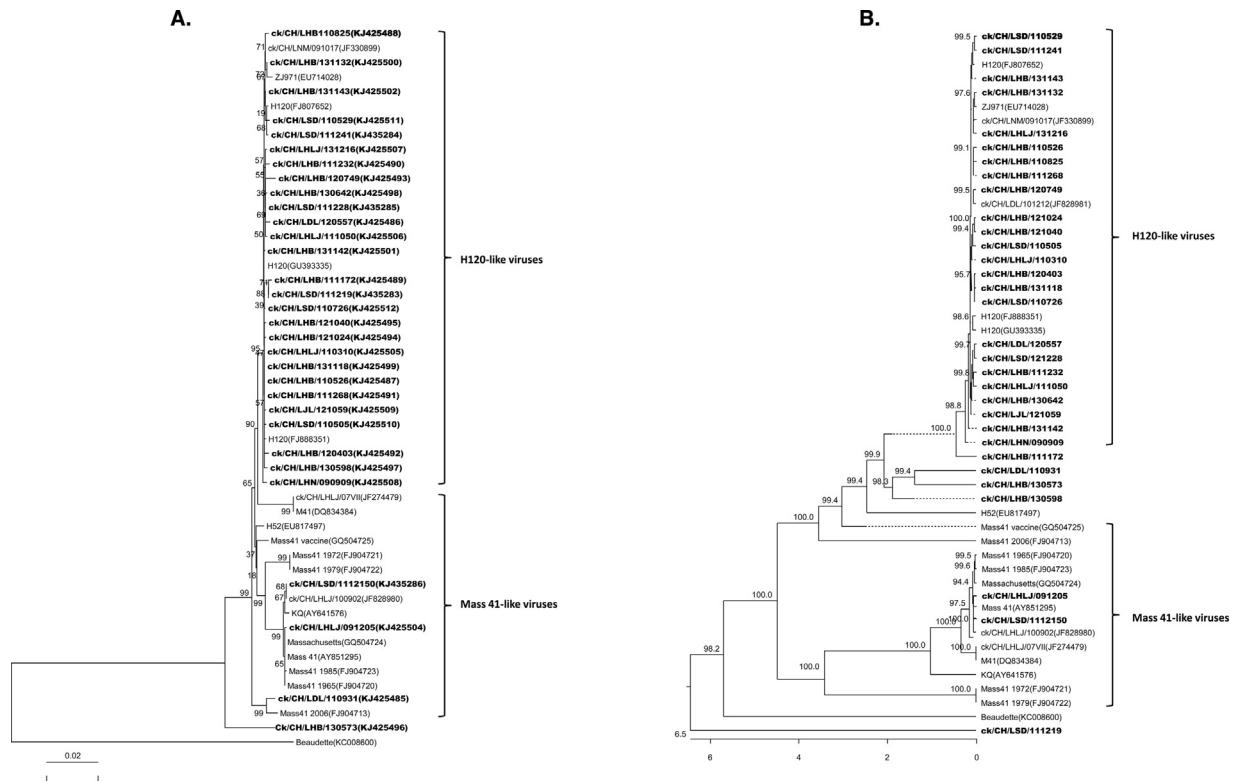
To further identify the recombinant events, the BLASTN program (Liu et al., 2013) was used to search GenBank for IBV sequences that were homologous to those of the five IBV isolates, ck/CH/LHB/111172, ck/CH/LSD/111219, ck/CH/LHB/130598, ck/CH/ LDL/110931, and ck/CH/LHB/130573. The Similarity Plot of the complete genomic sequences (Lole et al., 1999), the nucleotide similarities and phylogenetic trees for each deduced recombinant fragment were analyzed with the MEGA 5.0 program (Schierup and Hein, 2000).

All of the 31 complete genomic sequences reported herein have been deposited in the National Center for Biotechnology Information's GenBank database, and the accession numbers are list in Fig. 1A.

