AVIAN DISEASES 56:15–28, 2012

Genetic Diversity of Avian Infectious Bronchitis Coronavirus in Recent Years in China

Huijie Ma, Yuhao Shao, Chuyang Sun, Zongxi Han, Xiaoli Liu, Hongbo Guo, Xiaozhen Liu, Xiangang Kong, and Shengwang Liu[A]

Division of Avian Infectious Diseases, State Key Laboratory of Veterinary Biotechnology, Harbin Veterinary Research Institute, Chinese Academy of Agricultural Sciences, Harbin 150001, People's Republic of China

Received 23 May 2011; Accepted and published ahead of print 18 August 2011

SUMMARY. Fifty-six isolates of avian infectious bronchitis virus (IBV) were obtained from different field outbreaks in China in 2010, and they were genotyped by comparison with 19 reference strains in the present study. The results showed that LX4-type isolates are still the predominant IBVs circulating in chicken flocks in China, and these isolates could be grouped further into two clusters. Viruses in each cluster had favored amino acid residues at different positions in the S1 subunit of the spike protein. In addition, a recombination event was observed to have occurred between LX4- and tl/CH/LDT3/03I-type strains, which contributed to the emergence of a new strain. The most important finding of the study is the isolation and identification of Taiwan II–type (TW II–type) strains of IBV in mainland China in recent years. The genome of TW II–type IBV strains isolated in mainland China has experienced mutations and deletions, as demonstrated by comparison of the entire genome sequence with those of IBV strains isolated in Taiwan. Pathogenicity testing and sequence analysis of the 3′ terminal untranslated region revealed that TW II–type IBV strains isolated in mainland China have a close relationship with the embryo-passaged, attenuated TW2296/95.

RESUMEN. Diversidad genética del coronavirus la bronquitis infecciosa aviar en los años recientes en China.

Se obtuvieron cincuenta y seis aislamientos del virus de la bronquitis infecciosa aviar (IBV) de diferentes brotes de campo en China en el 2010, y se caracterizaron genéticamente mediante su comparación con 19 cepas de referencia en el presente estudio. Los resultados mostraron que cepas del tipo LX4 siguen siendo los virus de bronquitis que circulan predominantemente en las parvadas de pollos en China, y estas cepas podrían ser agrupadas en dos grupos. Los virus en cada grupo presentaban residuos de aminoácidos específicos en diferentes posiciones dentro de la subunidad S1 de la proteína de la espícula. Además, se observó un evento de recombinación que se ha producido entre las cepas LX4 y las cepas del tipo tl/CH/LDT3/03I, lo que contribuyó a la aparición de una nueva cepa. El hallazgo más importante de este estudio es el aislamiento e identificación de las cepas tipo Taiwán II (TW II-type) del virus de la bronquitis infecciosa en la China continental en los últimos años. El genoma de las cepas TW II aisladas en la parte continental de China ha experimentado mutaciones y deleciones, como lo demuestra la comparación de la secuencia de sus genomas con los de las cepas del virus de la bronquitis aisladas en Taiwán. Las pruebas de patogenicidad y análisis de la secuencia de la región terminal 3' no traducida, revelaron que las cepas de tipo TW II aisladas en la parte continental de China tienen una estrecha relación con la cepa atenuada en embriones de pollo TW2296/95.

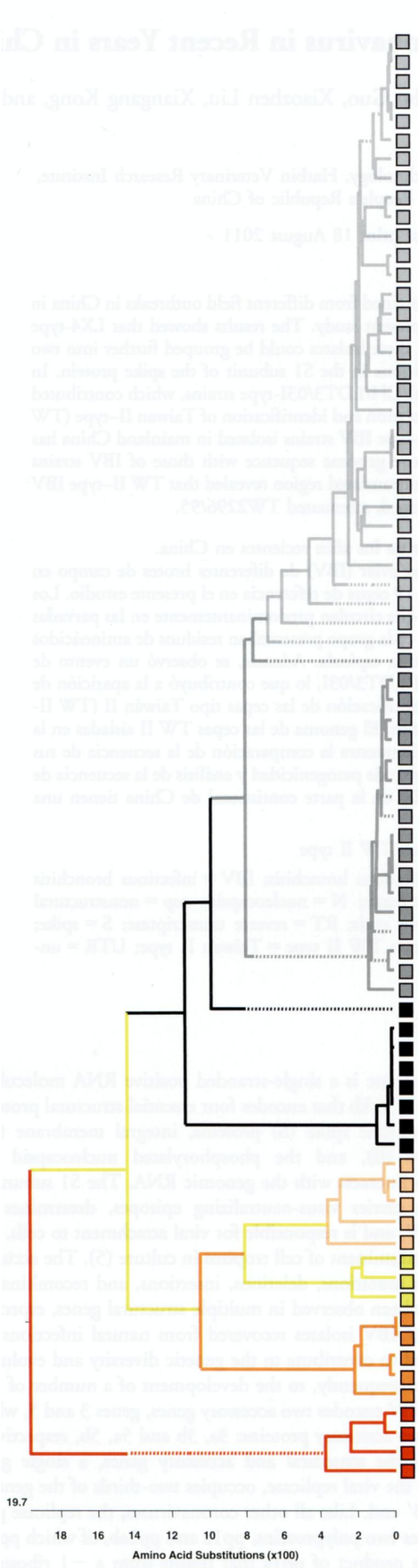
Key words: genetic diversity, avian infectious bronchitis coronavirus, emergence, TW II type

Abbreviations: E = envelope; EID_{50} = 50% embryo infectious doses; IB = infectious bronchitis; IBV = infectious bronchitis virus; M = membrane; Mass = Massachusetts; MHV = mouse hepatitis coronaviruses; N = nucleocapsid; nsp = nonstructural protein; PBS = phosphate-buffered saline; RACE = rapid amplification of cDNA ends; RT = reverse transcriptase; S = spike; S/P = serum to positive; SPF = specific-pathogen-free; TCoV = turkey coronavirus; TW II type = Taiwan II type; UTR = untranslated region

Coronaviruses are enveloped, positive-stranded RNA viruses that cause respiratory and enteric disease in a variety of avian and mammalian species, including humans, cattle, pigs, dogs, cats, mice, rats, rabbits, chickens, turkeys, and bats (49). On the basis of their serologic cross-reactivity and, more important, their genome organization, coronaviruses had been grouped recently into three genera, *Alphacoronavirus*, *Betacoronavirus*, and *Gammacoronavirus* (13,62). Avian infectious bronchitis virus (IBV), together with the genetically closely related turkey coronavirus (4,10,24,25), pheasant coronavirus (11), and recently identified coronaviruses from several species of wild birds (34,63), a beluga whale (*Delphinapterus leucas*; 50), and an Asian leopard (*Prionailurus bengalensis*; 18), form the genus *Gammacoronavirus*. Numerous variants and serotypes of IBV have been, and continue to be, discovered in poultry flocks worldwide (7,8,15,16,19,23,26,57). They cause infectious bronchitis (IB), a disease that is responsible for economic losses in the poultry industry throughout the world (9).

