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A 15-year analysis of molecular epidemiology of avian infectious bronchitis coronavirus in China

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ABSTRACT

A comprehensive study of the epidemiology and pathogenicity of infectious bronchitis virus (IBV) in China was carried out by molecular characterization of the S1 gene from 46 isolates obtained for this study and 174 reference strains isolated over a 15-year period. Nine types were found according to sequence analysis and phylogenetic study of the S1 gene. The co-circulation of multiple IBV types and the ongoing emergence of IBV variants are the epidemiological challenges in China. Factors contributing to the continual emergence include mutations, insertions and deletions in the S1 protein genes; recombination between local IBV strains circulating in chicken flocks in China; and recombination between local strains and vaccine strains. Vaccination-challenge analysis between circulating field strains and Mass-type H120 vaccine indicated the need to develop new vaccines from local IBV strains. These results also emphasize the importance of continued IBV surveillance in China.

1. Introduction

Coronaviruses (CoVs) cause a variety of clinical diseases in a wide range of species (Enjuanes et al., 2000). Currently, coronaviruses are classified into three genuses based on antigenic and genetic relatedness. The first to be discovered was infectious bronchitis coronavirus (IBV), which is a Gamma coronavirus (de Groot et al., 2008). IBV is a major poultry pathogen, and is probably endemic in all regions with intensive poultry production. All IBV strains are capable of infecting a large range of epithelial surfaces of the chicken. Different isolates vary in their ability to replicate in non-respiratory tissues, with some producing clinical disease in non-respiratory tissues, most notably the kidney and proventriculus (Liu et al., 2006a; Yu et al., 2001). The IBV genome is an approximately 27.6-kb single-stranded positive RNA molecule that encodes four structural proteins, spike (S), membrane (M), small membrane (E), and nucleoprotein (N). In addition, the genome also encodes the replicase complex that carries out the unique discontinuous transcription process that results in a nested set of six 3′ coterminal subgenomic mRNAs (Stern and Kennedy, 1980; Stern et al., 1982).

At least three major factors contribute to the genetic diversity of coronaviruses. First, the inaccuracy of the coronavirus RNA-dependent RNA polymerase makes the mutation rate approximately 1 per 1000–10,000 nucleotides replicated, making the viral genome particularly plastic (Duffy et al., 2008; Jenkins et al., 2002). Second, coronaviruses undergo a high frequency of homologous RNA recombination as a result of their possible unique random template switching during RNA replication (Lai, 1992; Pasternak et al., 2006). Third, the largest coronavirus genomes (26.4–31.7 kb) give this viral family extra flexibility to accommodate and modify genes (Masters, 2006). These three factors have not only led to a diversity of strains and genotypes from one coronavirus species, but also generated new species that can adapt to new hosts and ecological niches (Woo et al., 2006). IBV strains are also continuously evolving through point mutations, insertions, deletions and genome recombination, leading to emergence of new IBV serotypes or variants (Cavanagh et al., 1992a). Many variants are better adapted or show increased virulence in IB outbreaks. The design of appropriate control programs has been complicated by the large number of IBV sero- or genotypes that have been identified worldwide (Cavanagh, 2007), and the low degree of cross-protection observed among IBV serotypes (Cowen and Hitchner, 1975).

Nucleotide sequencing and genetic analysis provide a fast and accurate method for classification and prediction of IBV serotypes, and are a powerful instrument for monitoring phylogenetic and epidemiological evolution of IBV subtypes (Adzhar et al., 1997;

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Cavanagh and Davis, 1986; Cavanagh et al., 1988, 1992b; Dolz et al., 2008; Koch et al., 1990; Liu et al., 2006a; Mase et al., 2004; Moore et al., 1998). Sequence analysis has shown that some emergent viruses spread rapidly to other geographic areas and become established, while others remain restricted to the region of origin (Cavanagh et al., 1992b). The factors that determine the spreading ability and fitness of new emergent strains are poorly understood. Consistent molecular surveying of IBV in specific regions may reveal molecular features that contribute to these viral qualities.

IBV has been diagnosed in China since the early 1980s by pathogenic studies and serological techniques. Originally, serotype determination, and antigenic and immunogenic characterizations of IBV were studied using monoclonal antibodies against eight strains isolated between 1986 and 1995 from broilers and layers at eight different farms in four provinces in China (Wu et al., 1998). Since the original identifications, many strains of IBV have been isolated in China, and IB is one of the major infectious diseases of poultry farms. These findings indicate the need for in-depth studies to determine epidemiological conditions, and to improve the efficacy of vaccination and control programs. In addition, the abundance of IBV strains provides a unique opportunity for detailed genetic studies on the prevalent IBV genotypes in China, and the dynamics of the viral subpopulations that are required for a deep understanding of IBV evolution and epidemiology.

In this study, 46 IB viruses were isolated from clinical outbreaks in China in 2008, and characterized by S1 gene sequencing. To increase our insight into the epidemiology of IBV, we used all Chinese IBV isolates whose entire S1 gene was available in the GenBank database (totally 174 field strains) to investigate the evolutionary process of IBV in China from 1995 to 2009.

2. Materials and methods

2.1. Chick embryos and chickens

Fertile white leghorn specific pathogen-free (SPF) chicken eggs and chicks were obtained from the Laboratory Animal Center, Harbin Veterinary Research Institute, the Chinese Academy of Agricultural Sciences, China. The birds were maintained in isolators with negative pressure, and food and water were provided *ad libitum*.

The study was approved by the Animal Welfare Committee of Heilongjiang Province, China.

2.2. Viruses and vaccines

In 2008, IBV was isolated from kidney, proventriculus and trachea samples from 189 broiler or layer flocks suspected to have IB, covering most of the chicken-raising regions of China. Forty-six IBV were isolated from these samples (Table 1). For virus isolation, samples of tissue were pooled and 10% weight/volume tissue suspensions were made in 0.1% phosphate-buffered saline (PBS) containing 100 μg penicillin and 100 μg streptomycin/ml. After 12 h at 4 °C, 200 μl of suspension supernatant was inoculated into the allantoic cavity of 9-day-old to 11-day-old SPF embryos. Each sample was used to inoculate three to five eggs, which were incubated at 37 °C and candled daily. One to five blind passages were performed until between 2 and 7 days after inoculation, when characteristic embryo changes such as the dwarfing, stunting, or curling of embryos were observed (Liu et al., 2006b). Allantoic fluids were harvested from inoculated eggs and tested for IBV by electron microscopy.

Commercial IB vaccine virus H120 was obtained in freeze-dried 1000-dose vials that each contained at least 10^3 egg infective dose 50% (EID_{50}) per dose (*isbi*® BIO H120; batch No. F43862). Inoculates were prepared in distilled water just prior to use and contained at least $10^{3.0}$ EID_{50}/ml of H120 virus.

2.3. Electron microscopy

Samples of allantoic fluids after egg passages were submitted for electron microscopy. Briefly, after low-speed centrifugation at 1500 × *g* for 30 min (AllegraTM 21R centrifuge; Beckman, USA), supernatants from 1.5 ml of allantoic fluid were centrifuged at 12,000 × *g* for 30 min. The resulting pellet was resuspended in a minimal volume of deionized water and examined by negative contrast electron microscopy (JEM-1200, EX).

