Origin and characteristics of the recombinant novel avian infectious bronchitis coronavirus isolate ck/CH/LJL/111054

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ABSTRACT

Recombination among infectious bronchitis viruses (IBVs), coupled with point mutations, insertions, and deletions that occur in the genome, is thought to contribute to the emergence of new IBV variants. In this study an IBV, ck/CH/LJL/111054, was isolated from a H120-vaccinated chicken, which presented with a suspected IBV infection. Phylogenetic analysis of the S1 subunit sequence confirmed that strain ck/CH/LJL/111054 is of the Connecticut-type; however, further extensive full-length genomic analysis identified the occurrence of recombination events. Therefore, strain ck/CH/LJL/111054 may have originated from recombination events between Conn- and Mass-like strains at three recombination breakpoints: two located within the nsp3 gene sequence and one in the nsp12 gene sequence. Further, the uptake of the 5′ untranslated regions, nsp2, parts of nsp3, nsp4–11, and parts of nsp 12 from Mass-like virus by ck/CH/LJL/111054 might have resulted in changes in viral replication efficiency rather than antigenic changes, via cross-neutralization analysis with the H120 strain. Recombination events coupled with the accumulation of mutations in the ck/CH/LJL/111054 genome may account for its increased virulence in specific-pathogen free chickens.

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1. Introduction

Avian infectious bronchitis virus (IBV), a gamma-coronavirus in the *Coronaviridae* family (Carstens, 2010), is an enveloped, positive-sense, single-stranded, RNA virus with a genome size of approximately 27 kb and causes a highly contagious upper-respiratory tract disease in domestic chickens, which is characterized by coughing, sneezing and tracheal rales. The 5′ two-thirds of the IBV genome encodes two polyproteins (1a and 1ab) that contain proteins necessary for RNA replication, whereas the 3′ one-third encodes several structural proteins: the surface spike glycoprotein (S), and small envelope (E), membrane (M), and nucleocapsid (N) proteins. It is generally believed that the S1 subunit of S glycoprotein contains hypervariable regions that play a role in attachment to host receptors, membrane fusion, and entry into the host cell. The S1 subunit of S glycoprotein carries conformationally dependent virus-neutralizing and serotype-specific determinants, and is a determinant of cell tropism. Additional accessory protein genes are located in the 3′ region of the genome in an IBV species-specific position.

Since first described in 1936, IBV had undergone a high rate of genetic change that has contributed to the appearance of diverse subpopulations of the virus that continually emerge to form new variants of existing strains as well as completely new types. The Massachusetts (Mass) and Connecticut (Conn) strains are two of the most well characterized and important IBV types and were first isolated in the 1930s and 1956, respectively, in the United States of America (Cavanagh and Gelb, 2008). In addition, Mass-type viruses, which cause heavy economic losses to the poultry industry, have also been detected up to the present time in several European and Asian countries, including China, although at different frequencies (Jackwood, 2012). In contrast, Conn-type viruses, which show only 95.4% of nucleotides homology in the S1 subunit sequence with Mass-type viruses and are in different serotypes from the Mass-type viruses, circulate mainly in the United States of America and Central America (Jackwood, 2012).

Generally, point mutations, insertions, and deletions that occur in the S1 subunit of the spike protein in IBV strains contribute to the existence of many serotypes and the emergence of variants

that have been isolated worldwide (Cavanagh and Gelb, 2008). Consequently, genotyping and phylogenetic analysis of IBV are typically focused on the S1 subunit sequence. However, drawing conclusions based on a single gene sequence, and sometimes even a partial gene sequence, requires caution, as the correct molecular phylogeny of viruses can only be demonstrated by analyzing complete genomic sequences because IBV mutates at a high frequency due to the high error rate of RNA polymerization. Like that of other coronaviruses (CoVs), a unique feature of IBV genetics is the high frequency of RNA recombination events in its natural evolution (Liu et al., 2013b). Recombination among CoVs is an attribute of the genus and is thought to contribute to the emergence of new IBV variants (Masters, 2006; Kusters et al., 1989; Wang et al., 1993; Liu et al., 2013b). Recombination events can occur between field strains and also between field and vaccine strains (Masters, 2006; Kusters et al., 1989; Wang et al., 1993; Liu et al., 2013b). In the present study, we isolated an IBV strain, ck/CH/LJL/111054, and performed genomic sequence analysis that revealed evidence of multiple recombination events between Mass- and Conn-like strains. Coupled with the tendency for mutations, our results raise concerns of the likelihood of future outbreaks of new IBV serotypes or variants due to incorrect usage of multiple vaccines and highlights the need for continuous viral surveillance.