[A]Corresponding author. E-mail: swliu@hvri.ac.cn

The IBV genome is a single-stranded positive RNA molecule of approximately 27.6 kb that encodes four essential structural proteins: the three membrane spike (S) proteins, integral membrane (M), small envelope (E), and the phosphorylated nucleocapsid (N) protein, which interacts with the genomic RNA. The S1 subunit of the S protein carries virus-neutralizing epitopes, determines the serotype of IBV, and is responsible for viral attachment to cells. It is also a major determinant of cell tropism in culture (5). The accumulation of point mutations, deletions, insertions, and recombination events that has been observed in multiple structural genes, especially the S1 gene, of IBV isolates recovered from natural infections has been considered to contribute to the genetic diversity and evolution of IBV, and, consequently, to the development of a number of IBV serotypes (6). IBV encodes two accessory genes, genes 3 and 5, which both express two accessory proteins: 3a, 3b and 5a, 5b, respectively. In addition to the structural and accessory genes, a single gene, which encodes the viral replicase, occupies two-thirds of the genome closest to the 5′ end. Like all other coronaviruses, the replicase gene of IBV expresses two polyproteins, pp1a and pp1ab, of which pp1ab is an extension product of pp1a that results from a −1 ribosomal



IBV Isolates	Type	Province	Vaccines used for immunization	Organs used for virus isolation	Type of Chicken	Accession number
ck/CH/LHN/101208	LX4 (Cluster I)	Henan	H120	Kidney	Layer	JF330856
ck/CH/LHN/101209		Henan	H120+H52	Kidney	Layer	JF330857
ck/CH/LHN/101211		Henan	H120	Kidney	Layer	JF330859
ck/CH/LHN/101210		Henan	H120	Kidney	Layer	JF330858
ck/CH/LJS/101110		Jiangsu	H120	Kidney	Layer	JF330872
ck/CH/LSD/100505		Shandong	H120	Kidney	Broiler	JF330889
ck/CH/LSD/100205		Shandong	H120	Kidney	Broiler	JF330881
ck/CH/LSD/101221		Shandong	H120	Kidney	Broiler	JF330893
ck/CH/LDL/101215		Dalian	H120+Ma5	Kidney	Layer	JF330849
ck/CH/LLN/101213		Liaoning	H120+Ma5	Proventriculus	Layer	JF330879
ck/CH/LSD/100311		Shandong	H120	Kidney	Broiler	JF330884
ck/CH/LHB/100904		Hebei	H120+4/91	Kidney	Layer	JF330851
ck/CH/LHB/100906		Hebei	H120	Kidney	Broiler	JF330852
ck/CH/LSD/100102		Shandong	H120	Kidney	Broiler	JF330880
ck/CH/LSD/100409		Shandong	H120	Kidney	Broiler	JF330903
ck/CH/LJS/101117		Jiangsu	H120	Proventriculus	Layer	JF330901
ck/CH/LSD/100312		Shandong	H120	Kidney	Broiler	JF330885
ck/CH/LSD/101224		Shandong	H120	Kidney	Broiler	JF330896
ck/CH/LJL/101131		Jilin	H120	Kidney	Layer	JF330866
ck/CH/LSD/100305		Shandong	Non-vaccinated	Kidney	Layer	JF330883
ck/CH/LJL/100501		Jilin	H120+Ma5	Kidney	Layer	JF330860
ck/CH/LJL/100513		Jilin	H120	Kidney	Layer	JF330862
ck/CH/LJL/100713		Jilin	H120	Proventriculus	Layer	JF330864
ck/CH/LJL/100512		Jilin	H120	Kidney	Layer	JF330861
ck/CH/LHLJ/100309		Heilongjiang	H120_Ma5	Kidney	Layer	JF330854
ck/CH/LJL/100918		Jilin	H120+Ma5	Proventriculus	Layer	JF330865
ck/CH/LJL/100515		Jilin	H120	Kidney	Broiler	JF330863
ck/CH/LGS/100521		Gansu	H120+H52	Liver	Layer	JF330850
ck/CH/LSD/101222		Shandong	H120	Kidney	Broiler	JF330894
ck/CH/LJL/101150		Jilin	H120	Kidney	Broiler	JF330868
LX4		Xinjiang	H120	Kidney	Layer	AY189157
QX/BV		Shandong	NA	Proventriculus	Broiler	AF193423
ck/CH/LJS/101237		Jiangsu	H120+Ma5	Trachea	Layer	JF330878
ck/CH/LJS/101116	LX4 (Cluster II)	Jiangsu	H120	Kidney	Black-Boned	JF330900
ck/CH/LSD/101223		Shandong	H120	Kidney	Broiler	JF330895
ck/CH/LJS/101113		Jiangsu	H120	Kidney	Broiler	JF330875
ck/CH/LSD/101225		Shandong	H120+Ma5	Kidney	Broiler	JF330897
ck/CH/LSD/101139		Shandong	H120	Kidney	Broiler	JF330890
ck/CH/LSD/101216		Shandong	H120	Kidney	Broiler	JF330891
ck/CH/LJS/101109		Jiangsu	H120	Kidney	Layer	JF330871
ck/CH/LSD/100412		Shandong	H120	Proventriculus	Broiler	JF330904
ck/CH/LJS/101105		Jiangsu	H120	Kidney	Broiler	JF330867
ck/CH/LJS/101108		Jiangsu	H120	Kidney	Layer	JF330870
ck/CH/LJS/101111		Jiangsu	H120	Kidney	Layer	JF330873
ck/CH/LJS/101106		Jiangsu	H120	Kidney	Broiler	JF330869
ck/CH/LJS/101112		Jiangsu	H120	Kidney	Layer	JF330874
ck/CH/LJS/101114		Jiangsu	H120	Kidney	Broiler	JF330876
ck/CH/LSD/101115		Shandong	H120	Kidney	Broiler	JF330877
ck/CH/LSD/100315		Shandong	H120	Kidney	Broiler	JF330886
ck/CH/LHB/100908		Hebei	H120	Lung	Broiler	JF330853
ck/CH/LSD/100408		Shandong	H120	Kidney	Broiler	JF330902
ck/CH/LHB/090914	TI/CH/LDT3/03	Hebei	H120+Ma5	Kidney	Layer	HM194648
ck/CH/LSD/100504		Shandong	H120	Kidney	Broiler	JF330888
ck/CH/LSD/100208		Shandong	H120	Kidney	Broiler	JF330882
ck/CH/LSD/100318		Shandong	H120	Kidney	Broiler	JF330887
ck/CH/LSD/101217		Shandong	H120	Kidney	Broiler	JF330892
ck/CH/LHN/091025		Henan	Non-vaccinated	Trachea	Broiler	HM194673
TI/CH/LDT3/03		Guangdong	Non-vaccinated	Heart + Lung	Teal	AY702975
TW2296/95	TW-II	Taiwan	Yes	NA	Broiler	AY606321
ck/CH/LHB/100801		Hebei	H120+Ma5	Proventriculus+Kidn	Layer	JF330898
ck/CH/LJL/090608		Jilin	H120	Proventriculus	Broiler	HM194676
3263/04		Taiwan	Yes	NA	Broiler	EU822338
T07/02		Taiwan	Yes	Kidney	Fighting chicken	AY606322
3376/06	TW-I	Taiwan	Yes	Trachea	Broiler	CQ229244
3071/03		Taiwan	Yes	Kidney	Broiler	AY606319
3051/02		Taiwan	Yes	Kidney	Broiler	AY606318
ck/CH/LDL/101212	Mass	Dalian	H120+Ma5	Proventriculus	Layer	JF330848
H120		-	-	-	-	FJ807652
Ck/CH/LHLJ/100902		Heilongjiang	H120+Ma5	Trachea	Layer	JF330855
M41		America	Non-vaccinated	Trachea	Layer	X04722
Beaudette		America	Non-vaccinated	Trachea	Layer	NC_001451
T3	CK/CH/LDL/97I	Shandong	H120	Proventriculus	Layer	AF227438
CK/CH/LDL/97I		Dalian	H120	Proventriculus	Layer	DQ068701
2992/02		Taiwan	Yes	Kidney	Broiler	AY606323
3374/05		Taiwan	Yes	NA	Broiler	EU822337

shift mechanism. During or after synthesis, the two polyproteins are cleaved by two types of virus-encoded proteinase, which usually results in 16 nonstructural proteins (nsp 1–16); IBV lacks nsp 1 and thereby encodes nsp 2–16 (2,49).