2.4. RT-PCR amplification and sequencing

Total RNA was extracted from infected allantoic fluid with TRIzol reagent (Invitrogen, Grand Island, USA) according to the manufacturer's instructions. First-strand cDNA was synthesized using the antisense oligonucleotide N(−). PCR conditions include 5 min incubation at 95 °C followed by 30 cycles at 94 °C for 1 min, annealing at 50 °C for 1 min, and 72 °C for 2 min. A final extension step was performed at 72 °C for 10 min. Two sense oligonucleotides, IBV-257 (5′-TATTGATTAGAGATGTGG-3′) and S1Oligo5′ (5′-TGAAAACTGAACAAAAGAC-3′), were used with S1Oligo3′ (5′-CAT AACTAACATAAGGGCAA-3′) or IBV-275 (5′-GTATGTACTCATCTG-TAAC-3′) as antisense primers in PCR amplification (Liu et al., 2005). PCR products were analyzed on 1% agarose gels and sequenced. PCR products were also sequenced after cloning into the pMD18-T vector (TaKaRa, Dalian, China). Each region of the S1 gene in each IBV isolate was sequenced at least three times and the consensus sequence was determined.

2.5. Phylogenetic analysis of the S1 protein genes

The nucleotide and amino acid sequences of the S1 gene of the IBV isolates were assembled, aligned, and compared with reference IBV strains using MEGALIGN program in DNAStar. Phylogenetic analysis of the deduced S1 amino acid sequences was performed with the neighbour-joining method of DNAStar software (Liu et al., 2008b), and validated using Megaware (Liu et al., 2009c) and PAUP version 4.08b for the Macintosh (Johannesen et al., 2009; Swofford, 1999). The S1 sequence of the first Chinese IBV strain was published in 1995 in the GenBank database, and all 174 Chinese IBV isolates (Fig. 1) since 1995 whose entire S1 gene was available were selected as reference strains for the phylogenetic analysis. Ten vaccine strains were also selected (Liu et al., 2006b). The backgrounds of the selected IBV reference strains and their accession numbers are in Electronic Supplementary Material.

2.6. Amino acid sequence comparison and analysis

The 174 reference field strains and ten vaccine strains were used to determine mutations, insertions and deletions in S1 gene with those of the 46 new IBV isolates in this study. The S1 genes of IBV isolates of same type were compared, and representatives selected for further comparison were assembled, aligned and compared. Furthermore, NCBI Basic Local Alignment Search Tool (BLAST) searches were conducted using the entire S1 gene of isolates W118, X, WF, LC2, SH3, SAIB4, SAIBwj, CK/CH/LHB/08I and CK/CH/LAH/08I based on locally aligned sequences and phylogenetic analysis.

In addition, the nucleotide sequences of S1 gene of strains W118, CK/CH/LGD/04III and 4/91 vaccine strain were selected for comparison of gene recombination according to the BLAST searching results. Accurate estimation of pairwise comparison

Table 1
Epidemiology information for Chinese field IBV isolates isolated in this study.

IBV isolates	Province (city)[a]	Vaccines used for vaccination	Organs[b] used for virus isolation	Production type
ck/CH/LAH/08I	Anhui (Liuan)	4/91	Kidney	Broiler
ck/CH/LAH/08II	Anhui (Hefei)	4/91	Trachea	Broiler
ck/CH/LCQ/08I	Chongqing	28/86	Kidney	Broiler
ck/CH/LCQ/08II	Chongqing	28/86+Ma5	Kidney	Broiler
ck/CH/LDL/08I	Dalan	28/86+Ma5	Kidney	Layer hens
ck/CH/LDL/08II	Dalan	H120+Conn	Kidney	Layer hens
ck/CH/LDL/08III	Dalan	28/86+Ma5	kidney	Broiler
ck/CH/LDL/08IV	Dalan	H120	Proventriculus	Layer hen
ck/CH/LGS/08I	Gansu (Linxia)	4/91	Trachea	Broiler
ck/CH/LHB/08I	Hebei (Shijiazhuang)	28/86+Ma5	Kidney	Layer hen
ck/CH/LHLJ/08I	Heilongjiang (Zhaiyuan)	H120	Kidney	Broiler
ck/CH/LHLJ/08II	Heilongjiang (Zhaodong)	H120	Proventriculus	Layer hen
ck/CH/LHLJ/08III	Heilongjiang (Qiqihaer)	H120+28/86	Proventriculus	Layer hen
ck/CH/LHLJ/08IV	Heilongjiang (Yichun)	Ma5+28/86	Kidney	Layer hen
ck/CH/LJL/08I	Jilin (Changchun)	Non-vaccinated	Kidney + Trachea	Layer hen
ck/CH/LJL/08II	Jilin (Dehui)	H120	Proventriculus	Layer hen
ck/CH/LJL/08III	Jilin (Changchun)	H120	Kidney	Layer hen
ck/CH/LJL/08IV	Jilin (Taolan)	H120	Kidney	Layer hen
ck/CH/LJS/08I	Jiangsu (Xuzhou)	H120	Kidney	Broiler
ck/CH/LJS/08II	Jiangsu (Pizhou)	H120	Kidney	Broiler
ck/CH/LLN/08I	Liaoning (Shenyang)	Non-vaccinated	Proventriculus	Broiler
ck/CH/LLN/08II	Liaoning (Anshan)	H120	Proventriculus	Layer hen
ck/CH/LSD/08I	Shandong (Shouguang)	Ma5+H120	Kidney	Broiler
ck/CH/LSD/08II	Shandong (Laiyang)	Ma5+H120	Trachea	Broiler
ck/CH/LSD/08III	Shandong (Gaomi)	H120	Kidney	Broiler
ck/CH/LSD/08IV	Shandong (Shouguang)	H120	Kidney	Broiler
ck/CH/LSD/08V	Shandong (Yingli)	4/91	Kidney	Broiler
ck/CH/LSD/08VI	Shandong (Daotian)	H120	Kidney	Broiler
ck/CH/LSD/08VII	Shandong (Daotian)	H120	Kidney	Broiler
ck/CH/LSD/08VIII	Shandong (Liulv)	H120	Kidney	Broiler
ck/CH/LSD/08IX	Shandong (Gaomi)	H120+28/86	Kidney	Broiler
ck/CH/LSD/08X	Shandong (Changle)	H120+28/86	Kidney	Broiler
ck/CH/LSD/08XI	Shandong (Daotian)	H120+28/86	Trachea	Broiler
ck/CH/LSD/08XII	Shandong (Qufu)	Ma5+28/86	Kidney	Broiler
ck/CH/LSD/08-1	Shandong (Pingdu)	H120	Kidney	Broiler
ck/CH/LSD/08-2	Shandong (Zhilan)	H120	Kidney	Broiler
ck/CH/LSD/08-3	Shandong (Laiyang)	H120	Kidney	Broiler
ck/CH/LSD/08-4	Shandong (Gaomi)	H120	Proventriculus	Broiler
ck/CH/LSD/08-5	Shandong (Hanting)	Ma5+28/86	Kidney	Broiler
ck/CH/LSD/08-6	Shandong (Changle)	H120	Kidney	Broiler
ck/CH/LSD/08-7	Shandong (Zouping)	H120	Proventriculus	Broiler
ck/CH/LSD/08-8	Shandong (Weifang)	4/91	Kidney	Broiler
ck/CH/LSD/08-9	Shandong (Wanfu)	H120	Kidney	Broiler
ck/CH/LSD/08-10	Shandong (Shouguang)	H120	Trachea	Broiler
ck/CH/LSD/08-11	Shandong (Yingli)	Ma5+4/91	Kidney	Broiler
ck/CH/LSD/08-12	Shandong (Wanfu)	H120	Proventriculus	Broiler

[a] Province (city) where the viruses were isolated.
[b] Kidney: swollen kidney, proventriculus: swollen proventriculus, trachea: hemorrhagic trachea.

had been conducted and nucleotide identities between strains 4/91 and W118, and 4/91 and CK/CH/LGD/04III had been calculated based upon sequences that are finely aligned by software CLUSTAL W and are manually adjusted from mistakes.

2.7. GenBank accession numbers

All S1 nucleotide sequences from the IBV isolates reported here have been submitted to the GenBank database and assigned the accession numbers GQ258302–GQ258347.