**Table 1**  
Characteristics of the IBVs included in the present study<sup>a</sup>.

IBV strain	Year	Province	Day	Vaccine used for immunization	Organ used for virus isolation	Type of chicken
ck/CH/LHLJ/091205	2009	Heilongjiang	20	H120	Trachea	Broiler
ck/CH/LHN/090909	2009	Henan	18	H120	Trachea	Broiler
ck/CH/LDL/110931	2011	Dalian	30	H120	Proventriculus	Layer
ck/CH/LHB/110526	2011	Hebei	20	H120	Kidney	Layer
ck/CH/LHB/110825	2011	Hebei	25	H120	Kidney	Layer
ck/CH/LHB/111172	2011	Hebei	24	H120	Kidney	Layer
ck/CH/LHB/111232	2011	Hebei	18	H120	Proventriculus	Layer
ck/CH/LHB/111268	2011	Hebei	25	H120	Proventriculus	Broiler
ck/CH/LHLJ/110310	2011	Heilongjiang	6	H120	Proventriculus + Kidney	Broiler
ck/CH/LHLJ/111050	2011	Heilongjiang	46	H120	Proventriculus	Layer
ck/CH/LSD/110505	2011	Shandong	21	H120	Kidney	Broiler
ck/CH/LSD/110529	2011	Shandong	15	H120	Proventriculus	Broiler
ck/CH/LSD/110726	2011	Shandong	13	H120	Kidney	Broiler
ck/CH/LSD/111219	2011	Shandong	34	H120	Kidney	Layer
ck/CH/LSD/111241	2011	Shandong	26	H120	Proventriculus	Layer
ck/CH/LSD/1112150	2011	Shandong	23	H120	Proventriculus	Broiler
ck/CH/LDL/120557	2012	Dalian	14	H120	Proventriculus	Layer
ck/CH/LHB/120403	2012	Hebei	45	H120	Proventriculus	Broiler
ck/CH/LHB/121024	2012	Hebei	25	H120	Trachea	Layer
ck/CH/LHB/121040	2012	Hebei	25	H120	Kidney	Broiler
ck/CH/LHB/120749	2012	Hebei	23	H120	Proventriculus	Broiler
ck/CH/LJL/121059	2012	Jilin	35	H120	Proventriculus	Broiler
ck/CH/LSD/121228	2012	Shandong	20	H120	Kidney	Layer
ck/CH/LHB/130573	2013	Hebei	24	H120	Proventriculus + Trachea	Broiler
ck/CH/LHB/130598	2013	Hebei	18	H120	Proventriculus	Broiler
ck/CH/LHB/130642	2013	Hebei	25	H120	Proventriculus	Broiler
ck/CH/LHB/131118	2013	Hebei	20	H120	Proventriculus	Broiler
ck/CH/LHB/131132	2013	Hebei	15	H120	Proventriculus	Broiler
ck/CH/LHB/131142	2013	Hebei	23	H120	Proventriculus	Broiler
ck/CH/LHB/131143	2013	Hebei	15	H120	Proventriculus	Broiler
ck/CH/LHLJ/131216	2013	Heilongjiang	7	H120	Proventriculus + Kidney	Broiler

<sup>a</sup> IBV strains ck/CH/LHLJ/091205 and ck/CH/LDL/090909 were isolated in 2009 (Sun et al., 2011).



**Fig. 1.** Phylogenetic trees constructed from the nucleotide sequences of the S1 subunit gene (A) and the complete genomes (B) of our 31 Mass genotype viruses and 19 reference strains of infectious bronchitis viruses. The trees were computed using the neighbor-joining method. The significance of the tree topology was assessed by 1000 bootstrapping calculations. Sequences of the viruses in this study are indicated in bold. GenBank accession numbers are indicated in parentheses.

#### 2.4. Virus cross-neutralization tests

The titers of the aforementioned five isolates and four deduced parental viruses (H120 and Connecticut vaccine strains and the ck/CH/LHB/100801 and tl/CH/LDT3/03 field strains) were determined using 10-day-old embryonated chicken eggs. The 50% embryo infectious dose (EID<sub>50</sub>) was calculated by the method of Reed and Muench (1938). The  $\beta$  VN method with constant virus and diluted serum was employed in SPF chicken embryos for serotyping.

#### 2.5. Experimental design

One hundred and forty day-old SPF White Leghorn chicks were divided into groups of ten birds (14 groups) and placed into molded plastic isolators. Food and water were provided *ad libitum*. Chickens in groups 1, 3, 5, 7, 9, and 11 were inoculated with the H120 vaccine by ocular nasal application at 1 day of age at a dose of  $\log_{10}^4$  EID<sub>50</sub>/0.1 ml per chick. Birds in groups 2, 4, 6, 8, 10, 12, 13, and 14 were mock-inoculated with sterile allantoic fluid. Blood samples were collected individually from each bird at days 4, 8, 12, 16, and 20 PI. At 20 days PI, birds in groups 1 and 2, 3 and 4, 5 and 6, 7 and 8, 9 and 10, and 11 and 12 were challenged by ocular nasal application with  $10^6$  EID<sub>50</sub>/0.1 ml of IBV strains ck/CH/LHB/111172, ck/CH/LSD/111219, ck/CH/LHB/130598, ck/CH/LDL/110931, ck/CH/LHB/130573, and M41, respectively, while birds in group 13 were inoculated with the H120 vaccine, and birds in group 14 were mock-inoculated with sterile allantoic fluid. The nasopharyngeal swabs were collected individually from each bird in all groups at days 5, 10, 15, and 20 PI to detect virus shedding by virus recovery and subsequent RT-PCR detections as previously described (Liu et al., 2009). A positive sample was recorded if the specific lesions were observed and the RT-PCR amplification was positive. In addition, blood samples were also collected individually from birds in all groups at days 4, 8, 12, 16,

and 20 post challenge. IBV-specific antibodies in serum samples were tested using a commercial total antibody ELISA (IDEXX Corporation, Westbrook, ME, USA) according to the manufacturer's instructions. The birds in each group were examined daily for signs of infection for 30 days post challenge.