2. Materials and methods

2.1. Viral isolation and H120 vaccine

Tissue samples of swollen proventriculus were collected from a 26-day-old layer in Jilin province, China, in 2011. The chicken flocks were vaccinated against IBV with a commercially available, live attenuated, H120 vaccine at 2 days-old and received a booster vaccination at 16 days-old. Some of the chickens showed early signs of respiratory disease at 24 days-old. Post-mortem examinations were performed and the gross lesions were evaluated. The gross examination showed mild to severe tracheitis and proventriculitis. The morbidity was 20% and the mortality was nearly 10%.

For virus isolation, the samples were prepared as 10% w/v tissue suspensions in 0.1% phosphate-buffered saline (PBS), clarified by centrifugation at 1500g at 4 °C for 10 min, and filtered through 0.22-μm membrane filters (Millipore products division, Bedford, MA, USA) before inoculation into the allantoic cavities of 9- to 11-day-old embryos of specific pathogen-free (SPF) chickens. Two passages were performed and characteristic embryo changes, such as dwarfing, stunting, or curling of the embryos, were observed between post-inoculation days 2 and 7. The virus isolate was designated as ck/CH/LJL/111054. In addition, the vaccine strain, H120, was used in this study. The embryo-propagated viral stocks of the ck/CH/LJL/111054 and H120 strains 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 as previously described (Liu et al., 2013b).

2.2. Eggs and chickens

White Leghorn SPF chickens and fertile chicken eggs were obtained from the Laboratory Animal Center, Harbin Veterinary Research Institute, China. All experimental procedures were approved by the Ethical and Animal Welfare Committee of Heilongjiang province, China.

2.3. Cross virus neutralization tests

Cross virus neutralization tests were performed using anti-sera against strains ck/CH/LJL/111054 and H120 to determine their antigenic relationship. The viral titers of the ck/CH/LJL/111054 and H120 strains were determined by inoculation at 10-fold dilutions into groups of five 10-day-old embryonated chicken eggs as described previously (Liu et al., 2013a). The 50% embryo infectious dose (EID_{50}) was calculated using the methods of Reed and Muench (1938).

Cross virus neutralization tests were performed using constant (10^2 EID_{50}) viral titers and diluted serum against ck/CH/LJL/111054 and H120, respectively, in SPF chickens embryos for serotyping (Liu et al., 2013b). The end-point of each serum sample was calculated using the methods of Reed and Muench (1938).

2.4. Sequencing and phylogenic analysis of IBV strain ck/CH/LJL/ 111054

Fifteen specific primer sets spanning the entire viral genome were used for amplifying the complete genome of ck/CH/LJL/ 111054, as described previously (Liu et al., 2013b). The 3′ and 5′ ends of the viral genomes were confirmed by rapid amplification of cDNA ends (RACE) using a 3′/5′ RACE kit (Takara Bio Inc., Shiga, Japan) according to the manufacturer's instructions (Liu et al., 2013b). Viral RNA was extracted from 400 μL of ck/CH/LJL/111054 viral stock, using TRIzol reagent (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's instructions. The PCR products were cloned into the pMD 18-T vector (Takara Bio Inc.) following the manufacturer's instructions and each fragment of the viral genome was sequenced at least five times to determine a consensus sequence. Prediction of the open reading frames (ORFs) was performed using the Vector NTI Advanced 10 bioinformatics software package (Invitrogen) and the sequences were analyzed using Lasergene DNAStar DNA & Protein Sequence Analysis software (version 7; Lasergene Corp, Madison, WI, USA). The pairwise nucleotide identity was determined using Vector NTI Advanced 10 software and multiple sequence alignments were generated using Clustal-W nucleotide sequence alignment algorithm (Liu et al., 2013a).

Twenty reference strains which the S1 subunit sequences were available and 53 IBV reference strains which the entire genomic sequences were available in the GenBank database (www.ncbi.nlm. nih.gov/genbank/) had been selected for genomic pairwise comparison and phylogenetic analysis by using the neighboring-joining method of DNAStar software. The results were validated and confirmed by constructing maximum likelihood phylogenetic trees with 100 bootstrap replicates under the transversion model (TVM)+I+Γˆ4, using PhyML 3.0 software (Guindon et al., 2010) and using MEGA4.0 version at bootstrap value 1000 replicates (Liu et al., 2013b). The selected IBV reference strains and their accession numbers are shown in Fig. 1A and B, respectively.