IBV has been diagnosed in China since the early 1980s by pathogenic studies and serologic techniques, and Massachusetts (Mass) type live IB attenuated vaccine as well as inactivated or killed oil-emulsion vaccines have been applied to prevent and control the disease. Despite extensive vaccination, IBV infection has remained a problem in the Chinese poultry industry (26). Since the early 1980s, Chinese isolates of IBV have been characterized molecularly and grouped into at least nine populations and a couple of variants (26,55). Some of the populations or variants are indigenous to China; however, some of them, such as the LX4 type, are also found on other continents (30). LX4 genotype IBV was proposed to have originated in China in the mid-1990s. It is not only the most frequently detected type of IBV on nearly all Chinese chicken farms, but the rapid spread of this IBV type across Europe has also caused heavy economic losses (30), despite the widespread use of live vaccines. Furthermore, new strains of IBV have been isolated continuously in China over time, and outbreaks caused by IBV have been increasing annually. In view of the increasing epidemiologic importance of IBV in China, constant surveillance for the detection of IBVs that are infecting poultry populations currently and further characterization of IBV isolates were considered necessary. The objectives of this study were to report the isolation and characterization of IBV field isolates from a variety of clinical respiratory conditions. The genetic relationships of our isolates with previously known IBV strains were established to investigate the evolution and molecular characteristics of the virus, in addition to the detection of overwhelming field strains and new variants from outside the immediate region of interest.

MATERIALS AND METHODS

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

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*.

Field samples and virus isolation. In the course of our continuous surveillance activities for IBV in China, most flocks had been vaccinated against IB with commercial live attenuated vaccines. Nasal swabs and tissue samples from kidney, proventriculus, trachea, cecal tonsil, liver, and lung were analyzed from 216 flocks of cockerels, broilers, pullets, layers, and breeders suspected of IB infection. The samples covered most of the chicken-raising regions of China and were collected between January and December 2010 (Fig. 1). Nearly all the chickens that were suspected of IB infection showed early signs of respiratory disease, including gasping, coughing, sneezing, and tracheal rales at the onset of disease. Postmortem findings from affected birds included mild to severe tracheitis, marked airsacculitis, nephritis, and proventriculitis. The morbidity ranged from 5% to 70%, and the mortality varied between 5% and 30%. In layer flocks, mortality, egg drop, and deformed eggs were the most prevalent signs documented. The tissue samples from chickens from the same flock were pooled together.

For virus isolation, the samples were prepared as 10% weight/volume tissue suspensions in 0.1% phosphate-buffered saline (PBS), clarified by centrifugation at 1500 × *g* at 4 C for 10 min, and filtered through 0.22-μm membrane filters (Millipore, Bedford, MA) before inoculation into the allantoic cavities of 9-to-11-day-old embryos of SPF chickens. Nasal swabs in 1 × PBS were clarified directly by low-speed centrifugation at 1500 × *g* at 4 C for 10 min and filtered through 0.22-μm membrane filters. Three to five eggs were used for each sample. The inoculated eggs were incubated at 37 C and candled daily. Three to five blind passages were performed until the characteristic embryo changes, such as dwarfing, stunting, or curling of embryos, were observed between 2 and 7 days after inoculation, according to a previous report (44).

Cloning and sequencing of the S1 gene of IBV isolates. A reverse transcriptase (RT)-PCR protocol that has been described previously was used for the amplification of the S1 gene (1). Briefly, viral RNA was extracted from 200 μl of infected allantoic fluid using TRIzol reagents (Invitrogen, Grand Island, NY), following the manufacturer's protocol. For the first cDNA strand, a mixture containing 20 μM of the reverse primer N (−), 20 units of RNasin (Invitrogen), 0.5 mM each dNTP, 8 μl of 5× buffer, and the RNA template was first incubated at 70 C for 5 min and transferred immediately to ice for another 5 min. Subsequently, 100 U of Moloney-Murine leukemia virus RT (Invitrogen) was added to make a final volume of 40 μl. The reaction was run at 37 C for 2 hr, followed by 72 C for 10 min, and transferred immediately to ice for 5 min. The PCR was performed in a 50-μl reaction containing 2 μl of first strand cDNA; 15 nmol of downstream and 15 nmol of upstream primer; 5 μl of 10× PCR buffer (Mg^{2+} Plus, TaKaRa, Shiga, Japan); 4 μl of 2.5 mmol dNTPs; 2 U of *Taq* polymerase (TaKaRa); and 18 μl of water. The reaction was conducted at 95 C for 5 min; 30 cycles of 94 C for 1 min, 50 C for 1 min, 72 C for 2 min; and a final extension step of 72 C for 10 min. Two sense oligonucleotides, IBV-257 and S1Oligo5′, were used with S1Oligo3′, IBV-212 or IBV-275 as antisense primers in PCR amplification. The sequences and locations of the primers used in this study are presented in Table 1.

A product of about 1700 bp was generated, which was detected by ethidium bromide staining. The DNA generated by PCR amplification was cloned using a T-tailed vector, pMD18-T (TaKaRa), and transformed using JM109 competent cells (TaKaRa) according to the manufacturer's instructions. Each region of the S1 gene in each IBV isolate was sequenced at least three times, and the consensus sequence was determined.

Analysis of the S1 gene. The nucleotide and amino acid sequences of the S1 gene of the IBV isolates were assembled, aligned, and compared with those of other reference IBV strains using the MegAlign program in DNAStar. Phylogenetic analysis of the nucleotide sequences of the S1 gene was performed with the Clustal V method of DNAStar software (26) and validated using Megaware (47) and PAUP version 4.08b for the Macintosh (33,56). A total of 19 IBV reference strains were selected for phylogenetic analysis of the S1 gene in this study. The IBV strains LX4 and QX were selected as representatives of Chinese LX4-type strains (44,61). Three IBV strains, ck/CH/LHB/090914, ck/CH/LHN/091025, and tl/CH/LDT3/03, were selected as representatives of Chinese tl/CH/LDT3/03-type strains (42). Beaudette, M41, and H120 were selected as representatives of Mass-type strains. Seven IBV strains, TW2595/96, 2296, 3263, T07, 3376, 3071, and 3051, that belonged to Taiwan I–type (TW I–type) and Taiwan II–type (TW II–type) strains (27,28,58) were also selected for phylogenetic analysis in

←

Fig. 1. Phylogenetic relationships, based on the sequence of the S1 subunit of the S gene of IB vaccine strains and IBV field strains isolated in China (the first 1629 nucleotides, starting at the AUG translation initiation codon, of the S gene), obtained using the MegAlign program in DNAStar with the Clustal V method (Han *et al.* (26)). The IBV isolates obtained in the present study are in bold. The different colors of squares represent different types of IBV. The province in China where the viruses were isolated is represented. H120 + Ma5 indicate that prime-boost vaccination was used. NA means not available.

Table 1. Oligonucleotide primers used for amplification of the S1 gene of the IBV isolates and for the genome amplification of the ck/CH/LHB/100801 strain of IBV in this study.