### 3. Results

#### 3.1. Virus detection, isolation, and typing

Using RT-PCR, we found 522 IBV-positive samples among the tissue and organ samples collected from 1717 chicken flocks that were suspected to be infected with IBV. Four hundred and eighteen IBVs were isolated from the 522 RT-PCR positive samples. Twenty-nine out of the 418 IBV isolates tested positive for the Mass genotype by primer-specific RT-PCR detection (Cavanagh et al., 1999). Most of the isolates were LX4-type. Other types of IBV isolates included tl/CH/LDT3/03-, TW I- and 4/91-like IBVs (data not shown). The 29 Mass genotype IBV isolates and our two previous Mass type IBV strains (Sun et al., 2011) were used for subsequent complete genomic sequencing.

#### 3.2. Sequence comparison and phylogenetic analysis

As shown in Supplementary Table 1, the complete genomes of 21 isolates each contained 27,630 nucleotides, excluding the poly-A tail at their 3' ends, which is similar to that of the H120 vaccine strain. The genomes of two isolates had 27,473 nucleotides, which is similar to that of the M41 strain. IBV isolates ck/CH/LHB/131132 and ck/CH/LDL/120557 contained 27,632 nucleotides each, with two insertions found in the 5' untranslated region (UTR) compared with that of the H120 strain. The remaining six isolates, ck/CH/LSD/111241, ck/CH/LSD/121228, ck/CH/LDL/110931, ck/CH/





LHB/130573, ck/CH/LHB/130598, and ck/CH/LSD/111219, contained 27,604, 27,631, 27,582, 27,594, 27,609, and 27,654 nucleotides, respectively, with most of the differences (insertions/deletions) occurring in the 3' end of the genomes compared with those of either the H120 and M41 strains. Overall, the genomic organizations of the 31 viruses are as follows: 5'-UTR-Gene 1 (ORF1a, 1b)-S-Gene 3 (ORFs 3a, 3b, E)-M-Gene 5 (ORFs 5a, 5b)-N-UTR-3'.

All 31 IBV strains in this study were of the Mass genotype based on the S1 gene phylogenetic analysis (Fig. 1A). Of the 31 strains, the ck/CH/LHB/130573 strain was not grouped with either the H120 or M41 strains based on the S1 gene phylogenetic analysis. Among the 28 IBV strains that clustered with the H120 vaccine strain on the basis of their S1 genes, 24 clustered in parallel with H120 in the phylogenetic tree constructed using the complete genomic sequences. Less than two amino acid substitutions were found in the hypervariable regions I and II of S1 subunit between H120 and some of the H120-like IBV isolates (Supplementary Fig. 1). Two isolates, ck/CH/LHLJ/091205 and ck/CH/LSD/1112150, grouped with the M41 strain based on both S1 gene and the complete genomic sequences. The ck/CH/LDL/110931 was grouped with M41 based on S1 gene, however, it was not grouped with either the H120 or M41 strains based on the complete genomic sequences. The remaining four isolates, ck/CH/LHB/111172, ck/CH/LSD/111219, ck/CH/LHB/130598, and ck/CH/LHB/130573, did not cluster with either the H120 or M41 strains in the phylogenetic tree constructed using the complete genome sequences (Fig. 1B). The five isolates were used in a subsequent recombination analysis.

### 3.3. Recombinant analysis

As shown in Supplementary Table 2, the sequences of four isolates, ck/CH/LHB/111172, ck/CH/LHB/130598, ck/CH/LDL/110931, and ck/CH/LHB/130573, showed low similarity to those of H120 from the S1 gene to the 3' UTR of the genome. The region of the ck/CH/LHB/111172 isolate had a very similar sequence to that of ck/CH/LHB/100801, which is a Taiwan-II (TW-II) genotype virus (Ma et al., 2012). For the ck/CH/LSD/111219 isolate, only the nucleotide sequence encoding the S protein showed identity with that of the H120 strain, and, the remaining sequences showed a close relationship with those of the DY07 strain, which belonged to LX4-type and was isolated in China in 2007 (He et al., 2012). The nucleotide sequence from the M gene to the 3' end of the genome of isolate ck/CH/LHB/130598 was closely related to that of the 4/91 strain. We found that the sequence from the S gene to the 3' end of the genome in isolate ck/CH/LDL/110931 was closely related to the prototype Conn46 strain, while the region in isolate ck/CH/LHB/130573 shared a similar sequence identity with strain tl/CH/LDT3/03 (Liu et al., 2005).