To identify recombinant event, the complete genomic sequence of ck/CH/LJL/111054 were pairwise compared with those of Connecticut-type strains (Conn46 1972, Conn46 1983, Conn46 1991 and Conn46 1996) and Massachusetts-type strains (H120, M41, Beaudette, ck/CH/LNM/091017 and ck/CH/LHLJ/100902). In addition, the data sets were scanned using a Recombination Detection Program (RDP) v2, and similarity plot and bootscan analyses were performed using the Simplot program (Lole et al., 1999) to identify recombination events and recombination breakpoints (McKinley et al., 2011). Furthermore, a phylogenetic tree was reconstructed for each recombinant fragment to avoid phylogenetic biases derived from ignoring recombination (Schierup and Hein, 2000; Arenas and Posada, 2010). In addition, the population recombination rate was estimated by using the coalescent-likelihood programs in the LAMARC package (version 2.1.9, Kuhner, 2006; Kuhner and Smith, 2007). Rho (the population recombination rate) is presented as $4NrL$, where N is the population size, r is the recombination rate per site and L is the sequence length (number of nucleotides).
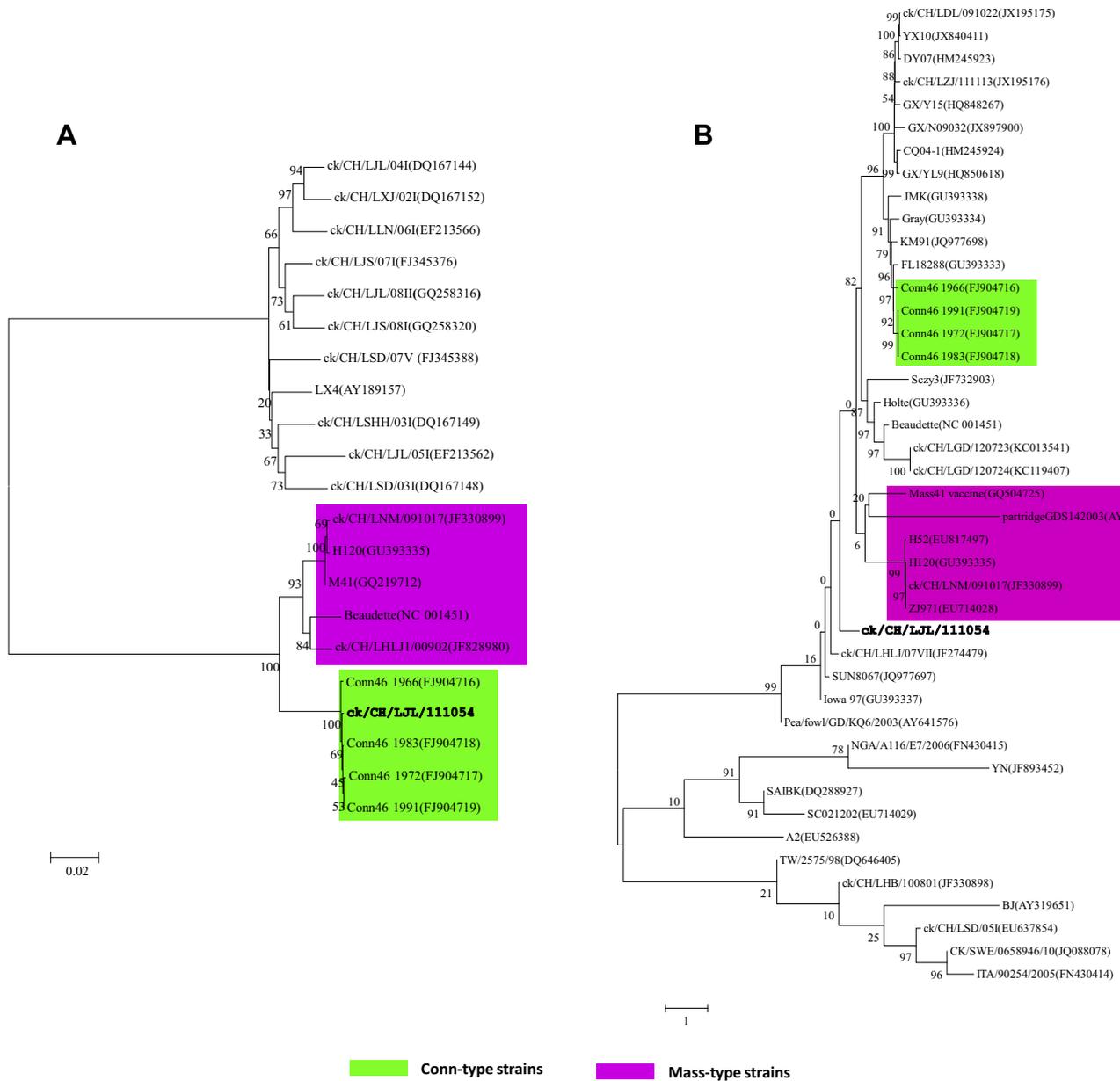
Fig. 1. Relationships among the ck/CH/LJL/111054, Conn-type, Mass-type and reference strains are illustrated with a maximum likelihood phylogeny unrooted tree, based on S1 nucleotides 1–1626 (A) and the full-length genomic sequences (B), respectively. The ck/CH/LJL/111054 isolate is shown in bold. The Conn- and Mass-type strains are shown in green and pink, respectively. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

2.5. Accession number

The genomic sequence of the IBV strains ck/CH/LJL/111054 was submitted to the GenBank database and assigned the accession number KC506155.