Primers	Sense[A]	Sequence (5′→3′)	Position in genome[B]
IBV-366	+	AACCCAAAAGATTACGCTGATGCTT	640–664
IBV-367	−	TACTGAAAAGTCTTTTGAATTCTGG	1887–1911
IBV-368	+	GATGTCTTGAAGCTGTTTCAATC	1828–1850
IBV-369	−	ACGCCATCTACAAGCACATTCTT	3817–3839
IBV-370	+	TATGTTAAGAAACATGGGCCAC	3685–3706
IBV-371	−	CCAGCAACTTCAGGAGACATAAATGTA	6405–6431
IBV-372	+	GTAAGAGACATAATTGGTATTG	6353–6374
IBV-373	−	GCAGTTTGTAAAATTAAAACTGCACC	9052–9077
IBV-377	+	CTTAATCTTGCTAATAATCATGAG	8971–8994
IBV-378	−	GCTGCATTAAAACAACAGTTGTACAC	10,632–10,657
IBV-379	+	GTGTGGGAAGTCTTTTCGACAAATATAC	10,534–10,561
IBV-380	−	GAACCTTTAAATTGACAACGTCC	12,100–12,122
IBV-381	+	CTCCTGATCAGGATTCTTATGGAGGAGC	12,011–12,038
IBV-382	−	ATATAACGCTCCATAACAGCCACAGG	14,835–14,860
IBV-383	+	CATTTTGGGTGCATGTGTTTTTGTAGATG	14,789–14,817
IBV-384	−	CAGATTGTGTAGTGTTTGTCACATGGTGTC	17,407–17,436
IBV-375	+	CCTTGGCATGTTATAAGACCAAGGATAG	17,346–17,373
IBV-376	−	CAGAATGGTATGCCATTAAAAGATGGAGCG	18,903–18,932
IBV-374	+	GTTCTGTATGATGATAGATATGGTGATTACC	18,780–18,810
IBV-105	−	TTATGTGTACTACTACCAAAGTGCC	20,388–20,412
IBV-257[C]	+	TATTGATTAGAGATGTGG	20,356–20,373
S1Oligo5′[C]	+	CATAACTAACATAAGGGCAA	22,002–22,021
S1Oligo3′[C]	−	TGAAAACTGAACAAAAGAC	20,302–20,320
IBV-212[C]	−	ATACAAAATCTGCCATAA	22,017–22,034
IBV-275[C]	−	GTATGTACTCATCTGTAAC	22,147–22,165
IBV-167	+	GCTTCTTGAGAA(T/C)CAGTTTTA	21,921–21,941
IBV-168	−	AGACGATCAACTTGTGCATCTG	22,952–22,973
IBV-182	+	GACATTTAC(C/G)(A/C)GCAACTTGA	22,921–22,940
IBV-183	−	AACATTAGTCTAGGCTGTGC	24,014–24,033
IBV-199	+	CAAGTTTTATTCCAAATTAT	23,961–23,980
IBV-200	−	TCGCCAGTGTCTACTGACTG	25,120–25,139
IBV-170	+	CCAAAGCGGAAATAAGAAAA	25,074–25,093
IBV-171	−	AACCAAGATGCATTTCCAGA	25,960–25,979
N (+)	+	GACGCCCCAGCGCCAGTCATTAAA	25,903–25,926
N (−)	−	ACGCGGAGTACGATCGAGGGTACA	27,484–27,507

[A]Negative-sense (−) or positive-sense (+) primer.
[B]The nucleotide positions correspond to those in the sequence of the IBV Beaudette genome, GenBank accession no. M95169.
[C]The oligonucleotide primers that were used for amplification of the S1 gene of the IBV isolates in this study.

this study. In addition, four CK/CH/LDL/97I-type IBV strains (26), T3, CK/CH/LDL/97I, 2992, and 3374, were selected as an outgroup (31) for phylogenetic comparison in this study. These selected IBV strains and their accession numbers are listed in Fig. 1.

Based on S1 sequences obtained, ck/CH/LHLJ/100902 and ck/CH/LDL/101212 were further examined with other pathogenic and nonpathogenic Mass-type strains, pathogenic M41 and vaccine H120 strains, each with different accession numbers, and several other nonpathogenic strains were selected as reference strains for phylogenetic analysis and pairwise comparison. The selected IBV strains and their accession numbers are listed in Fig. 2.

Genome cloning, sequence determination and analysis of IBV ck/CH/LHB/100801. In order to obtain more genetic information on the emergent TW II–type IBV strain isolated in mainland China, the entire genome of ck/CH/LHB/100801 was cloned and sequenced. The protocols for viral RNA extraction and RT-PCR of the entire genome of IBV strain ck/CH/LHB/100801 were used as described above. The primer N (−) was used for generation of the first cDNA strand. The primers used for PCR amplification are listed in Table 1. A cDNA clone representing the 5′ and 3′ ends of the IBV ck/CH/LHB/100801 genome had been synthesized according to the 5′ rapid amplification of cDNA ends (RACE) and 3′ RACE System, respectively (TaKaRa). The PCR was performed according to the instructions of the kits. The antisense primers had been designed on the basis of the sequences obtained above that were constant in strain ck/CH/LHB/100801. The primer used for amplifying the 5′ end of strain ck/CH/LHB/100801 was 5′-CAGCTATGGCAATGCGCAG-3′. The primer used for amplifying the 3′ end was 5′-GAGGAGAGGAACAATGCACA-3′. Eighteen overlapped PCR fragments, including the 5′ and 3′ ends, that spanned the entire viral genome were amplified using specific primer sets. The DNA generated by PCR amplification was cloned using a T-tailed vector, pMD18-T (TaKaRa), and transformed using JM109 competent cells (TaKaRa) according to the manufacturer's instructions. At least five clones of each fragment were sequenced, and the consensus sequence was determined. The sequences were analyzed using the Sequencher 4.5 sequence analyses program, and a single contiguous sequence that comprised the entire genome of IBV strain ck/CH/LHB/100801 was constructed.

The nucleotide and amino acid sequences of the entire genome of the IBV strain ck/CH/LHB/100801 were assembled, aligned, and compared with those of other reference IBV and turkey coronavirus (TCoV) strains using the MegAlign program in DNAStar. Open reading frames were determined using the Gene Runner program version 3.00 (http://www.generunner.com), and the sequences were analyzed using Lasergene DNAStar (version 7, Lasergene Corp., Madison, WI). A total of 21 IBV reference strains for which the entire genomic sequences were available in the GenBank database were selected for phylogenetic analysis in this study. Seven strains of TCoV

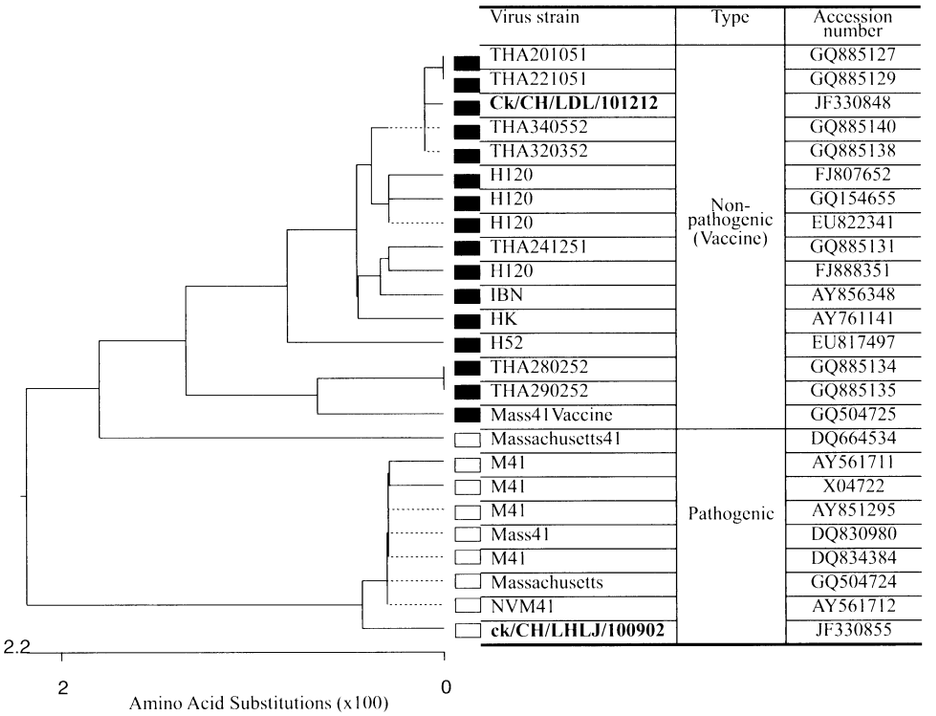


Fig. 2. Phylogenetic relationships, based on the sequence of the S1 subunit of the S gene of isolates ck/CH/LHLJ/100902 and ck/CH/LDL/101212, and other Mass-type strains (the first 1650 nucleotides, starting at the AUG translation initiation codon, of the S gene), obtained using the MegAlign program in DNAStar with the Clustal V method (Han *et al.* (26)). The Mass-type pathogenic M41 and vaccine H120, each with different accession numbers, and several nonpathogenic strains, were selected as reference strains in construction of the phylogenetic tree. The isolates ck/CH/LHLJ/100902 and ck/CH/LDL/101212 obtained in this study are in bold.

were used as an outgroup (31) in construction of the phylogenetic tree, based on the fact that TCoV has a similar sequence to IBV (4,24,41). The selected avian coronavirus reference strains and their accession numbers are shown in Fig. 3.