SimPlot analysis confirmed the aforementioned results, and it was clearly shown that the five IBV isolates arose from recombination events from a template switch (Figs. 2A, 3A, 4A, 5A and 6A). Isolate ck/CH/LHB/111172 emerged from recombination events between H120- and ck/CH/LHB/100801-like viruses, and two recombination breakpoints were observed, which were located at nucleotides 24143–24158 (at the 3' end of the 3b gene) and 25527–25535 (at the 5' end of the 5a gene) (Fig. 2B). Isolate ck/CH/LSD/111219 originated from multiple recombination events between H120- and DY07-like viruses and two breakpoints were located at nucleotides 18123–18133 (nsp14) and 24075–24079 (at the 5' end of the 3b gene) (Fig. 3B). The ck/CH/LHB/130598 isolate originated from a recombination event between H120- and 4/91-like viruses, with the breakpoint located at nucleotides 25348–25358 (at the intergenic UTR between the M gene and gene 5)

(Fig. 4B). Isolates ck/CH/LDL/110931 and ck/CH/LHB/130573 originated from H120- and Conn46- (Conn-) like viruses and H120- and tl/CH/LDT3/03-like viruses, with breakpoints at nucleotides 21488–21528 and 2167221684, respectively (both at the 3' end of the S1 gene) (Figs. 5B and 6B).

In addition, data from the phylogenetic trees (Figs. 2C, 3C, 4C, 5C and 6C) and similarity analysis (Figs. 2D, 3D, 4D, 5D and 6D) using the corresponding gene fragments confirmed these results and strongly suggested that the five Mass genotype IBVs originated from recombination events.

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

As illustrated in Table 2, remarkable cross-neutralization of H120 was observed with three isolates, ck/CH/LHB/111172, ck/CH/LSD/111219, and ck/CH/LHB/130598, and between the three isolates themselves. In contrast, the three isolates did not neutralize the ck/CH/LHB/100801, Connecticut, and tl/CH/LDT3/03 strains. For the ck/CH/LDL/110931 and ck/CH/LHB/130573 isolates, virtually no cross-neutralization was observed with either Mass serotype strains or other serotypes, which confirmed the absence of an antigenic relationship between isolates ck/CH/LDL/110931 and ck/CH/LHB/130573 and the other strains investigated in this study, indicating that isolates ck/CH/LDL/110931 and ck/CH/LHB/130573 represent two novel serotypes.

### 3.5. Protection provided by the H120 vaccine

As illustrated in Table 3, no overt disease was observed in chicks in the ck/CH/LDL/110931- and H120-inoculated groups. However, mild clinical signs, such as listlessness, huddling, and ruffled feathers, were observed in some of the chicks inoculated with strains ck/CH/LHB/111172, ck/CH/LSD/111219, ck/CH/LHB/130598, ck/CH/LHB/130573, and M41 from days 3 to 10 PI. The antibodies were detected in birds of all groups (except the control group) after 8 days PI. Challenge viruses were re-isolated from oropharyngeal samples of all or most of the chicks inoculated with our five strains, the H120 vaccine virus, and the M41 strain on days 5 and 10 PI. Sixty and forty percent of the chicks inoculated with strains ck/CH/LDL/110931 and ck/CH/LHB/130573, respectively, were still shedding viruses on day 15 PI.

In general, H120 vaccination provided complete clinical protection against challenge with the ck/CH/LHB/111172, ck/CH/LSD/111219, ck/CH/LHB/130598, ck/CH/LDL/110931, and M41 strains. However, 30% of vaccinated chicks showed clinical signs after challenge with strain ck/CH/LHB/130573. A significant reduction of challenge virus replication was achieved by vaccination using H120 vaccines for the viruses ck/CH/LHB/111172, ck/CH/LSD/111219, ck/CH/LHB/130598, and M41, indicating that H120 vaccination provided good protection. In contrast, most of the birds vaccinated with H120 vaccines were shedding viruses after ck/CH/LDL/110931 and ck/CH/LHB/130573 challenge on days 5 and 10, suggesting that H120 vaccination did not provide significant protection against these two viruses.