2.6. Pathogenicity studies

Thirty 1-day-old SPF White Leghorn chicks were used to assess the pathogenicity of the ck/CH/LJL/111054. Three groups of ten chickens were kept in isolators with negative pressure. Chickens in groups 1 and 2 were inoculated oculonasally with, respectively, a dose of $\log 10^{5.5}$ ck/CH/LJL/111054 and $\log 10^{5.3}$ H120 EID_{50} per chick. Birds in group 3 were mock-inoculated with sterile allantoic fluid and served as the control. Birds in each group were examined daily for signs of infection through post-inoculation day 30. Blood samples were collected from all birds in each treatment group at post-inoculation days 4, 8, 12, 16, 20, and 24. Serum samples were

assayed using a commercial total antibody enzyme-linked immunosorbent assay kit (IDEXX Laboratories Inc., Westbrook, ME, USA) according to the manufacturer's instructions. Each sample was tested in triplicate.

3. Results

3.1. Isolate ck/CH/LJL/111054 belongs to Conn type IBV

The S1 subunit sequence of isolate ck/CH/LJL/111054 had 99.6%, 99.6%, 99.9%, and 99.9% identities to those of the IBV/Conn/66, IBV/Conn/72, IBV/Conn/83, and IBV/Conn/91 strains (McKinley et al., 2011), respectively. Thus, the ck/CH/ LJL/111054 isolate was clearly of the Conn genotype, although it also shared 95.5% and 94.5% identities with those of Mass-type M41 and H120, respectively. In addition, IBV Mass-type M41 and H120 had not more 94.5% and 94.4% identities with those of Conn-type strains.

3.2. Isolate ck/CH/LJL/111054 possibly originated from recombination between the Conn- and Mass-type strains

To examine sequence characteristics not only in S1 subunit, but also over the entire length of the genome, we sequenced the full-length genome sequence of isolate ck/CH/LJL/111054, which was assembled into one contiguous sequence to represent the entire viral genome. A sequence of 27,632 nucleotides was obtained from ck/CH/LJL/111054, excluding the poly-A tail at the 3′ end. The coding capacity and organization of the ck/CH/LJL/111054 genome was similar overall to those of other IBVs. However, the phylogenetic analysis using S1 subunit sequence of the ck/CH/LJL/111054 was different than the phylogenetic analysis using the full-length genomic sequences (Fig. 1B). The nucleotide identities between isolate ck/CH/LJL/111054 and strains IBV/Conn/66, IBV/Conn/72, IBV/Conn/83, and IBV/Conn/91 were 96.3%, 96.4%, 96.4%, and 96.3%, respectively, which was lower than those of the S1 subunit sequence, which reportedly exhibited great genetic variation in the IBV genome and used to determine IBV type (Cavanagh and Gelb, 2008). However, isolate ck/CH/LJL/111054 shared genomic nucleotide identities of 91.1% and 95.5% with strains M41 and H120, respectively. Therefore, the similarity between ck/CH/LJL/111054 and H120 was close to that between isolate ck/CH/LJL/111054 and Conn-type strains.

We conducted a pairwise comparison of the genomic sequence of the isolate ck/CH/LJL/111054 with four Conn-type strains, Conn46 1966, Conn46 1972, Conn 46 1983, and Conn46 1991, and two Mass-types, M41 and H120. From the 5′ untranslated region (UTR) to genome position 2754 in the non-structural protein 3 (nsp3) sequence of strain ck/CH/LJL/111054 showed a much closer relationship and greater nucleotide sequence identity to that of Mass-type H120 than to the Conn strains (Supplementary file 1). However, the ck/CH/LJL/111054 isolate fell into the same group as Conn strains from location 2772 to 3649 in the phylogenetic tree and shared 100% identity with those of the Conn-type, in contrast to 77.4% and 87.6% identities with those of Mass-type H120 vaccine and M41 strains, respectively. It is worth noting that the ck/CH/LJL111054 isolate from position 3657 to 13,332 had a nearly identical sequence (99.9%) to that of H120 and was different from those of the M41 and Conn strains (89.6% and 91.3%, respectively). Interestingly, the ck/CH/LJL/111054 isolate had more than 99.5% nucleotide identity with the Conn strains from nt 13346 to the 3′ end of the genome, including the 3′ end of genes 1, S, 3, M, the 5th accessory protein gene, N, and 3′ UTR. Here, we investigated the presence of three recombination breakpoints, two in nsp3 and one in nsp12 (Supplementary files 2 and 3), in which the first recombination breakpoint was confirmed by Simplot analyses (Fig. 2). The phylogenetic analysis also showed the same results as the sequence identity analysis using the corresponding gene fragments (Fig. 3). These data strongly suggested that ck/CH/LJL/111054 arose from a homologous RNA recombination event from multiple template switches between Mass- and Conn-like viruses. These results implied that template switches occurred within the nsp3 and nsp12 sequences.