In addition, accurate estimation and comparison of the nucleotide sequence of the proximal 3′ one-third of the genome of IBV strain ck/CH/LHB/100801 was conducted with available sequences of pathogenic and attenuated TW2296/95, ck/CH/LJL/090608, CK/CH/LSD/05I, CK/CH/LHLJ/04V P3, and P110 as reference strains, respectively, using Clustal W. The 5′ two-thirds of the genome sequence of IBV strain ck/CH/LHB/100801 was compared with that of the TW2575/98 strain because TW2575/98 was the only strain that was closely related to Taiwan strains and had its entire genomic sequence in the GenBank database. These sequences were edited manually and adjusted for errors. Mutations, deletions, and insertions were determined according to the phylogenetic analysis and the results of pairwise comparison.

GenBank accession numbers of IBV sequences. The entire S1 gene nucleotide sequence, including the cleavage site, of each of the 56 IBV isolates in this study was deposited in GenBank with the accession numbers listed in Fig. 1. The full sequence of IBV strain ck/CH/LHB/100801 was also submitted to GenBank with the accession numbers listed in Fig. 1.

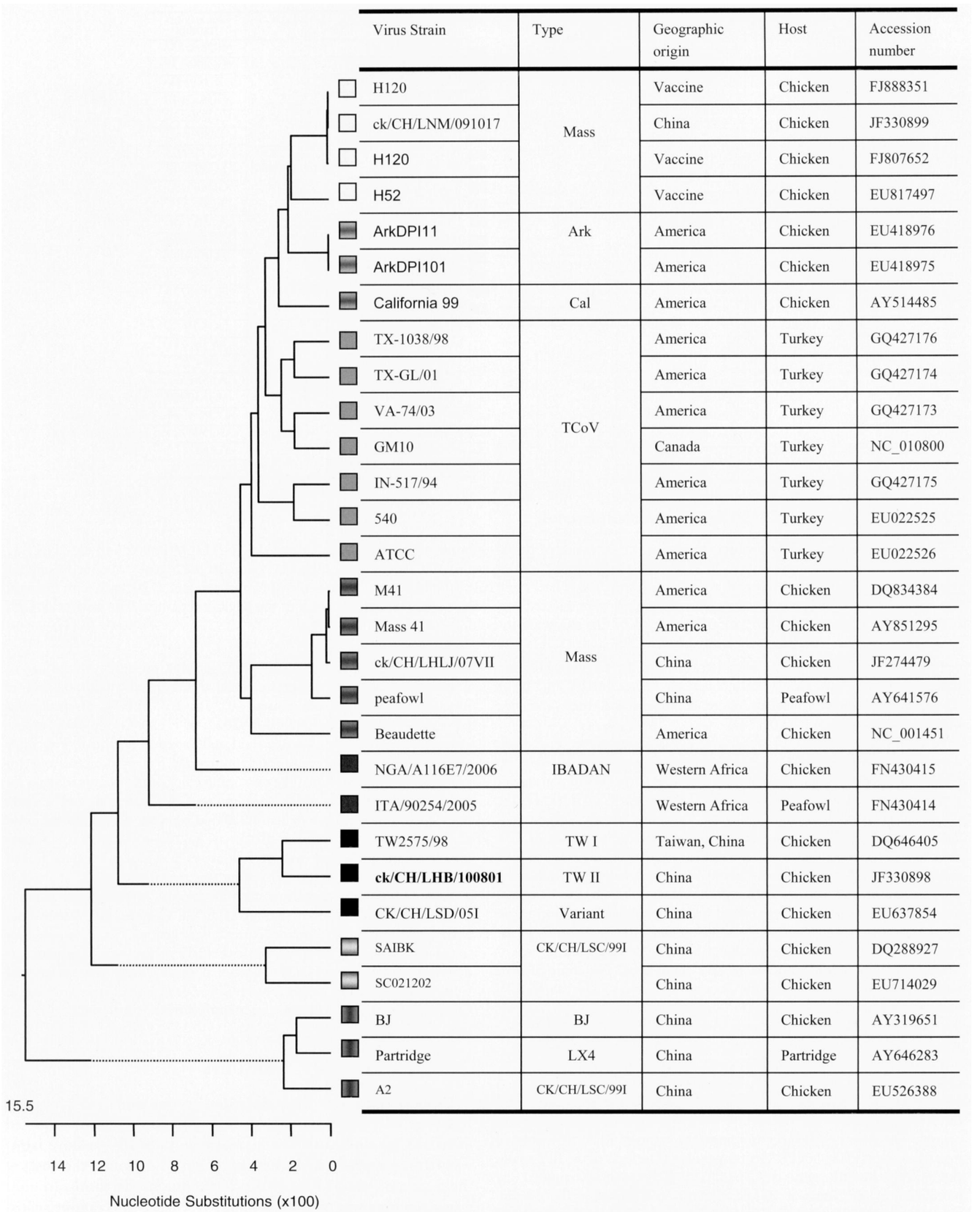
Pathogenicity study. The IBV isolate obtained in this study, ck/CH/LHB/100801, was used as the challenge virus in a pathogenicity study. A commercial Mass-type vaccine, H120, was used as the attenuated virus control, and IBV strain ck/CH/LDL/091022 (55) was used as the pathogenic control. The dose of vaccine was adjusted to contain $10^{4.8}$ 50% embryo infectious doses (EID_{50}) of H120 virus per dose. The titers of the IBV strains ck/CH/LHB/100801 and ck/CH/LDL/091022 were determined by inoculation of the viruses at 10-fold dilutions into groups of five 10-day-old embryonated chicken eggs. The EID_{50} was calculated by the method of Reed and Muench (51). An inoculum containing $10^{4.8–5.0}$ EID_{50}/100 μl was prepared and used to infect chicken poults experimentally.

Sixty 1-day-old white leghorn SPF chickens were used to assess the pathogenicity of the ck/CH/LHB/100801 strain. Four groups of 15 chickens were kept in isolators with negative pressure. At the age of 3 days, groups 1 to 3 were inoculated intranasally with 0.1 ml of inoculators per chick containing $10^{4.8}$ to $10^{5.0}$ EID_{50} of strains ck/CH/LHB/100801, ck/CH/LDL/091022, or H120. Group 4 was mock-inoculated with sterile allantoic fluid and served as a control. Five birds each from groups 1–4 were killed humanely 5 days postchallenge for postmortem examination. The remaining chicks were examined daily for signs of infection, including gasping, coughing, sneezing, and tracheal rales, for 25 days after inoculation. Blood samples were collected from all birds in each treatment group at 5, 10, 15, 20, and 25 days postinoculation. Serum was stored at −70 C until enzyme-linked immunosorbent assay (ELISA) testing was performed.