2.8. Experimental design

The pathogenicity of representatives of nearly all the known types of IBV in China had been reported and the protection against these types of IBV by commercially available H120 vaccine have been evaluated (Liu et al., 2006b, 2008a, 2009c). IBV isolates ck/CH/LAH/08II and ck/CH/LHB/08I in this study showed high diversity with the known IBV strains by comparison of the S1 protein gene. Hence, these two isolates were selected for further study on pathogenicity and vaccination-challenge test using commercially available H120 vaccine.

One hundred, day-old SPF white leghorn chicks were divided into five groups of 20 birds each and housed in different isolators. Chickens in groups 1 and 3 were vaccinated with IBV vaccine H120 oculo-nasally and intra-tracheally. Birds in groups 2, 4 and 5 were mock-inoculated with sterile allantoic fluid. Blood samples from 10 birds from each group were collected at 4, 8, 12 and 16 days after vaccination and the serum was stored at −70 °C before enzyme-linked immunosorbent assay (ELISA) testing. At 20 days post-inoculation, birds in groups 1 and 2 were challenged with IBV isolates ck/CH/LAH/08II, and groups 3 and 4 with isolates ck/CH/LHB/08I by oculonasal application with dose of $\log10^5$ EID_{50} per chick (Table 2), while the negative control birds in group 5 were mock-inoculated again with sterile allantoic fluid. Ten birds each from groups 1–5 were killed humanely at 5 days post-challenge. The lung, caecal tonsil, trachea and kidney were collected for virus recovery. Blood samples from the remaining birds in each group were collected at

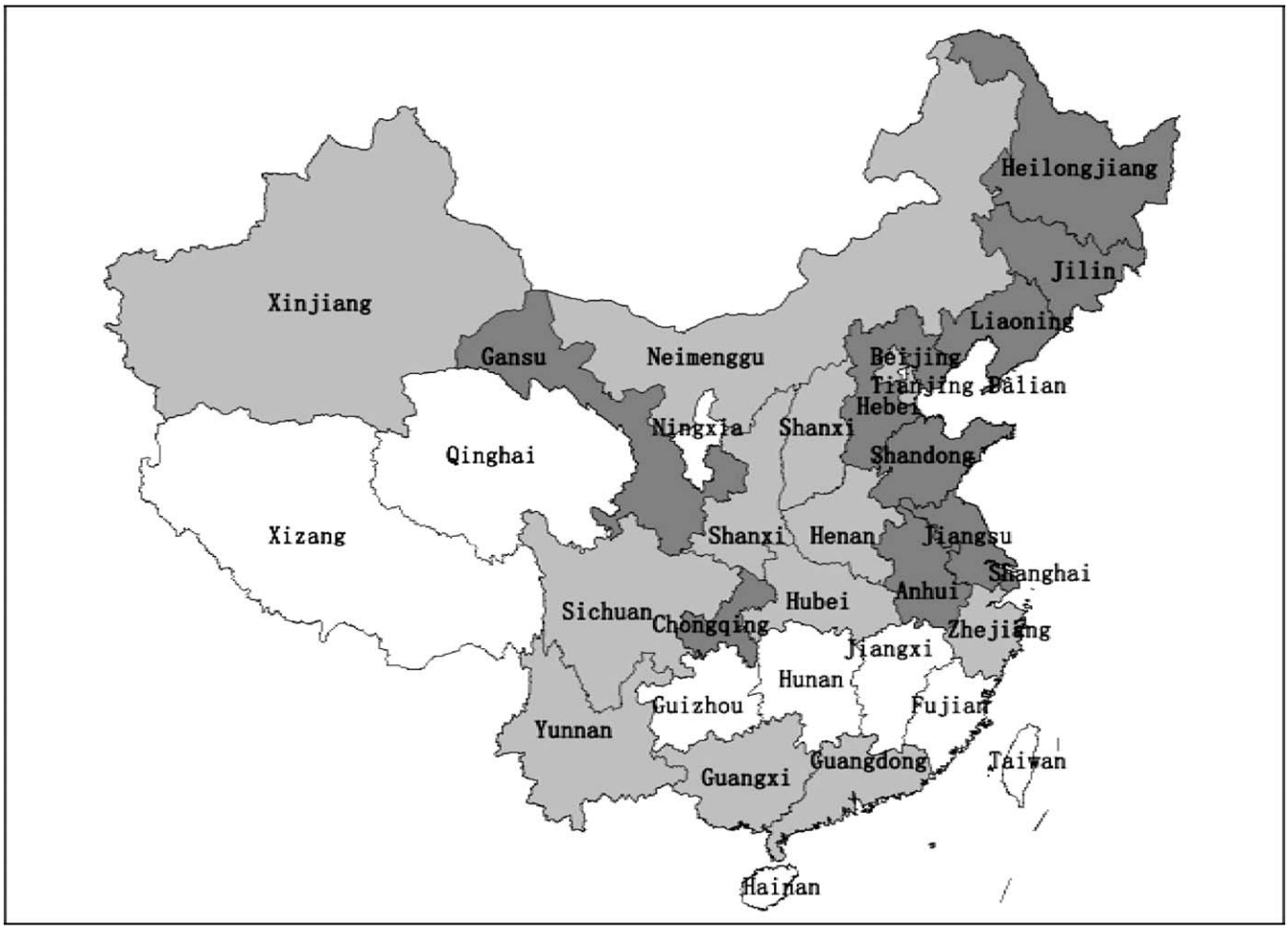

Fig. 1. Geographical locations of Chinese provinces or cities from where IBV strains were obtained over 15 years. The light grey indicated isolates found in the GenBank. The dark grey reflected the virus isolated both in the study and isolates found in the GenBank.

3, 6, 9, 12 and 16 days after challenge and serum stored at −70 °C before ELISA testing. The chicks were examined daily for signs of infection for 30 days after inoculation.

2.9. Virus recovery and RT-PCR detection

All tissues collected post-challenge were used for virus recovery as described above. Viral samples containing 10,000 U penicillin and 10,000 μg streptomycin were used to inoculate at least four SPF embryos via the allantoic cavity (0.2 ml per egg). Eggs were candled daily, and allantoic fluid from two of the inoculated embryos was collected 72 h postinoculation for RT-PCR, and the remaining embryos were examined 1 week later for characteristic IBV lesions such as the dwarfing, stunting, or curling of embryos.

RNA was extracted from 200 μl of allantoic fluid from each inoculated embryo, and RT was used to generate cDNA using IBV oligonucleotide N(−), which is specific to the 3′ untranslated region (UTR) (Liu et al., 2006b). Oligonucleotides N(−) and N(+) were used to amplify a fragment of approximately 1600 bp with most of the N gene and parts of the 3′-UTR. The PCR products were analyzed on 1% agarose gels.

2.10. Total antibody ELISA

Serum samples were assayed using a commercial total antibody ELISA kit (IDEXX Corporation, Westbrook, Maine, USA) according to the manufacturer's instructions. Each sample was tested in triplicate. Serum-to-positive ratios (S/P ratios) were calculated as described previously (de Wit et al., 1998; Liu et al., 2009b). Individual serum titers were calculated from S/P ratios, evaluated as positive or negative, and expressed as $OD_{650\ nm}$ values according to the manufacturer's instructions.

3. Results

3.1. S1 gene phylogeny and sequence comparison revealed nine IBV types circulating in China

This study examined 46 kidney, proventriculus and trachea tissue samples that were positive for IBV. When tissue samples or viral isolates from the samples were inoculated into embryos, typical signs of pathology, including embryo dwarfing and death, were observed at different passages (Liu et al., 2009b). Typical coronaviruses were detectable by electron microscopy.