The population recombination rate was estimated using the LAMARC package. The maximum likelihood estimates (MLE) of θ and r were 2.377914 and 0.000590, respectively, and the sequence length of ck/CH/LJL/111054 is 27636 bp, therefore, the Rho calculated by Lamarc is 38.77, which is actually a high recombination rate.

3.3. The genetic similarities of ck/CH/LJL/111054 with other Conn type viruses

The percent nucleotide similarities between strains ck/CH/LJL/111054 and H120 at the 5′ end of the genome (from the 5′ UTR to genome position 2754) were 99.4% and 99.8%, respectively, indicating that other than recombination events, the ck/CH/LJL/111054 isolate experienced multiple mutations in the genome over time. In addition, the Conn-like ck/CH/LJL/111054 sequences in both the S1 subunit sequence (Table 1) and full-length genome was closer to those of Conn 46 1983 and 1991, especially a 3-bp nucleotide insertion at genome position 20,521–20,525 among ck/CH/LJL/111054, Conn 46 1983, and Conn 46 1991, by comparison to those of Conn 46 1966 and 1973.

3.4. The ck/CH/LJL/111054 isolate is serologically different from Mass-type IBV

The last dilution of each serum against ck/CH/LJL/111054 and H120, which protected 50% of the embryos against 10^2 EID$_{50}$ of strains ck/CH/LJL/111054 and H120, was tested at 1:158.68 and 1:81.00, respectively. However, serum against strain ck/CH/LJL/111054 could not neutralize 10^2 EID$_{50}$ of H120 and vice versa, indicating that the ck/CH/LJL/111054 isolate was antigenically distinct from the IBV Mass serotype. Due to the unavailability of the Conn-type virus and the serum against this type of virus in our laboratory, we could not serologically identify the antigenic changes of the ck/CH/LJL/111054 isolate after recombination and evolution over time, by comparison with the Conn-type virus.

3.5. Virulence and seroconversion

Mild clinical signs were observed in some of the ck/CH/LJL/111054-infected chicks from post-inoculation days 3 to 10, including listlessness, huddling, and ruffled feathers. None of the chicks died during the experiment and no overt disease was observed in chicks of the H120-inoculated and control groups. In addition, chicks inoculated with strains ck/CH/LJL/111054 and H120 were negative for IBV antibodies until post-inoculation day 8. At each time point from post-inoculation day 8 onwards, the ck/CH/LJL/111054-inoculated chicks had comparable, but slightly higher seroconversion rates than those inoculated with strain H120 (Table 2).