Serum antibody detection. Serum samples were assayed using a commercial total antibody ELISA (IDEXX Corporation, Westbrook, ME) according to the manufacturer's instructions. Each sample was tested in triplicate. Serum-to-positive (S/P) ratios were calculated as described previously (14,43). Individual serum titers were calculated from these S/P ratios, evaluated as positive or negative, and expressed as OD_{650nm} values according to the manufacturer's instructions.

RESULTS

Clinical signs and virus isolation. In this study, 56 isolates of IBV were obtained from samples from 216 poultry farms suspected of IB in China in 2010. The mortality on these IBV-positive farms was 10% to approximately 30% in the first 5 days after the onset of the disease on most of the IBV-positive commercial farms. In most cases, nephritis was observed in both vaccinated and nonvaccinated flocks and was characterized by enlarged, pale kidneys, frequently with urate deposits in the tubules, severe dehydration, and weight loss. In addition to kidney damage, respiratory signs and lesions were also found on these farms, and most cases were complicated by other



Virus Strain	Type	Geographic origin	Host	Accession number
H120	Mass	Vaccine	Chicken	FJ888351
ck/CH/LNM/091017		China	Chicken	JF330899
H120		Vaccine	Chicken	FJ807652
H52		Vaccine	Chicken	EU817497
ArkDPI11	Ark	America	Chicken	EU418976
ArkDPI101		America	Chicken	EU418975
California 99	Cal	America	Chicken	AY514485
TX-1038/98	TCoV	America	Turkey	GQ427176
TX-GL/01		America	Turkey	GQ427174
VA-74/03		America	Turkey	GQ427173
GM10		Canada	Turkey	NC_010800
IN-517/94		America	Turkey	GQ427175
540		America	Turkey	EU022525
ATCC		America	Turkey	EU022526
M41	Mass	America	Chicken	DQ834384
Mass 41		America	Chicken	AY851295
ck/CH/LHLJ/07VII		China	Chicken	JF274479
peafowl		China	Peafowl	AY641576
Beaudette		America	Chicken	NC_001451
NGA/A116E7/2006	IBADAN	Western Africa	Chicken	FN430415
ITA/90254/2005		Western Africa	Peafowl	FN430414
TW2575/98	TW I	Taiwan, China	Chicken	DQ646405
ck/CH/LHB/100801	TW II	China	Chicken	JF330898
CK/CH/LSD/05I	Variant	China	Chicken	EU637854
SAIBK	CK/CH/LSC/99I	China	Chicken	DQ288927
SC021202		China	Chicken	EU714029
BJ	BJ	China	Chicken	AY319651
Partridge	LX4	China	Partridge	AY646283
A2	CK/CH/LSC/99I	China	Chicken	EU526388

Fig. 3. Phylogenetic relationships, based on the entire genomic sequence of avian coronaviruses, including IBV and TCoV strains, obtained using the MegAlign program in DNAStar with the Clustal V method (Han *et al.* (26)). The IBV isolate ck/CH/LHB/100801 is in bold. The different colors of squares represent different types of IBV.

infections such as airsacculitis caused by *Escherichia coli* infection. This was most severe on broiler farms, with a higher mortality rate in contrast to the layer farms. In most cases on layer farms, slight decreases in productivity were observed but no obvious clinical signs were identified. An IBV isolate, ck/CH/LHB/100801, was detected in a broiler flock in Hebei province, China, where the birds were suffering from nephropathogenic lesions and proventriculitis. Two IBV isolates, ck/CH/LDL/101212 and ck/CH/LHLJ/100902, were isolated from laying hens in Dalian and Heilongjiang provinces, respectively, in China, where the birds were suffering from nephropathogenic lesions and respiratory signs, respectively. In addition, the diseased birds in both of the flocks were suffering from proventriculitis. The backgrounds of the remaining IBV isolates in this study are listed in Fig. 1. The diagnosis was based on electron microscope examination of allantoic fluid at different passages, which showed that all isolates had typical coronavirus morphology and that the samples were free of other agents such as Newcastle disease virus (results not shown).

Sequence analysis showed that the LX4-type IBVs could be grouped further into two separate genetic clusters. Phylogenetic analysis based on the nucleotide sequences of the S1 gene of 56 IBV isolates obtained in this study and 19 reference strains of IBV showed that the IBV strains isolated most recently are mainly of the LX4 type (49/56; Fig. 1). Similar to our previous result, the LX4 type of IBV could be separated mainly into two distinct genetic clusters, identified as cluster I and cluster II. The analysis and comparison of nucleotide and deduced amino acid sequences of the S1 gene of 33 isolates in cluster I with those of 18 isolates in cluster II were performed. The nucleotide and amino acid identities of the S1 gene sequences among the 33 IBV isolates ranged from 92.8% to 100% and 93.1% to 100%, respectively. Within the cluster II group, all the 18 IBV isolates shared 97% to 99.9% and 95.5% to 99.8% nucleotide and amino acid similarities to each other, respectively, regardless of whether they had been isolated from kidney, proventriculus, or lung. The minimum amino acid divergence observed between isolates of the two clusters was 90.9%. Furthermore, LX4-type isolates in clusters I and II had different favored amino acids at different positions, as listed in Table 2, although some of the positions had a mixture of amino acid residues, further confirming the existence of subpopulations in LX4-type viruses. In addition, no insertions or deletions were observed within S1 gene sequences of the 49 Chinese LX4-type isolates obtained in this study, except for the ck/CH/LHLJ/100713 isolate, which showed two deletions at amino acid positions 202 and 203 in the S1 subunit of the spike protein. The amino acid residue at position 202 was serine and that at 203 was valine or isoleucine in the S protein of other LX4-type IBVs.

The S1 sequence of IBV isolate ck/CH/LSD/100408 is a mosaic. When we conducted the pairwise comparison between the S1 genes of IBV LX4- and tl/CH/LDT3/03-type isolates in this study, we found that one IBV isolate, ck/CH/LSD/100408, which was grouped into cluster II of LX4-type isolates in the phylogenetic tree, had the sequences of both LX4- and tl/CH/LDT3/03-type IBV isolates at different parts of the S1 gene. The S1 gene of two IBV isolates, ck/CH/LSD/101223 and ck/CH/LSD/100504, which were the representatives of LX4- and tl/CH/LDT3/03-type IBV isolates, were selected to compare with that of ck/CH/LSD/100408. As shown in Fig. 4, the 5′ region of the S1 gene sequence of isolate ck/CH/LSD/100408 was the same as that of strain ck/CH/LSD/101223, and the 3′ region showed the same sequence as that of ck/CH/LSD/100504, indicating that isolate ck/CH/LSD/100408 may have originated from S1 gene recombination between ck/CH/LSD/101223- and ck/CH/LSD/100504-like viruses. In order to study this apparent shift in the homology within the S1 region further, S1 phylogenetic studies were conducted in both of the regions of S1 separately, using all of the LX4- and tl/CH/LDT3/03-type IBV strains in this study. In the 5′ region, which comprises the first 1398 nucleotides, isolate ck/CH/LSD/100408 grouped with the LX4 type (Fig. 5a); however, the ck/CH/LSD/100408 isolate grouped together with tl/CH/LDT3/03-type strains in the 3′ region, which comprises nucleotides 1437–1649 (Fig. 5b). These findings provided important evidence that the S1 sequence of ck/CH/LSD/100408 is a mosaic that has been descended from two putative parents, the ck/CH/LSD/101223- and ck/CH/LSD/100504-like viruses. A conserved region, GCTATTTTAGATAC, located between bases 1399 and 1436, downstream of the hypervariable region III (HVR) in the S1 gene (Fig. 4), was identified and assumed to be the crossover site of the recombinant event.