Phylogenetic analysis based on both S1 nucleotide and deduced amino acid sequences of the 220 Chinese isolates and 10 vaccine strains showed that most of the Chinese isolates were from nine distinct genetic groups or types (Fig. 2a). Type I included 119 of the field isolates, isolated between 1995 and 2009, and grouped with the LX4 strain (LX4-type), most of which were isolated in recent years. The type II group contained 27 field isolates from 1999 to 2009, and showed maximum nucleotide and amino acid identity with CK/CH/LSC/99I strain (CK/CH/LSC/99I-type). Most isolates in this group were isolated in the Sichuan province. Group III

Table 2
Serological and virus recovery results post-challenge with ck/CH/LAH/08II and ck/CH/LHB/08I.

Group [a]	Vaccinated	Challenged	Dose, median embryo infectious doses (log 10)[b]	Morbidity	Mortality	Antibody[c]								Virus recovery[d]			
						Days post vaccination				Days post challenge							
						4	8	12	16	3	6	9	12	16	Trachea	Kidney	Lung	Caecal tonsil
1	Yes	Yes, with ck/CH/LAH/08II	5.0	0/10	0/10	0/10	2/10	9/10	10/10	10/10	10/10	10/10	10/10	10/10	4/10	2/10	2/10	4/10
2	No	Yes, with ck/CH/LAH/08II	5.0	10/10	1/10	0/10	0/10	0/10	0/10	0/10	0/9	9/9	9/9	9/9	8/10	4/10	5/10	8/10
3	Yes	Yes, with ck/CH/LHB/08I	5.0	10/10	3/10	0/10	4/10	10/10	10/10	10/10	9/9	8/8	7/7	7/7	10/10	3/10	1/10	6/10
4	No	Yes, with ck/CH/LHB/08I	5.0	10/10	7/10	0/10	0/10	0/10	0/10	0/10	5/9	4/5	4/4	3/3	10/10	10/10	6/10	10/10
5	No	No	–	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] Twenty chicks per group.
[b] Dose per chick (ck/CH/LAH/08II and ck/CH/LHB/08I), 100 μl.
[c] Number seroconverted/number inoculated.
[d] Two procedures were used for virus recovery after challenge. First, lesions in embryos that had been inoculated with individual trachea or kidney tissue samples were observed. Second, RT-PCR using oligonucleotide primers N(+) and N(−) on RNA from the allantoic fluid of the same eggs was conducted. The results from the two procedures were identical. Data are number of chicks that showed positive results after challenge/number of chicks that survived after challenge.

comprised 16 field viruses isolated between 1995 and 2008 and grouped with tl/CH/LDT3/03 strain (tl/CH/LDT3/03-type). Type IV contained seven field isolates that shared 99% nucleotide sequence identity. Six of these were isolated between 1997 and 2001 on the Liaotung Peninsula and all showed a close relationship to the CK/CH/LDL/97I strain (CK/CH/LDL/97I-type). An additional two types, BJ-type and CK/CH/LHLJ/95I-type, were found, containing seven and six isolates, respectively.

In addition, 19 of the 220 field isolates, isolated between 1995 and 2008, were grouped with the Massachusetts (Mass)-type vaccine strains in the phylogenetic tree. Eight of the IBV isolates, collected from 1998 to 2007, were in a unique genetic group that was closely related to non-authorized vaccines (J9, JAAS and Jilin) from Australia. In particular, the IBV isolate TA03 showed maximum nucleotide and amino acid identities with the 4/91 strain. An additional 13 isolates could not been grouped into any of the IBV types.

3.2. LX4-type IBVs showed slight genetic diversity in the S1 gene

In this study, 119 Chinese IBV isolates were grouped as LX4-type (Liu and Kong, 2004) by phylogenetic analysis, which was more than 50% of the total 220 IBV strains (Fig. 2b). This type was found circulating in most of the H120-vaccinated flocks, in nearly all Chinese regions (Table 1 and Electronic Supplementary Material), indicating that infection with the LX4-type of IBV was common. The IBV LX4-type isolates shared more than 94% and 95% nucleotide and amino acid similarities, respectively, but mutations, insertions and deletions in the S1 gene were still found among these strains (Fig. 3). The average nucleotide and amino acid identities were not more than 77% and 78%, respectively, between the Chinese LX4-type strains and the Mass-type vaccine strains.

3.3. Mass-type IBV is endemic even though the same vaccines have been used for years

Mass-type IBVs were the third most predominant IBV type in circulation in Chinese chicken flocks, according to our analysis. In total, 19 Mass-type IBVs have been isolated since 1995 in different regions in China. Many of the chickens from which the isolates were obtained had been vaccinated with the H120 vaccine or a combination of vaccines including H120 and others such as 4/91 and 28/86. When the S1 genes of the Mass-type isolates were compared with each other and the Mass-type vaccine strains, point mutations were observed, but no insertions and deletions. S1 amino acid identities were greater than 95% among the Mass-type isolates, and between the isolates and vaccine strains. The previously reported single amino acid substitution at position 63 of the S1 subunit of the S protein was found only in isolates BJ9601, SD/97/01, ck/CH/LHLJ/07II and ck/CH/LHLJ/08II, all of which resulted in escape mutants of Mass 41 (Cavanagh et al., 1988).

3.4. 4/91 emerged in China and showed recombination with local isolates

TA03 was a 4/91-type isolate, and because the 4/91-type vaccine has been used in China for many years (Liu et al., 2009a) without official authorization, the emergence of IBV TA03 may reflect re-isolation of the vaccine strain. We compared the TA03 S1 gene sequence with the standard vaccine and pathogenic strains. The results showed that the S1 nucleotide sequence of TA03 S1 was 99.6% identical, and the amino acid sequence was 99.4% identical to the pathogenic 4/91 strain. However, similarity to the 4/91 vaccine strain was somewhat low, at 99.4% nucleotide and 98.9% amino acid identities. In contrast, TA03 had only 93.3% nucleotide