## 4. Discussion

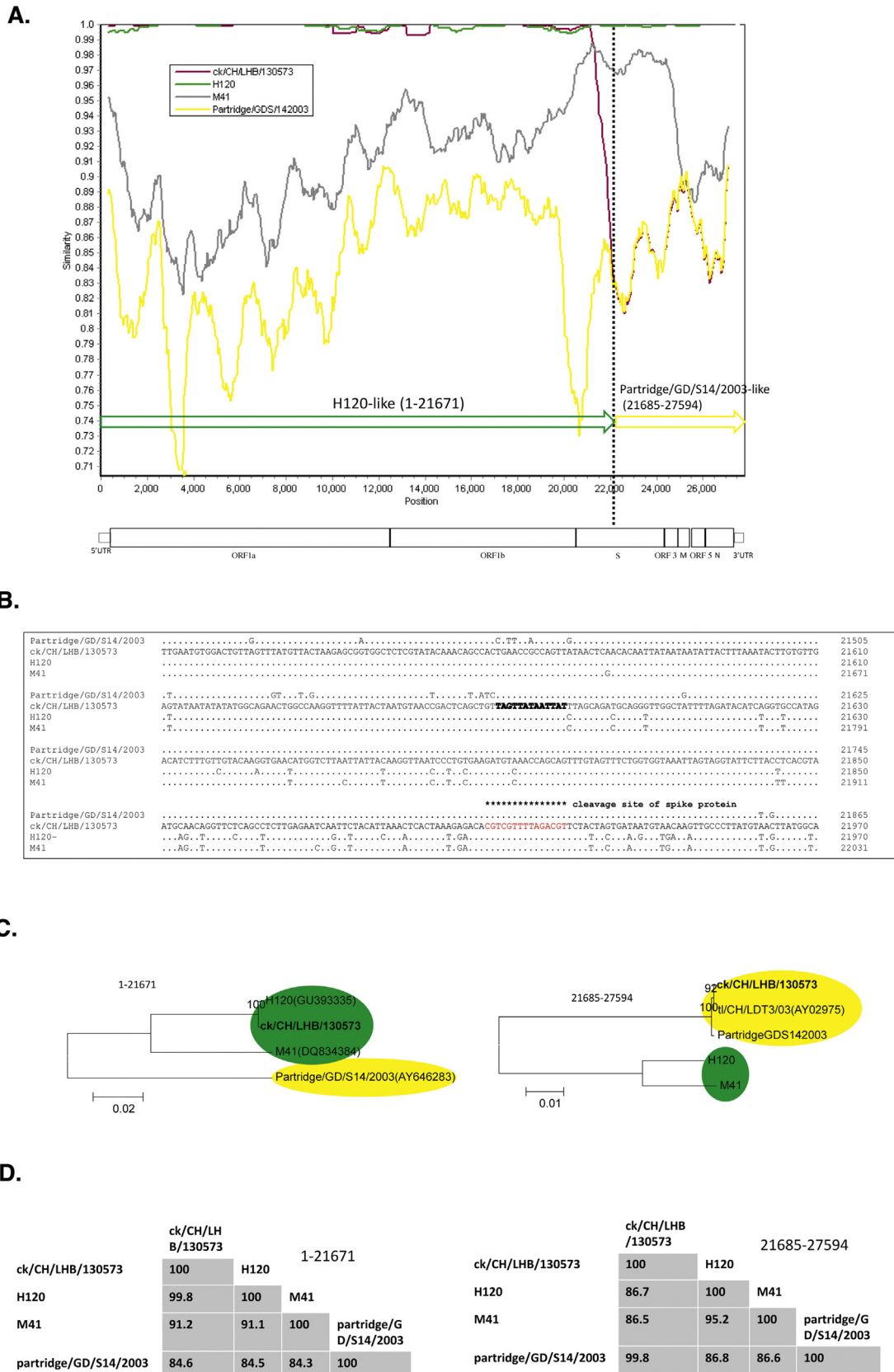
In this study, 418 IBVs were isolated using 10-day-old SPF chicken embryos from samples collected from 1717 chicken flocks ( $\approx 24.5\%$ ) suspected to be infected with IBVs, and 29 out of the 418 IBV isolates ( $\approx 7\%$ ) belonged to the Mass genotype. The lower proportion of the Mass genotype IBV in the samples collected in this study was not surprising because Mass-based live IBV vaccines











**Fig. 6.** Recombination analysis of the ck/CH/LHB/130573 isolate. The methods used for recombination analysis are similar as those of isolate ck/CH/LHB/111172 in Fig. 2. The sequences of Mass 41, H120, ck/CH/LHB/130573, and Partridge/GD/S14/2003 strains were compared and analyzed.

**Table 2**

Titers of reciprocal  $\beta$  virus neutralization tests (diluted serum, constant virus numbers).

Virus	Serum								
	1	2	3	4	5	6	7	8	9
1. ck/CH/LHB/111172	256	160.9	128	146.4	3.5	1.4	1.8	<1	<1
2. ck/CH/LSD/111219	224	512	177	128	3.6	1.6	1.4	1:1.4	1.4
3. ck/CH/LHB/130598	159	211	164	158	1.7	2	<1	<1	1.1
4. H120	218	292	218	362	14	5.8	1.2	<1	1.1
5. ck/CH/LDL/110931	1.4	3.2	1.4	1.1	201	4.5	<1	<1	1.8
6. ck/CH/LHB/130573	<1	2.5	<1	<1	6.9	181	2	<1	11.2
7. ck/CH/LHB/100801	<1	2.4	1.6	<1	1.8	2.8	256	1.4	2
8. Connecticut	1.5	2.2	2.8	2.9	1.4	2.2	<1	299	2.4
9. tl/CH/LDT3/03	<1	1.4	<1	<1	11.2	21	1.6	<1	216

63 of the S1 subunit of the spike protein in the M41 strain can result in escape mutants (Cavanagh et al., 1988). In this study, we did not find this substitution in the S1 subunit of our two M41-like isolates, indicating that the vaccination failure in the two flocks was not due to a mutation in the S1 gene at this site.