Both pathogenic and nonpathogenic Mass-type IBVs are circulating in chicken flocks in China. Two isolates, ck/CH/LDL/101212 and ck/CH/LHLJ/100902, were grouped with isolates of the Massachusetts serotype in this study. The ck/CH/LDL/101212 isolate was grouped with Mass-type vaccine strains (H120) in the phylogenetic tree (Fig. 2), and this is likely to indicate reisolation of the vaccine strain used in the immunization of these flocks. Interestingly, ck/CH/LDL/101212 shared 100% identity of the S1 gene nucleotide sequence with two IBV strains, THA320352 and THA340552, which had been isolated in Thailand. In contrast, isolate ck/CH/LHLJ/100902 clustered with the pathogenic Mass-type M41 strain (Fig. 2). Each mutation and resultant substitution in isolates ck/CH/LDL/101212 and ck/CH/LHLJ/100902 was compared with those of the H52, H120, and M41 strains. A single base mutation (C→T) was observed in isolate ck/CH/LDL/101212 that had not resulted in amino acid substitution when compared with the H120 virus, and this mutation was located at the 5′-terminal region of the S gene, at nucleotide 56 from the AUG start codon of the S1 gene. However, 16 out of 40 nucleotide mutations resulted in amino acid substitutions in the S1 gene of ck/CH/LDL/101212 compared with that of the pathogenic M41 strain, which provides evidence that isolate ck/CH/LDL/101212 represented reisolation of the vaccine strain. Further pairwise comparison of the S1 gene showed that isolate ck/CH/LHLJ/100902 had 40 nucleotide mutations in the S1 gene, of which 19 resulted in amino acid substitutions when compared with H120. Analysis of the sequences showed that three amino acid positions showed changes in the S1 gene of isolate ck/CH/LHLJ/100902 when compared with that of M41.

TW II–type strains of IBV have emerged in chicken flocks in mainland China. In this study, an IBV isolate, ck/CH/LHB/100801, was grouped as the TW II type by phylogenetic analysis and was closely related to TW2595/96 (27) and ck/CH/LJL/090608 (55), which were isolated respectively from Taiwan and Jilin province, China. Analysis of the S1 gene sequences showed that seven mutated amino acid positions were observed in the ck/CH/LHB/100801 isolate with respect to the TW2595/96 strain. Furthermore, each mutated residue was compared with the residue present in the same amino acid position in the S1 subunit of the S spike of the ck/CH/LJL/090608 strain. Four out of the seven mutated positions were shared with ck/CH/LJL/090608, but in addition three amino acid differences between isolates ck/CH/LHB/100801 and ck/CH/LJL/090608 were observed.

In order to obtain more information about the genetic relationship between ck/CH/LHB/100801 and TW II–type strains, the genome of isolate ck/CH/LHB/100801 was sequenced. The

Table 2. Comparison of amino acid preferences among the S1 genes of 51 LX4-type clusters I and II in this study.

Cluster[A]	23[B]	26	64	65	88	120	143	157	167	203	256
I	P(6/33)[C] S(27/33)	T(1/33) Deleted(2/33) N(32/33)	G(1/33) N(1/33) Q(1/33) P(1/33) R(2/33) S(27/33)	Y(1/33) W(1/33) Q(1/33) H(1/33) A(2/33) V(4/33) G(6/33) E(17/33)	L(3/33) S(5/33) P(25/33)	T(15/33) I(2/33) S(3/33)	I(1/33) T(1/33) A(13/33) S(31/33)	K(2/33) T(2/33) M(3/33) N(3/33) R(8/33) S(15/33)	L(1/33) S(5/33) F(27/33)	Deleted(1/33) I(4/33) V(28/33)	E(4/33) D(29/33)
II	S(1/18) P(17/18)	N(1/18) T(17/18)	G(1/18) K(1/18) S(1/18) E(15/18)	G(1/18) H(17/18)	L(18/18)	S(18/18)	T(18/18)	N(18/18)	F(1/18) L(17/18)	I(18/18)	E(18/18)

[A]The backgrounds of the viruses in clusters I and II, and the names of the 51 LX4-type IBV strains, are the same as in Fig. 1.
[B]The deduced amino acid positions correspond to those in the sequence of the IBV LX4 S1 gene, GenBank accession no. AY338732. Amino acid abbreviations: A, alanine; C, cysteine; D, aspartic acid; E, glutamic acid; F, phenylalanine; G, glycine; H, histidine; I, isoleucine; K, lysine; L, leucine; M, methionine; N, asparagine; P, proline; Q, glutamine; R, arginine; S, serine; T, threonine; V, valine; W, trytophan; Y, tyrosine.
[C]Number(s) of favored amino acid(s)/number(s) of compared amino acid(s).

remaining 3′ region of the genome of ck/CH/LHB/100801, from S2 to the 3′-untranslation region (3′-UTR), was compared with that of the TW2595/96 strain. Nucleotide mutations that had resulted in amino acid substitutions were identified in the S2, 3c (E), M, and 5a genes in ck/CH/LHB/100801. Table 3 summarizes the nucleotide mutations and resultant amino acid substitutions in the 3′ region of the genome for viruses ck/CH/LHB/100801, TW2595/96, and ck/CH/LJL/090608. Remarkably, a 49-bp deletion, located eight nucleotides downstream of the stop codon of the N protein gene, was found in the 3′-UTR of isolate ck/CH/LHB/100801. Two embryo-passaged, attenuated IBV strains, TW2595/96 and CK/CH/LHLJ/04V, had shown deletions in this region by nucleotide-based analysis (29,46). Surprisingly, ck/CH/LHB/100801 shared the deletion in this region with embryo-passaged, attenuated TW2595/96.

Given that the entire sequence of the genome of TW II–type viruses is not available, two IBV strains, TW2575/98 and CK/CH/LSD/05I, which had a close relationship with ck/CH/LHB/100801 in the phylogenetic tree constructed using entire genomic sequences, were selected for pairwise comparison of the 5′ two-thirds of the genome, which contains two overlapping replicase genes. The identity between ck/CH/LHB/100801 and TW2575/98 from nucleotide 1 to 20,317 in the genome was 95.2%, and that between ck/CH/LHB/100801 and CK/CH/LSD/05I was 91.3%. The nucleotide point mutations were found to be scattered in the 20,317-bp sequence when the three IBV strains were compared. In addition, a 15-bp nucleotide deletion located in nsp2, and another 3-bp deletion located in nsp4 were observed within the 20,317-bp sequence of the ck/CH/LHB/100801 isolate, when compared with that of the TW2575/98 strain. However, multiple deletions and a 3-bp insertion were also found in the CK/CH/LSD/05I strain, all of which were located in nsp2, when compared with the ck/CH/LHB/100801 isolate and TW2575/98.

IBV strain ck/CH/LHB/100801 had low virulence in SPF chickens. To characterize the pathogenicity of isolate ck/CH/LHB/100801 further, a pathogenic study was conducted using three different strains of IBV. The chickens challenged with strain ck/CH/LDL/091022 showed typical IB-induced disease. The diseased chicks exhibited slight respiratory clinical signs at about 4 to 15 days postchallenge. The clinical signs included tracheal rales, watery eyes, nasal mucus, and sneezing, and were similar to those caused by other IBV strains with affinity for the respiratory tract (55). Furthermore, ck/CH/LDL/091022 caused death 5 to 10 days postchallenge. Gross lesions of the dead chickens were mainly confined to the kidneys. The kidney parenchyma of the affected birds was pale, swollen, and mottled; the tubules and urethras were distended with uric acid crystals. Hemorrhagic lesions of the cecal tonsil and respiratory tract were also observed in some of the affected chickens. The clinical signs of the surviving inoculated birds tended to disappear gradually after 20 days of challenge. Similar to the negative control, the chickens inoculated with ck/CH/LHB/100801 or the H120 vaccine showed no clinical signs or gross lesions. Although ck/CH/LHB/100801 did not produce typical IB-induced disease, nearly all the chickens inoculated with ck/CH/LHB/100801 showed seroconversion by 10 days postchallenge, which was similar to the chickens challenged with ck/CH/LDL/091022 but different from those given H120. Table 4 summarizes the results of the challenge test using IBV ck/CH/LHB/100801, ck/CH/LDL/091022, and the H120 vaccine strain.