4. Discussion

By phylogenetic analysis of the S1 subunit sequence and full-length genomic sequence, and extensive pairwise comparisons of the genomic sequences of strain ck/CH/LJL/111054 with those of the Conn- and Mass-type strains, multiple recombinant events and three recombination breakpoints were identified during the evolution of the virus. The first recombination breakpoint was confirmed by the Simplot analyses. In addition, we surveyed the whole genome of the three virus strains by using the LAMARC package for calculating the recombination rate. The population recombination rate is 38.77, which is actually a high recombination rate, suggesting the recombinant events happened in the genome of IBV ck/CH/LJL/111054 strain. Like most other RNA viruses, template switching by RNA-dependent RNA polymerases is a mechanism that contributes to genetic recombination events and sequence diversity in coronaviruses (Lai, 1992). In this study, three recombination breakpoints were identified in the genome of ck/CH/LJL/111054 strain, two located within the nsp3 sequence and one within the nsp12 sequence. The recombination events leading to formation of the ck/CH/LJL/111054 sequence can be explained by two models. In the simplest scenario, recombination may have involved only two parental viral strains with RNA replication initiating in a Mass-like template of either negative or positive polarity and then switching of the polymerase-nascent cRNA complex to a Conn-like virus template, followed by double switches in the nsp3 and 12
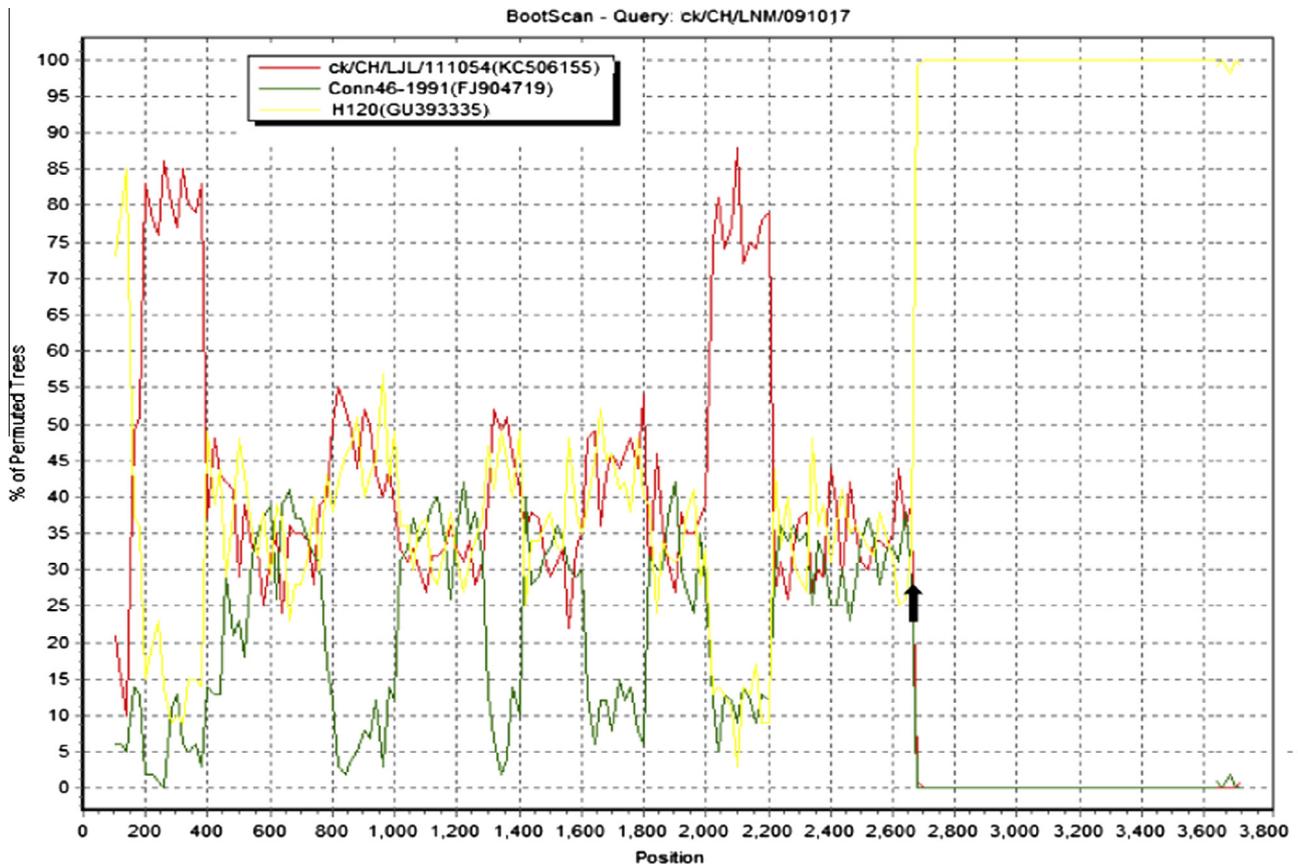
Fig. 2. Simplot analysis to detect recombination and estimate recombination breakpoints within the ck/CH/LJL/111054 genome. Similarity plot using ck/CH/LNM/091017 (Accession number: JF330899) as the query sequence. The arrows showed the deduced recombination breakpoint (2755–2771).

sequences, respectively. The first switch may have occurred at the beginning of the nsp3 sequence. Alternatively, a more complicated scheme was proposed. In this scheme, a Mass–Conn hybrid arose from a single switch at either of the three or two recombination breakpoints and spread among the chicken flocks and in turn experienced additional recombination events with another Conn-like virus.

The Mass-like sequences in the ck/CH/LJL/111054 genome showed high genetic similarities and were closely related to a Mass H120 vaccine, compared to that of strain M41, implicating a possible H120-related vaccine origin. Mass-type vaccines are very commonly used in chicken flocks in China and can persist in the field for long periods. Until now, no Conn-like viruses have been found in chicken flocks in China, however, Mass–Conn bivalent vaccines is used extensively throughout China, although it is not government approved. Due to the unavailability of the sequence of Conn-type vaccine strains so far, we cannot compare the sequences in the genome of isolate ck/CH/LJL/111054 with those of a Conn-type vaccine strain.