In this study, by complete genomic sequence comparison, SimPlot analysis, and phylogenetic and similarity analysis using the corresponding gene fragments, our results clearly suggested that five of the 31 Mass genotype IBVs originated from recombination events. An H120-like virus was predicted to be one of the parental viruses for each of the recombinant viruses. This is not surprising because the H120 vaccine virus is commonly used in China. In addition, the nucleotide sequences of S1 subunit of all the recombinant viruses was predicted to be derived from an H120-like virus because all the viruses were primarily screened by Mass type-specific RT-PCR, the primers for which targeted the S1 subunit region (Cavanagh et al., 1999). Five IBV strains, including DY07-, 4/91-, tl/CH/LDT3/03-, ck/CH/LHB/100801- and Conn-like viruses, were predicted to be parental viruses for each of the isolates. In China, DY07-, tl/CH/LDT3/03- and ck/CH/LHB/100801-like viruses have been found to be prevalent in chicken flocks in recent years (Liu et al., 2005; Han et al., 2011; Ma et al., 2012). Vaccine 4/91 was used in China for many years, although only a few field strains of this type were isolated. Interestingly, no Conn-like viruses have been isolated in China thus far; however, similar to a

previous report (Liu et al., 2014), the ck/CH/LDL/110931 isolate was found to be a naturally recombinant virus that arose from Conn-like and H120-like viruses.

The results in this study showed that the ck/CH/LHB/111172 and ck/CH/LSD/111219 isolates experienced multiple recombination events and two breakpoints were identified. The recombination events from which the two viruses resulted can be explained by two models, similar to that of ck/CH/LZJ/111113 (Liu et al., 2013). For the remaining three viruses, ck/CH/LDL/110931, ck/CH/LHB/130573, and ck/CH/LHB/130598, only one breakpoint was identified. The recombination event may have involved only two parental viral strains with RNA replication initiating in an H120-like template of either negative or positive polarity, followed by switching of the polymerase-nascent cRNA complex to a Conn-, tl/CH/LDT3/03-, and 4/91-like virus template, respectively.

It has already been demonstrated that there is a strong correlation between the S1 sequence, mainly in the hypervariable region (HVR) 1/2, and protective relatedness values obtained in VN tests (Ladman et al., 2006). In the present study, this correlation was confirmed by an analysis of the IBV strains ck/CH/LHB/111172, ck/CH/LSD/111219 and ck/CH/LHB/130598. However, two other strains, ck/CH/LDL/110931 and ck/CH/LHB/130573, although they shared nearly the same sequences in the HVR1/2 regions with that of the H120 strain, had different serotypes than the H120 strain and vaccination with the H120 strain did not provide protection against challenges with these two strains, as evaluated by tracheal virus shedding. It is believed that only a small number of amino acid changes in the S1 domain are sufficient to change the serotype of the virus and affect cross-protection (Cavanagh et al., 1992). We compared the amino acid sequences of the S1 subunit and found that a mutation at nucleotide 118 resulted in a change from a G residue in Mass viruses to a V residue in the ck/CH/LDL/110931 and ck/CH/LHB/130573 isolates. Alternatively, it was reported that amino acid differences were responsible for the different secondary structures of the S2 subunit of IBVs, which might result in different interactions between the S1 and S2 subunits and which might affect the conformation of the S1 subunit where serotype-specific epitopes are located, therefore accounting for serologic differences (Callison et al., 1999). We cannot conclude that the substitution in S1 and/or amino acid differences in the S2 subunits

**Table 3**

Results of vaccination-challenge tests using IBV strains ck/CH/LHB/111172, ck/CH/LSD/111219, ck/CH/LHB/130598, ck/CH/LDL/110931, ck/CH/LHB/130573, and M41.