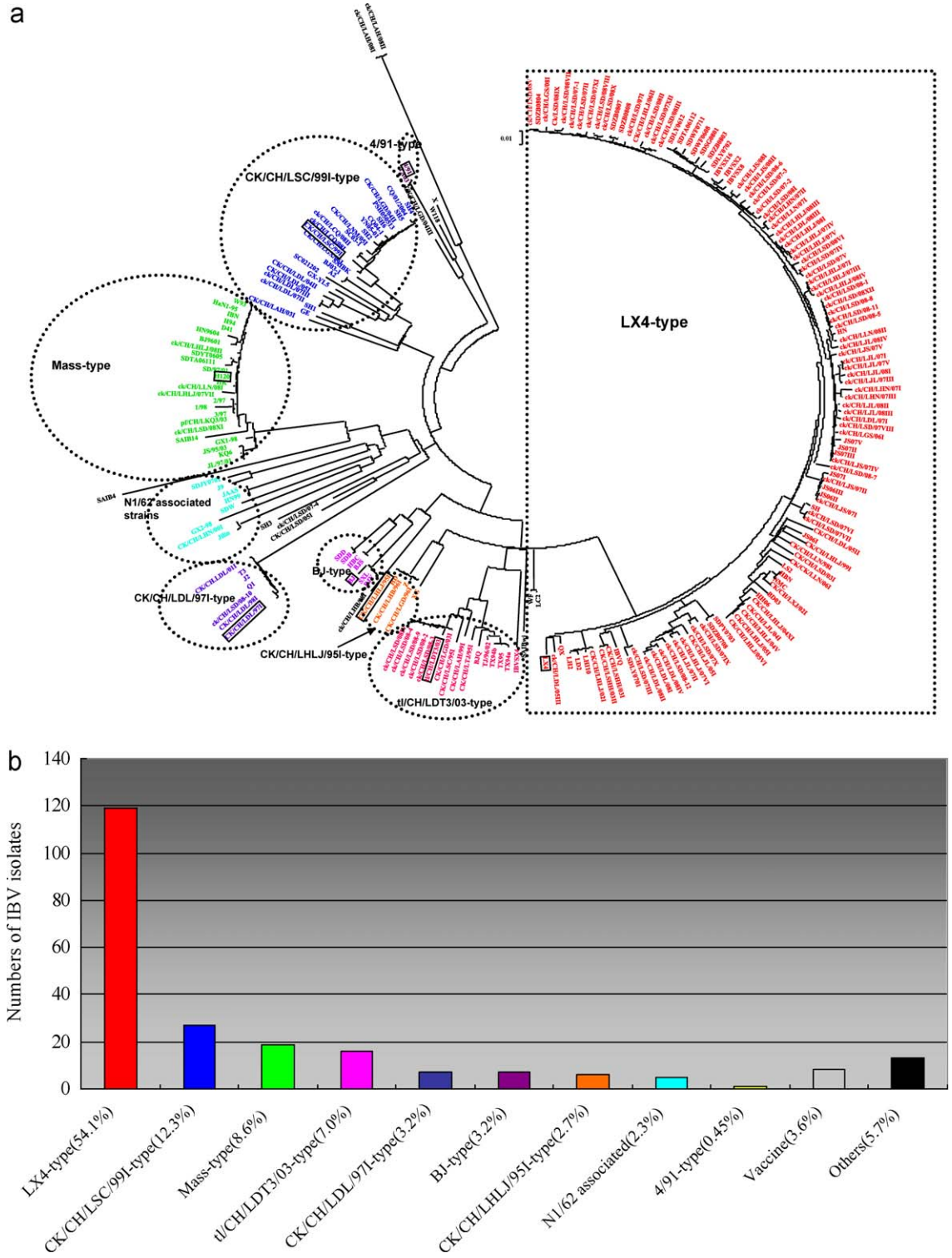

Fig. 2. Phylogenetic tree constructed based on the S1 subunit of the spike protein using neighbour-joining. The tree was rooted with the first 1572 nucleotides, starting with the ATG encoding the translation initiation codon (a). Each type of IBVs was grouped in one circle or box and the representative strains were boxed. The percentage of each type of IBV isolate is indicated (b).

identify, and 89.8% amino acid identity with a Japanese 4/91-type strain. Of the nine unique nucleotide mutations and six amino acid substitutions found in the S1 gene of TA03 when compared to the 4/91 pathogenic and vaccine strains (Table 3), TA03 shared three nucleotide mutations and three amino acid substitutions with the pathogenic strain, indicating that TA03 was not likely to be a re-isolate of the 4/91 vaccine strain.

NCBI Basic Local Alignment Search Tool (BLAST) searches were conducted using the entire S1 gene of isolate W118, revealing that W118 was most closely related to the 4/91-type IBV strain. As illustrated in Fig. 4, the S1 gene of W118 maintained its low identity with pathogenic 4/91 strain from the start codon to nucleotide 669, but from this point to the end, was 99.06% identical to the 4/91 pathogenic strain. A recombination event associated

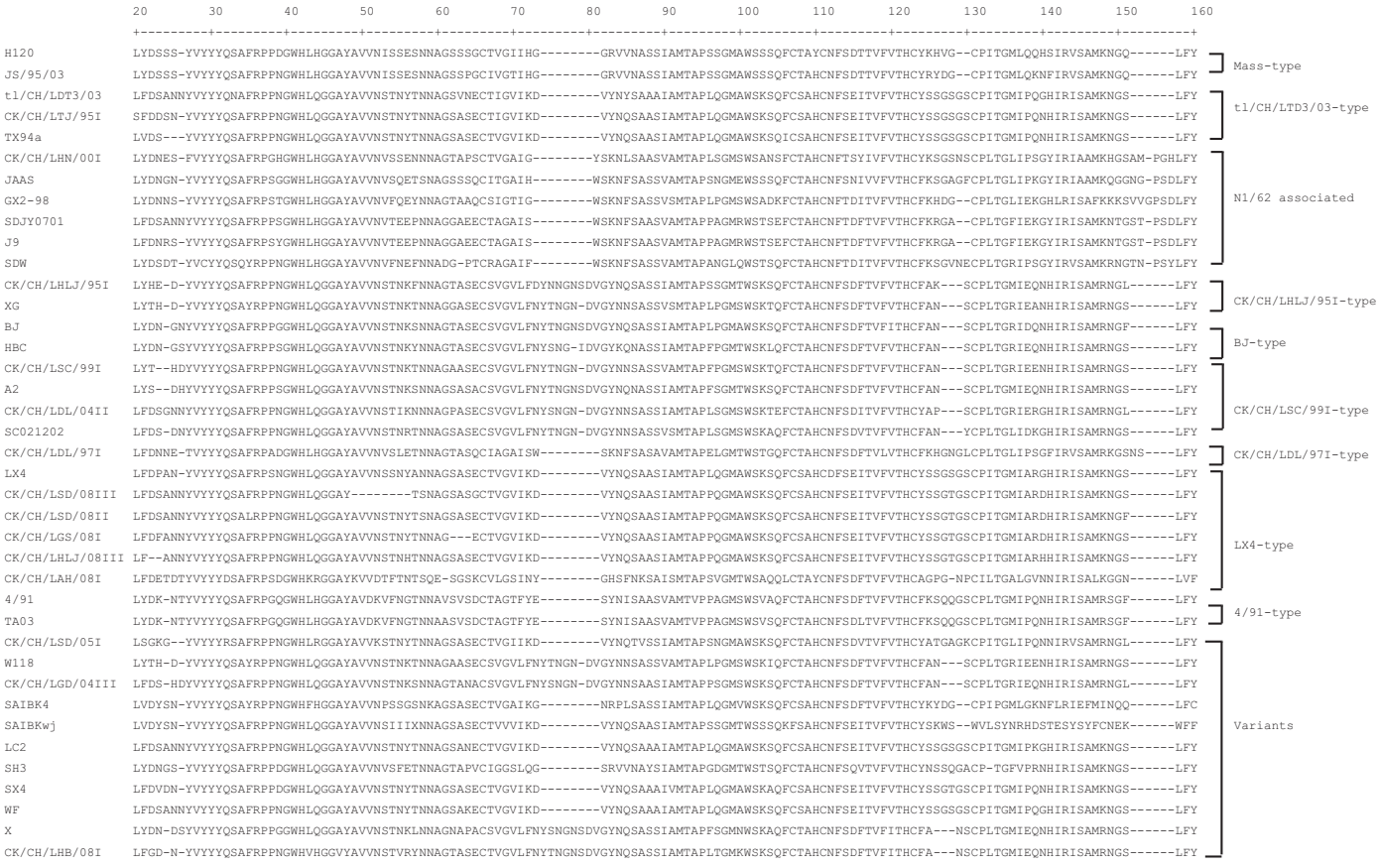

Fig. 3. Sequence alignment of the HVRs of S1 amino acid sequences from IBV variants. Deleted amino acids are represented as dashes. Variants with the same pattern of insertions and deletions are listed with only one representative. The positions of deduced amino acids start with the methionine encoded by the ATG start codon of the S1 gene.

with 4/91-type virus might account for the emergence of IBV W118.

Interestingly, the strain CK/CH/LGD/04III also had a 4/91-like sequence in the S1 gene. As illustrated in Fig. 5, CK/CH/LGD/04III had much lower nucleotide similarity (73.7%) to the 4/91 vaccine strain when the S1 708-bp 5′ terminal regions were compared, whereas identity with the 4/91-type vaccine strain was 100% for the 615 bp from nucleotides 709 to 1325. In contrast, when the 307 bp of the 3′ terminal region was compared, CK/CH/LGD/04III showed only 83.4% nucleotide identity with the 4/91 vaccine strain.