The 5′ UTRs in the IBV genome, like other CoVs, usually harbor important structural elements involved in replication and/or translation (Chang et al., 1994; Raman et al., 2003; Raman and Brian, 2005). The nsp3 and 5 sequences downstream of the 5′ UTR contain papain-like protein 2 proteases and the main protease, respectively, which cleaves nsp2, 3, 4, and 5–16, respectively (Masters, 2006). Nsp2, 4, and 6 contain hydrophobic residues predicted to play a role in anchoring the replication/transcription complex to the Golgi apparatus. Nsps 7, 8, 9, and 10 are reported to have RNA-binding activity, whereas nsp11/12 contains the RNA-dependent RNA-polymerase (Masters, 2006). Perhaps the acquisition of the 5′ UTR, nsp2, parts of nsp3, nsp4–11, and parts of nsp12 from

Mass-like virus by ck/CH/LJL/111054 alter the viral replication efficiency, and thus alter its pathogenity in chickens because it is reported that the replicase gene of avian coronavirus, infectious bronchitis virus, is a determinant of pathogenicity (Hodgson et al., 2004; Armesto et al., 2009). Therefore, investigations using reverse genetic systems should provide further insight into this issue and increase our understanding of IBV pathogenesis.

IBV pathogenicity was shown to be polygenic, involving the spike as well as replicase proteins (Brooks et al., 2004; Cavanagh et al., 1992; Fang et al., 2005; Jackwood et al., 2010) and the recombination between vaccine strains might result in a virus that acquired attenuated vaccine viral sequences, thus the outcome may likely be a relatively benign virus regardless of the type of parental viruses involved. It appeared that the emergence of most variants or new IBV types and outbreaks of new diseases are due to the accumulation of mutations in the regions that contain genes important for pathogenicity, especially the spike glycoprotein gene (McKinley et al., 2011). The ck/CH/LJL/111054 isolate likely originated from two vaccines by recombination events; however, the viral genome experienced mutations during its evolution. Although it is difficult to determine which mutation(s) play key roles in pathogenicity, some chickens showed clinical signs after challenge with strain ck/CH/LJL/111054, indicating altered virulence compared with the two possible parental vaccine viruses. However, this observation requires further investigation.

The ck/CH/LJL/111054 isolate was shown to be antigenically distinct from the Mass-type IBVs via cross neutralization analysis in this study. This was not unexpected because analysis of the S1 subunit sequence, which contains conformationally dependent virus-neutralizing and serotype-specific epitopes (Cavanagh et al., 1998), showed that strain ck/CH/LJL/111054 was a Conn-type,
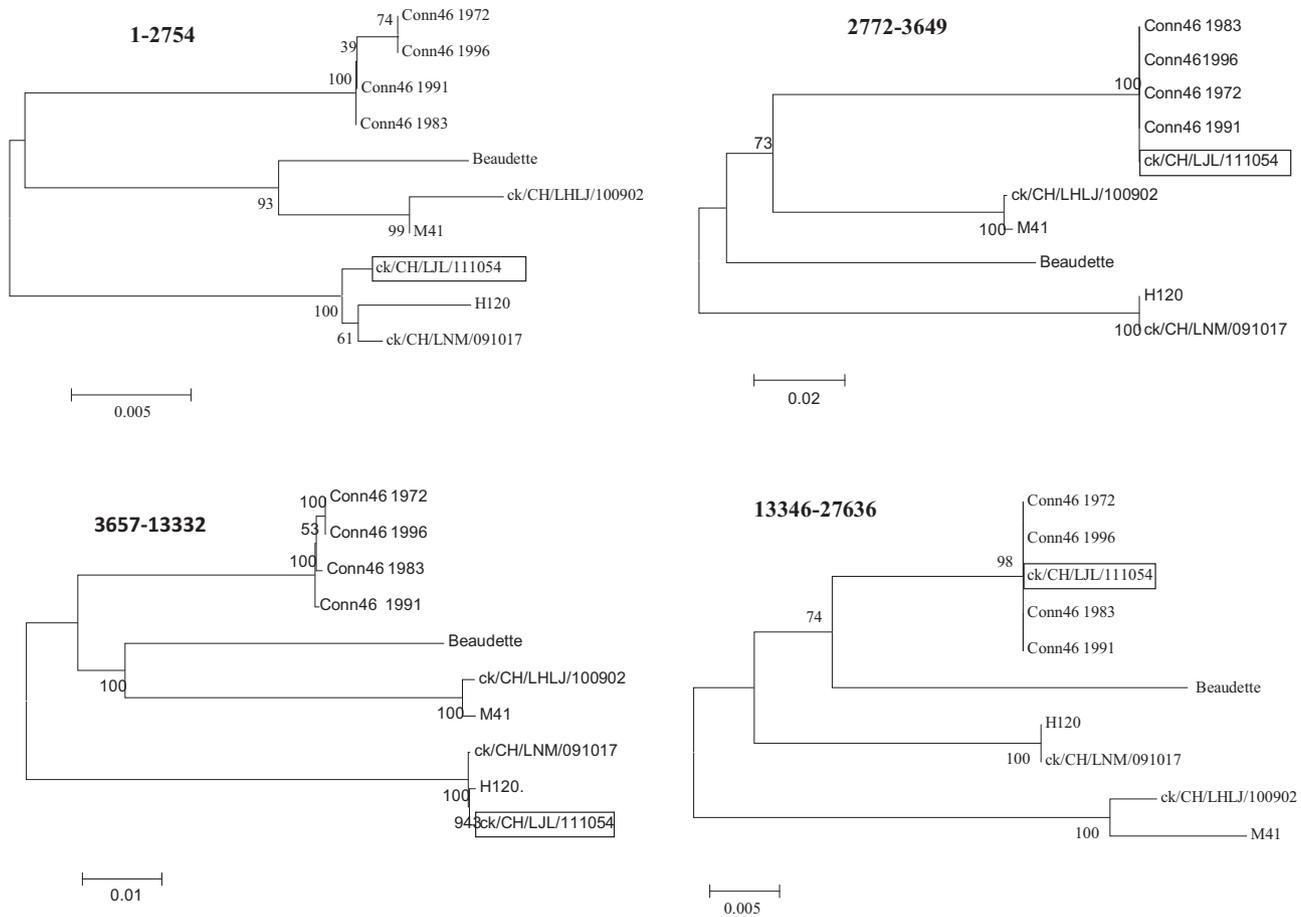
Fig. 3. Phylogenetic analysis of genome positions 1–2754, 2772–3649, 3657–13,332, and 13,346–27,636 among ck/CH/LJL/111054 and four Conn-type and five Mass-type strains. The trees were constructed using the neighbor-joining method.