Group <sup>a</sup>	Dose (log <sub>10</sub> EID <sub>50</sub> )	Morbidity (%)	Mortality (%)	Virus recovery <sup>b</sup>			
				5 d <sup>c</sup>	10 d	15 d	20 d
1 V-C	6.0	0/10 (0%)	0/10 (0%)	0/10 (0%)	0/10 (0%)	-	-
2 C	-	3/10 (30%)	0/10 (0%)	10/10 (100%)	10/10 (100%)	1/10 (10%)	1/10 (10%)
3 V-C	6.2	0/10 (0%)	0/10 (0%)	1/10 (10%)	1/10 (10%)	0/10 (0%)	-
4 C	-	1/10 (10%)	0/10 (0%)	10/10 (100%)	7/10 (70%)	1/10 (10%)	1/10 (10%)
5 V-C	6.2	0/10 (0%)	0/10 (0%)	0/10 (0%)	-	-	-
6 C	-	2/10 (20%)	0/10 (0%)	10/10 (100%)	7/10 (70%)	0/10 (0%)	-
7 V-C	6.0	0/10 (0%)	0/10 (0%)	7/10 (70%)	5/10 (50%)	0/10 (0%)	-
8 C	-	0/10 (0%)	0/10 (0%)	10/10 (100%)	8/10 (80%)	6/10 (60%)	0/10 (0%)
9 V-C	6.0	3/10 (30%)	0/10 (0%)	8/10 (80%)	7/10 (70%)	1/10 (10%)	0/10 (0%)
10 C	-	3/10 (30%)	0/10 (0%)	10/10 (100%)	9/10 (90%)	4/10 (40%)	2/10 (20%)
11 V-C	6.2	0/10 (0%)	0/10 (0%)	1/10 (10%)	0/10 (0%)	-	-
12 C	-	6/10 (60%)	0/10 (0%)	10/10 (100%)	7/10 (70%)	2/10 (20%)	0/10 (0%)
13	-	0/10 (0%)	0/10 (0%)	10/10 (100%)	9/10 (90%)	2/10 (20%)	0/10 (0%)
14	-	0/10 (0%)	0/10 (0%)	0/10 (0%)	0/10 (0%)	0/10 (0%)	0/10 (0%)

<sup>a</sup> Birds in Groups 1, 3, 5, 7, 9, and 11 were vaccinated (V) with the H120 vaccine and challenged (C) with IBV strains ck/CH/LHB/111172, ck/CH/LSD/111219, ck/CH/LHB/130598, ck/CH/LDL/110931, ck/CH/LHB/130573, or M41. Birds in Groups 2, 4, 6, 8, 10, and 12 were only challenged with IBV strains ck/CH/LHB/111172, ck/CH/LSD/111219, ck/CH/LHB/130598, ck/CH/LDL/110931, ck/CH/LHB/130573, or M41. Birds in Group 13 were only vaccinated with the H120 vaccine, and birds in Group 14 were not exposed to any viruses and served as negative controls.

<sup>b</sup> Two procedures were used for virus recovery after vaccination/challenge as described previously (Liu et al., 2014). First, embryos that had been inoculated with individual nasopharyngeal swab samples were observed for lesions. Second, RT-PCR using a pair of oligonucleotide primers, N(-) and N(+), was conducted on RNA recovered from allantoic fluid of the same eggs. The results from the two procedures were identical. Data represent the number of chicks that showed a positive result after challenge/the number of chicks used for attempted virus recovery after challenge.

<sup>c</sup> Days after vaccination/challenge.

contributed to the serotype change of the two isolates in this study, as this will require further investigations using reverse genetic and virus cross-neutralization tests.

Based on these findings, we conclude that extensive vaccination with modified live vaccines, such as H120, against different serotypes, in association with the high recombination and mutation abilities of IBV, has led to the broad dissemination of the vaccine viruses and to the emergence of novel serotypes. New strategies to effectively control IBV infections in chickens should be considered, especially the use of inactivated and/or genetically engineered vaccines.

### Competing interests

The authors declare that they have no competing interests.

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### Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at <http://dx.doi.org/10.1016/j.vetmic.2015.10.003>.