3.5. IBV variants have been continually isolated throughout China

Thirteen of the IBV strains, including three isolated in this work, did not cluster into any of the previously described types. Of these, CK/CH/LSD/05I was a variant that exhibits affinity for the respiratory tract (Liu et al., 2008a). ck/CH/LSD/07-4 originated from S1 gene recombination between CK/CH/LSD/05I-like and tl/CH/LDT3/03-like viruses (Liu et al., 2008b). Strains W118 and CK/CH/LGD/04III are described in the above section. The entire S1 sequence was used to screen the remaining 9, using BLAST to determine preliminary relatedness to previously published S1 sequences.

BLAST searches showed that isolate ck/CH/LHB/08I was most closely related to two Chinese IBV strains, A2 and BJ. Both were isolated before 2003 in Beijing (Liu et al., 2006a) and shared the highest nucleotide identity (87%) with ck/CH/LHB/08I, compared to less than 86% nucleotide identity between the other isolates and ck/CH/LHB/08I. BLAST searches of ck/CH/LAH/08I and ck/CH/LAH/08II S1 revealed that these two isolates were most closely related to a Chinese IBV X strain, although with less than 83% nucleotide identity. All three IBV isolates had the same S cleavage site sequence, Arg-Arg-Phe-Arg-Arg, which is the most common cleavage recognition site of Chinese IBV isolates (Liu et al., 2006a).

For the remaining six IBV strains, WF and LC2 were closely related to each other and comprise a unique branch in the phylogenetic tree.

Table 3
Pairwise comparison of nucleotides and amino acids of the S1 gene of Chinese IBV TA03 with 4/91 pathogenic and attenuated strains.

Strains	87 (29)	179 (60)	283 (95)	319 (107)	825 (275)	1491 (497)	1522 (508)	1574 (525)	1589 (530)
4/91 attenuated	C (Y)	T (V)	G (A)	T (F)	T (T)	C (N)	A (I)	A (Q)	C (P)
4/91 pathogenic	C (Y)	T (V)	T (S)	T (F)	T (T)	C (N)	G (V)	A (Q)	T (L)
TA03	T (Y)	C (A)	T (S)	C (L)	C (T)	T (N)	G (V)	G (R)	T (L)

We compared nucleotides and deduced amino acids (in parentheses) from the ATG encoding the translational start to the cleavage recognition site. No deletions or insertions were found in the S1 genes of the three IBV strains. Of the nine mutations, three (283, 1522 and 1589) were the same as the 4/91 pathogenic strain and the other six were different from both the 4/91 pathogenic and the attenuated strains. Three (87, 825 and 1491) of the mutations were synonymous and did not result in amino acid substitutions. GenBank accession numbers: 4/91 pathogenic strain, AF093794 and 4/91 attenuated strain, AF093793. Y, tyrosine; V, valine; A, alanine; S, serine; T, threonine; F, phenylalanine; L, leucine; N, asparagine; I, isoleucine; Q, glutamine; R, arginine; P, proline.

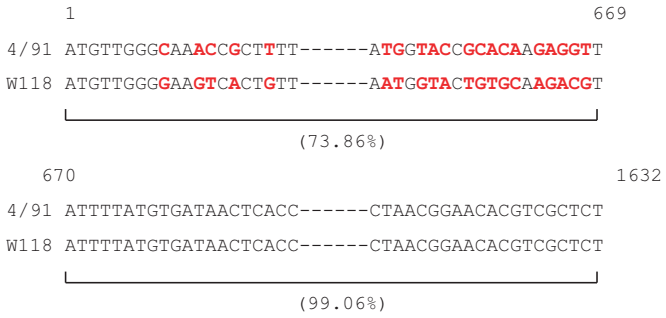

Fig. 4. Sequence and alignment of the S1 genes from the W118 and the pathogenic 4/91 strains. Nucleotides that are different between the two sequences are indicated in red. The positions of nucleotides begin with the ATG that encodes the start codon of S1. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of the article.)

They shared 92.8% S1 nucleotide identity with LX4-type QX. In addition, strain X had the highest nucleotide identity with BJ-type IBV strains at 86%. SH3 was closely related to IBV strains isolated in Taiwan and shared the highest nucleotide identity (92%) with strain TW2296/95. SAIB4 showed less than 82% S1 nucleotide identity with the published IBV strains, with the most closely related being a Mass-type strain, M41. SAIBwj had the highest nucleotide identity with three United Kingdom strains (UK/AV2150/07, NL/L-1449T/04 and NL/L-1449K/04), although nucleotide identity between SAIBwj and these strains was not more than 90%. Predicting the origin, and evaluating the potential harm to the Chinese poultry industry of these isolates was difficult because of limited background information about the vaccination program of the flocks from which they were isolated, and the morbidity and mortality caused by the viruses under field conditions.

3.6. Isolates ck/CH/LHB/08I and ck/CH/LAH/08II were nephropathogenic IBV strains

The virulence of the newly isolated ck/CH/LHB/08I and ck/CH/LAH/08II strains was evaluated using 15-day-old SPF chickens. All chicks exhibited severe clinical signs at 3–15 days after challenge. Clinical signs included tracheal rales, watery eyes, nasal mucus, and sneezing, similar to symptoms caused by other IBV strains (Liu et al., 2008a). In addition, some chicks died during the experiment (Table 2). Gross lesions of dead chicks were mainly confined to the kidneys, similar to effects from other IBV strains that are nephropathogenic to SPF chickens (Li and Yang, 2001; Liu et al., 2009a,c). No clinical signs and gross lesions were observed in the negative control group.

As summarized in Table 2, ck/CH/LHB/08I was detected in the trachea, kidney, and caecal tonsil of all birds at 5 days post-challenge, as assessed by recovery of the virus using 9-day-old embryos and subsequent RT-PCR. In addition, 6 out of 10 chicks were positive for virus in the lung. In contrast, some of the ck/CH/LAH/08II-challenged chickens were negative for virus recovery from the trachea and caecal tonsil, and ck/CH/LAH/08II was detected in the kidney and lung of less than 50% of these chickens. Moreover, more than half of the chickens challenged with ck/CH/LHB/08I exhibited seroconversion at 6 days post-challenge, but none of the nine chickens who survived challenged with ck/CH/LAH/08II showed seroconversion after 6 days. The virus was not detected in the trachea, kidneys, lung and caecal tonsil of unchallenged negative control birds, and none of the unchallenged chickens showed seroconversion (Table 2).

3.7. Mass-type H120 vaccine provided different protection against variants ck/CH/LHB/08I and ck/CH/LAH/08II

As summarized in Table 2, 100% of H120-vaccinated birds exhibited clinical respiratory signs and mild gross lesions after challenge with ck/CH/LHB/08I, and the clinical signs and gross lesions were similar to birds in the ck/CH/LHB/08I-challenged group. The clinical signs in the diseased birds tended to disappear gradually after 15 days of challenge, although three birds in the H120-vaccinated group died during the experiment. Interestingly, the rate of viral recovery from the respiratory tract of H120-vaccinated birds after challenge with ck/CH/LHB/08I was 100%, while more than 60% of the vaccinated chicks tested positive for caecal tonsil IBV at 5 days post-challenge. These results were in