Table 1
Pairwise comparison of nucleotide sequences of the S gene between Conn46 strains and isolate ck/CH/LJL/111054.

Strain	374[a]	986	3316	3322	3328	3333	3336	3348
Conn46 1966	G	T	T	C	A	T	C	A
Conn46 1972	G	T	T	C	A	T	C	A
ck/CH/LJL/ 111054	**A**	**G**	**C**	**T**	**G**	**G**	**T**	**T**
Conn46 1983	**A**	**G**	**C**	**T**	**G**	**G**	**T**	**T**
Conn46 1991	**A**	**G**	**C**	**T**	**G**	**G**	**T**	**T**

[a] Position in S1 subunit sequence. The nucleotides identical to those in strain ck/CH/LJL/111054 are highlighted in bold.

which is serotypically different from the Mass-type strains (Cavanagh and Gelb, 2008). Due to the unavailability of the Conn-type virus and serum against this type of viruses in our laboratory, we could not serologically identify the antigenic changes in isolate ck/CH/LJL/111054 after recombination and evolution over time, by comparisons with that of Conn-type viruses.

Recombination events contribute to the genetic diversity of CoVs and can lead to the emergence of new viruses and subsequent outbreaks of new diseases (Woo et al., 2009). High frequencies of recombination events between IBV vaccine and field strains have been reported (Masters, 2006; Kusters et al., 1989; Wang et al., 1993; Liu et al., 2013b). Herein, we isolated an IBV strain which may have originated from recombinant events between Mass- and Conn-like strains. The implication of our results emphasizes the importance of IBV surveillance in chicken flocks vaccinated with IBV live vaccines.

Acknowledgments

Table 2
Results of the pathogenicity study using two IBV strains.

Group[a]	Dose, median embryo infectious doses (log10)[b]	Morbidity (%)	Mortality (%)	Antibody (%)[c]				
				4[d]	8	12	16	20
CK/CH/LJL/111054	5.5	40	0	0/10(0)	3/10(30)	7/10(70)	8/10(80)	10/10(100)
H120	5.3	0	0	0/10(0)	2/10(20)	6/10(60)	7/10(70)	10/10(100)
Control	–	0	0	0/10(0)	0/10(0)	0/10(0)	0/10(0)	0/10(0)

[a] Ten chicks per group.
[b] Dose per chick, 100 μL.
[c] Number seroconverted/number inoculated.
[d] Days after challenge.

Appendix A. Supplementary material

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.meegid.2014.02.015.

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