### References

- Callison, S.A., Jackwood, M.W., Hilt, D.A., 1999. Infectious bronchitis virus S2 gene sequence variability may affect S1 subunit specific antibody binding. *Virus Genes* 19, 143–151.
- Cavanagh, D., Davis, P.J., Cook, J.K.A., Li, D., Kant, A., Koch, G., 1992. Location of the amino-acid differences in the S1 spike glycoprotein subunit of closely related serotypes of infectious bronchitis virus. *Avian Pathol.* 21, 33–43.
- Cavanagh, D., Davis, P.J., Mockett, A.P.A., 1988. Amino acids within hypervariable region 1 of avian coronavirus IBV (Massachusetts serotype) spike glycoprotein are associated with neutralization epitopes. *Virus Res* 11, 141–150.
- Cavanagh, D., Gelb Jr., J., 2008. Infectious Bronchitis, In: Saif, Y.M., et al. (Ed.), *Diseases of Poultry*, 12th edn. Blackwell, Ames, pp. 117–135.
- Cavanagh, D., Mawditt, K., Britton, P., Naylor, C.J., 1999. Longitudinal studies of infectious bronchitis virus and avian pneumovirus in broilers using type-specific polymerase chain reactions. *Avian Pathol.* 28, 593–605.
- Cook, J.K., Jackwood, M., Jones, R.C., 2012. The long view: 40 years of infectious bronchitis research. *Avian Pathol.* 41, 239–250.
- de Groot, R.J., Baker, S.C., Baric, R., Enjuanes, L., Gorbalenya, A.E., Holmes, K.V., Perlman, S., Poon, L., Rottier, P.J.M., Talbot, P.J., Woo, P.C.Y., Ziebuhr, J., Coronaviridae King, A.M.Q., Adams, M.J., Carstens, E.B., Lefkowitz, E.J. (Eds.), 2012. *Virus Taxonomy: Ninth Report of the International Committee on Taxonomy of Viruses*. Elsevier Academic Press, San Diego, pp. 774–796.
- de Wit, J.J., 2000. Detection of infectious bronchitis. *Avian Pathol.* 29, 71–93.
- Han, Z., Sun, C., Yan, B., Zhang, X., Wang, Y., Li, C., Zhang, Q., Ma, Y., Shao, Y., Liu, Q., Kong, X., Liu, S., 2011. 15-year analysis of molecular epidemiology of avian infectious bronchitis coronavirus in China. *Infect. Genet. Evol.* 11, 190–200.
- He, K., Li, M., Wei, P., Mo, M.L., Wei, T.C., Li, K.R., 2012. Complete genome sequence of an infectious bronchitis virus chimera between cocirculating heterotypic strains. *J. Virol.* 86, 13887–13888.
- Ladman, B.S., Loupos, A.B., Gelb Jr., J., 2006. Infectious bronchitis virus S1 gene sequence comparison is a better predictor of challenge of immunity in chickens than serotyping by virus neutralization. *Avian Pathol.* 35, 127–133.
- Lai, M.M., Cavanagh, D., 1997. The molecular biology of coronaviruses. *Adv. Virus Res.* 48, 1–100.
- Liu, S., Chen, J., Chen, J., Kong, X., Shao, Y., Han, Z., Feng, L., Cai, X., Gu, S., Liu, M., 2005. Isolation of avian infectious bronchitis coronavirus from domestic peafowl (*Pavo cristatus*) and teal (*Anas*). *J. Gen. Virol.* 86, 719–725.
- Liu, X., Ma, H., Xu, Q., Sun, N., Han, Z., Sun, C., Guo, H., Shao, Y., Kong, X., Liu, S., 2013. Characterization of a recombinant coronavirus infectious bronchitis virus with distinct S1 subunits of spike and nucleocapsid genes and a 3' untranslated region. *Vet. Microbiol.* 162, 429–436.
- Liu, S., Xu, Q., Han, Z., Liu, X., Li, H., Guo, H., Sun, N., Shao, Y., Kong, X., 2014. Origin and characteristics of the recombinant novel avian infectious bronchitis coronavirus isolate ck/CH/LJL/111054. *Infect. Genet. Evol.* 23, 189–195.
- Liu, S., Zhang, X., Gong, L., Yan, B., Li, C., Han, Z., Shao, Y., Li, H., Kong, X., 2009. Altered pathogenicity, immunogenicity, tissue tropism and 3'-7kb region sequence of an avian infectious bronchitis coronavirus strain after serial passage in embryos. *Vaccine* 27, 4630–4640.
- Lole, K.S., Bollinger, R.C., Paranjape, R.S., Gadkari, D., Kulkarni, S.S., Novak, N.G., Ingersoll, R., Sheppard, H.W., Ray, S.C., 1999. Full-length human immunodeficiency virus type 1 genomes from subtype C-infected seroconverters in India, with evidence of intersubtype recombination. *J. Virol.* 73, 152–160.
- Ma, H., Shao, Y., Sun, C., Han, Z., Liu, X., Guo, H., Liu, X., Kong, X., Liu, S., 2012. Genetic diversity of avian infectious bronchitis coronavirus in recent years in China. *Avian Dis.* 56, 15–28.
- Reed, L.J., Muench, H., 1938. A simple method of estimating fifty percent endpoints. *Am. J. Hyg.* 27, 493–497.
- Schierup, M.H., Hein, J., 2000. Consequences of recombination on traditional phylogenetic analysis. *Genetics* 156, 879–891.
- Sun, C., Han, Z., Ma, H., Zhang, Q., Yan, B., Shao, Y., Xu, J., Kong, X., Liu, S., 2011. Phylogenetic analysis of infectious bronchitis coronaviruses newly isolated in China, and pathogenicity and evaluation of protection induced by Massachusetts serotype H120 vaccine against QX-like strains. *Avian Pathol.* 40, 43–54.
- Tamura, K., Peterson, D., Peterson, N., Stecher, G., Nei, M., Kumar, S., 2011. MEGA5: molecular evolutionary genetics analysis using maximum likelihood, evolutionary distance, and maximum parsimony methods. *Mol. Biol. Evol.* 28, 2731–2739.