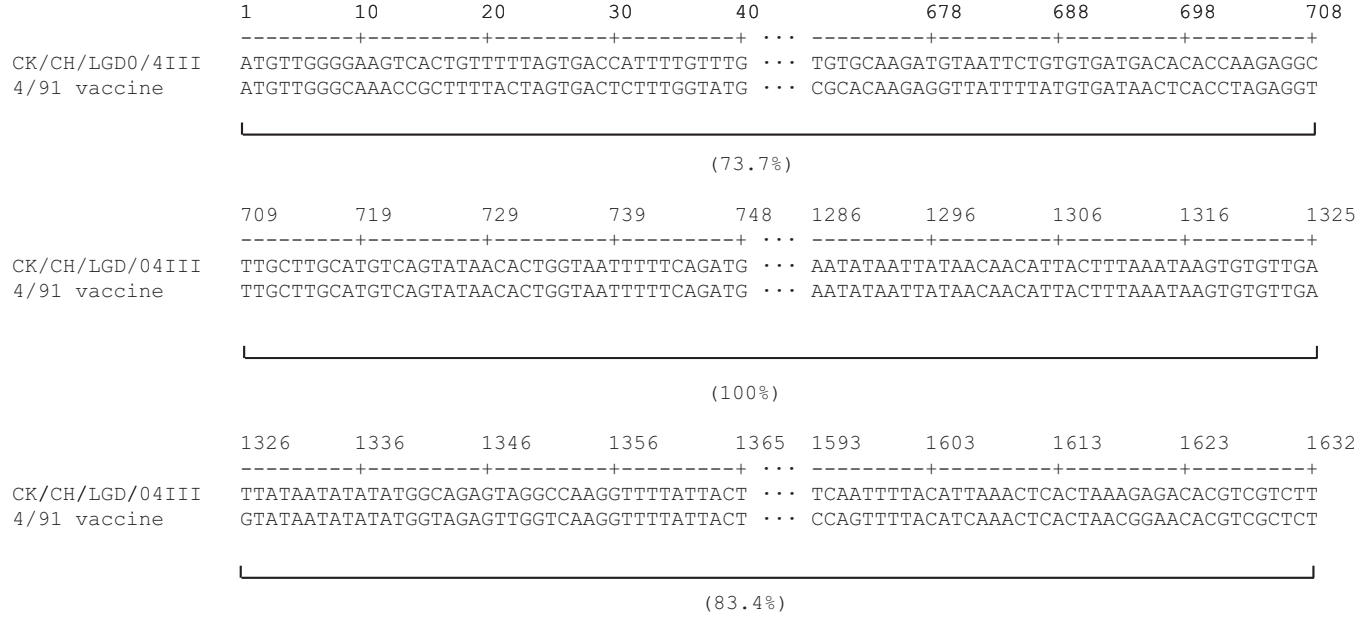

Fig. 5. Sequence alignment of S1 genes from the CK/CH/LGD/04III and the 4/91 vaccine strains. Deleted nucleotides are represented as dots. Nucleotide identities are in parentheses. The positions of nucleotides are given from the ATG that encodes the start codon of S1.

contrast to those obtained for H120-vaccinated birds challenged with ck/CH/LAH/08II. None of these birds showed clinical signs or death during the experiment, suggesting good clinical protection against isolate ck/CH/LAH/08II after vaccination with H120. However, offspring ck/CH/LAH/08II virus was recovered from 40% of the respiratory tract and caecal tonsils of vaccinated birds, indicating poor protection of these areas. Interestingly, the H120 vaccination provided better kidney protection against both ck/CH/LHB/08I and ck/CH/LAH/08II. Serum antibodies for all vaccinated birds, as detected by ELISA, are summarized in Table 2. Antibodies were not detected in non-vaccinated or unchallenged birds.

4. Discussion

In this study, we isolated 46 IBV strains from chicken flocks in various provinces or cities in China in 2008. To increase our insight into the comprehensive epidemiological situation and evolutionary process of IBV in China, we analyzed the S1 genes of all 174 Chinese IBV isolates with sequence information deposited in GenBank since 1995. Our phylogenetic analysis of S1 glycoprotein genes revealed that Chinese IBV strains could be at least classified into nine genetic groups. In addition, 13 IBV variants were also found to be circulating in vaccinated chicken flocks in recent years, which complicates the IBV epidemiology in China. Interestingly, we found that more than 50% of Chinese IBV strains were LX4-type, consistent with previous results (Liu and Kong, 2004; Liu et al., 2009a). LX4-type IBVs were first isolated in 1999 (Liu and Kong, 2004) and later found to be the predominant IBV type circulating in both laying hens and broilers in China (Liu et al., 2006b, 2009a). Recently, this type was reported to be one of the most important in Russia and other European countries (Bochkov et al., 2006; Dolz et al., 2008; Domanska-Blicharz et al., 2006; Gough et al., 2008; Worthington and Jones, 2006). The S1 gene of LX4-type strains showed slight variability among isolates obtained between 1995 and 2009 in China, suggesting that genetic change might not be a constant feature of IBV strains, as it is among mammalian coronaviruses (Masters, 2006). Nonetheless, we found some mutations, insertions and deletions in the S1 gene among LX4-type strains (Fig. 3). This opens the possibility that different LX4-type IBV strains evolved independently in different regions under various field conditions, although it is still possible that these viruses have same origin. Most of the isolates came from flocks vaccinated with Mass-type vaccines, which implies insufficient protection against the field strains. The poor relationship between the Chinese LX4-type field isolates and the Mass-type vaccine strains (average amino acid identity of 78%) could explain the failure of the Mass-type vaccination programs to control IBV in these flocks. Indeed, a cross-protection analysis between the LX4 and Mass-type H120 vaccine strains established this failure. Because of the large amounts of LX4-type IBV isolated in various parts of China, and the possibility of different virulence from different IBVs (Cavanagh and Gelb, 2008), the pathogenicity of newly emerged viruses and cross-protection from Mass-type vaccines must be reevaluated.

The presence and the spreading of various IBV types in different continents or regions is an interesting aspect of IBV epidemiology. Approximately 20 emergent types in North America have not spread to other continents. Similarly, the European, Australian, and Asiatic types of IBVs, have apparently not spread. This is the case for the CK/CH/LSC/99I-type IBV in this study. Almost all IBV strains isolated from Sichuan province (including isolates from Chongqing City) were CK/CH/LSC/99I-type. Sichuan is one of the provinces with a high density of chicken farms in China, so this result is significant for vaccination programs in this region. In contrast, other types of IBV strains were found to co-circulate in China. The spreading of a virus from one area to another could be due, at least in part, by the illegal trading of chickens or by the use of attenuated vaccines. Recently, wild birds have been of concern as natural IBV carriers, since infected birds carry the viruses from one area to another (Cavanagh, 2007; Liu et al., 2005; Shimazaki et al., 2009; Sun et al., 2007).

CK/CH/LDL/97I-type IBV is a new IBV type that first appeared in 1995 in China (Liu et al., 2009c; Yu et al., 2001). This isolate seems to have high affinity for the proventriculus of chicken and is implicated in a novel pathogenicity associated with transmissible proventriculitis (Yu et al., 2001), even though few experimental proventriculitis models have been generated using this IBV. The CK/CH/LDL/97I-type IBV is associated with IB outbreaks from 1997 to 2001 in Liaotung Peninsula (Liu et al., 2009c; Yu et al., 2001). In this study, a CK/CH/LDL/97I-type IBV isolate, ck/CH/LSD/08-10, was isolated from the trachea of a H120-immunized broiler in Shandong Province. Interestingly, unlike other CK/CH/LDL/97I-type strains, ck/CH/LSD/08-10-infected chickens did not show proventriculitis under field conditions. Importantly, this type of IBV has been isolated again in immunized chicken flocks after it "disappeared" for 7 years. Furthermore, the pathogenicity and tissue tropism of ck/CH/LSD/08-10 appears to have changed under field conditions (Liu et al., 2009c; Yu et al., 2001). Thus, its characteristics should be analyzed further.

The third major type of IBV circulating in China is Mass-type, which is a pandemic type that is frequently isolated from chicken flocks in China, even those vaccinated with H120 or other Mass-type vaccines. This also occurs in other countries (Dolz et al., 2008; Rimondi et al., 2009; Roussan et al., 2009) and is expected, because vaccine strain re-isolation is possible when extensive vaccination programs use attenuated strains. However, vaccination is not likely to be the only possibility for Mass-type virus circulation in China. In this study, even though S1 nucleotide and amino acid identities were greater than 95% among Mass-type isolates, and between isolates and vaccine strains, sequence comparison and analysis showed discrepancies in the S1 genes for most of the isolates and vaccine strains. Hence, circulation between field Mass-type isolates in vaccinated and non-vaccinated chickens flocks cannot be excluded and may indicate a vaccination failure in these flocks. Alternatively, molecular studies have shown that only a few changes in S1 spike protein amino acid composition can result in immune failure, even when the majority of the virus genome remains unchanged (Cavanagh et al., 1992b). Amino acid changes may result from immunological pressure caused by the widespread use of vaccines (Cavanagh et al., 2005) and recombination after mixed infections, which allows other field strains to emerge. In this study, we found four IBV strains (BJ9601, SD/97/01, ck/CH/LHLJ/07II and ck/CH/LHLJ/08II) with an amino acid substitution at position 63 of the S1 subunit of the S protein that resulted in escape mutants (Cavanagh et al., 1988).

One of the most challenging aspects of IBV epidemiology in China is the emergence of 4/91-type IBV isolates and new isolates resulting from recombination between 4/91-type and Chinese local strains. IBV strains of the 4/91-type, which are also known as 793/B, were first reported and characterized in Britain in 1991 (Adzhar et al., 1997; Gough et al., 2008). Both viral and serological surveys have shown that the 4/91-type has become one of the predominant European types, with high incidences of infection in layer and broiler chickens worldwide (Capua et al., 1999; Cavanagh et al., 2005; Cook et al., 1996; Dolz et al., 2008; Gough et al., 1992; Meulemans et al., 2001; Roussan et al., 2009; Worthington et al., 2008). In this study, we found that TA03 had low sequence similarity to the Japanese 4/91 field strain (Shimazaki et al., 2008), indicating minimal likelihood that TA03 originated in Japan. In contrast, TA03 showed close relationship with 4/91 European strains, and is more closely related to the pathogenic strain than the vaccine strain. Three amino acids at positions 95, 508 and 530

were different between a UK/4/91 field isolate (4/91 pathogenic) and its embryo-passaged, attenuated derivative (4/91 vaccine) (Cavanagh et al., 2005). Interestingly, TA03 shared the same three amino acids with the 4/91 pathogenic strain, even though these changes are not associated with pathogenicity (Shimazaki et al., 2009). It has been suggested that replication of 4/91 strains in chickens and embryonated eggs is associated with position 95, particularly alanine in the vaccine strain and serine in the chick-passaged strain (Cavanagh et al., 2005). For TA03, amino acid 95 was serine, the same as the chick-passaged isolate. These results might suggest that TA03 has adapted to propagate in chickens. However, we cannot conclude that IBV TA03 is not associated with the 4/91 vaccine. The background of the commercial chickens from which TA03 was isolated and the pathogenicity of TA03 strain was unclear. Hence, the origin of the Chinese 4/91-type isolates, especially their relation to the vaccine strain, requires further investigation.

In this work, we found 4/91-like sequences in the S1 genes of two IBV strains (W118 and CK/CH/LGD/04III) clustered in unrestricted regions. Alignment and comparison of S1 gene suggested that recombination events associated with 4/91 might have been involved in the origin and evolution of Chinese local IBV strains W118 and CK/CH/LGD/04III. Because TA03, W118 and CK/CH/LGD/04III were isolated from different regions, and the challenges created by these types of viruses might be substantial. Hence, a dynamic investigation of the prevalence of the 4/91-type virus is necessary for a deeper understanding of its evolution and epidemiology in China.

Another challenging aspect of IBV epidemiology in China is the continual emergence of IBV variants in different regions in China, which complicates disease control. In this study, using comparison of S1 genes, we discovered 13 IBV variants from 1995 to 2009. Nearly all had unique deletion and insertion patterns in S1, especially in the hypervariable regions (Fig. 3), indicating possible different origins for these strains. From S1 gene sequence analysis, we hypothesized that some originated from recombination events between local IBVs, and between local and vaccine strains, indicating that the widespread use of live virus vaccines may contribute to the origin and genetic evolution of IBV. However, because of limited background information, the exact origin of most of the IBV variants is still unclear. Predicting the origin of these IBV variants using whole genome sequence analysis might provide more information.

The IBV strains, as a group, infect a large range of epithelial surfaces, from the top to the bottom of the chicken. Isolates differ in the extent of replication in non-respiratory tissues, and some produce clinical disease in non-respiratory tissues, like the kidney and proventriculus. We found that both ck/CH/LHB/08I and ck/CH/LAH/08II caused nephrosis after infection of 15-day-old chickens, even though ck/CH/LAH/08II was isolated from the trachea of an infected broiler. Similar mortalities were also observed after inoculating 15-day-old SPF chickens with the two Chinese IBV isolates (Table 2), comparing the outcome to other nephropathogenic IBV isolates (Li and Yang, 2001; Liu et al., 2009a). Although this is not a true reflection of the situation in the flocks, the results confirmed that the two isolates are nephropathogenic.

Although genotyping based on the S1 gene has been useful for determining the epidemiology of field IBVs, and for predicting the effectiveness of vaccines against field isolates, vaccination-challenge is the only way to establish the actual protective potential of a given vaccine strain for a field isolate (Hofstad, 1981). Only the Mass-type vaccine is officially authorized in China, so its protection against heterologous isolates must be evaluated. In this study, we determined the ability of the H120 vaccine to protect against two IBV variants, ck/CH/LHB/08I and ck/CH/LAH/08II, in a vaccination-challenge test. Vaccination reduced, and in many cases prevented, replication of ck/CH/LAH/08II in lung, kidney, caecal tonsil, and the respiratory tract. However, H120 vaccination did not provide respiratory protection against ck/CH/LHB/08I, since virus was recovered from 100% of the respiratory tracts of ck/CH/LHB/08I-challenged chickens. ck/CH/LHB/08I could be a exceptionally virulent strain for chickens. Nonetheless, according to the Pharmacopoeia's reference standards depending on the country (de Wit et al., 1998; Liu et al., 2006b; Terregino et al., 2008), H120 shows low effectiveness against ck/CH/LHB/08I and ck/CH/LAH/08II. The protection is especially poor since IBV infection of very young layer hens can produce permanent damage to the genital tract that is evident only several weeks after infection, when the birds come into lay and show reduced egg production and quality (Cavanagh and Gelb, 2008), or an increased incidence of false layers is observed in the flock. Vaccination with only Mass-type vaccines does not appear to provide enough protection against various IBV types and the variants indigenous to China (Liu et al., 2006b, 2008a, 2009c). The results presented here are additional evidence for the poor protection against challenge with a heterologous IBV strain. Although vaccination with various types of vaccines may improve protection efficacy (Terregino et al., 2008), the Mass-type vaccine is the only vaccine officially authorized in China, suggesting a vaccine from Chinese local IBV strains should be developed and IB control programs in China should be adjusted.

Some IBV strains from China were not included in this study because their entire S1 protein genes were not sequenced (Wu et al., 1998) or only partly sequenced (Li and Yang, 2001). In addition, only strains isolated before April 2009 were available in the database. In conclusion, the detailed epidemiological information in this work is of significance for vaccination programs in China. Dynamic surveillance of IBV is important, even though the complicated epidemiology of the multiple IBV types and variants co-circulating in any specific region in China makes designing molecular diagnostic techniques difficult. Rapid, accurate and sensitive methods for diagnosis of IBV in China must be investigated in future studies.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.meegid.2010.09.002.

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