DISCUSSION

LX4-type strains of IBV were first isolated in China (61), and they are now established in some other Asian (40) and European countries (30). The LX4-type strain has been the predominant genotype circulating in chicken flocks in China from 1995 to the present (26,55,66). Using phylogenetic analysis of the S1 gene of newly isolated IBV strains in China, our results in this study complement the previous finding that the LX4 genotype can be grouped into two clusters (55). Importantly, we also extended the finding to demonstrate that viruses in clusters I and II had different favored amino acid residues at different positions in the S1 subunit of the spike protein, although some of the positions had a mixture of amino acid residues.

In China, the only vaccine approved by the Agriculture Ministry belongs to the Mass type of virus, and these have been used in the prevention and in controlling the incidence of the disease. Despite extensive vaccination using this type of vaccine, not only has the existence of antigenic groups divergent from the Mass type, such as the LX4 type, already been demonstrated in the country (26,44,55), but molecular studies in this and other studies (26,55) have shown that Mass-type strains are isolated frequently from vaccinated chicken flocks. This also occurs in other countries (16,52,53) and is expected, because vaccine strain reisolation is possible when extensive vaccination programs use attenuated strains. This is the case for the IBV isolate ck/CH/LDL/101212 in this study, which

Table 2. Extended.

271	282	291	331	353	369	382	393	394	395	402	422	497
L(1/33) A(3/33) T(29/33)	N(11/33) T(22/33)	H(1/33) N(6/33) S(26/33)	R(23/33) S(5/33) K(2/33) M(2/33) I(1/33)	T(33/33)	N(11/33) S(22/33)	S(4/33) R(29/33)	S(33/33)	M(1/33) T(1/33) R(1/33) S(30/33)	S(1/33) D(1/33) M(1/33) T(30/33)	V(33/33)	T(5/33) M(28/33)	F(6/33) L(10/33) V(17/33)
A(18/18)	N(18/18)	N(18/18)	S(18/18)	A(18/18)	S(18/18)	I(18/18)	R(18/18)	T(18/18)	N(18/18)	I(18/18)	T(18/18)	V(18/18)

had only one nonsynonymous mutation, at position 56 relative to the start codon of the S1 gene, in comparison with the H120 vaccine strain. However, vaccination is not likely to be the only explanation for the circulation of Mass-type viruses. Isolate ck/CH/LHLJ/100902 had the closest relationship with the pathogenic M41 strain in phylogenetic analysis and in pairwise sequence comparison as well, in comparison with the H120 and H52 vaccine strains. Therefore, it can be concluded that isolate ck/CH/LHLJ/100902 has a close relationship with but differed, at least in part, from M41. The origin of and protection offered by Mass-type vaccines against isolate ck/CH/LHLJ/100902 are unclear at present; analysis of the full-length genomic sequence and vaccination-challenge tests are required to answer these questions.

Extensive homologous and heterologous recombination events have been documented in both human and animal coronaviruses, and these have led to the generation of various genotypes and strains within each coronavirus species. It has been found that as many as 25% of mouse hepatitis coronaviruses (MHV) were recombinants, and the S gene of MHV has a frequency threefold that of the *pol* gene (20,21,48). In the case of IBV, there has been little information published. However, genetic recombination is known to be one of the important mechanisms of evolution of IBV and is involved in the generation of new variant viruses in the field (7). Putative genetic recombination in the S gene of IBV has been documented in different field isolates, including the Japanese strain KB8523, European isolates D207 and 6/82, American PP14, SE17, CU-T2,

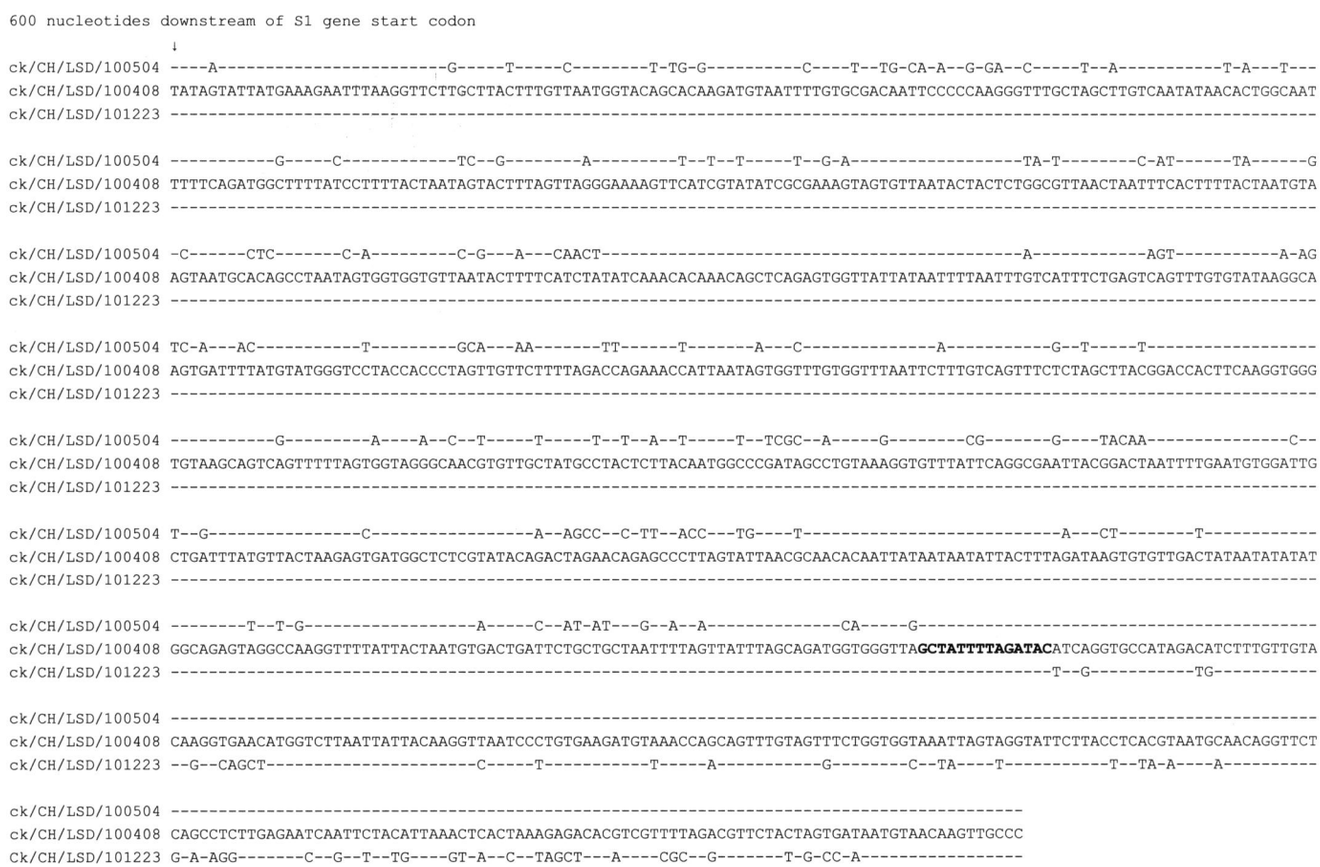


Fig. 4. Nucleotide alignment of the partial S1 genes of IBV isolates ck/CH/LSD/100504, ck/CH/LHB/091223, and ck/CH/LSD/100408. Dashes indicate the nucleotides identical to those of ck/CH/LHB/090804. Potential crossover regions are in bold.

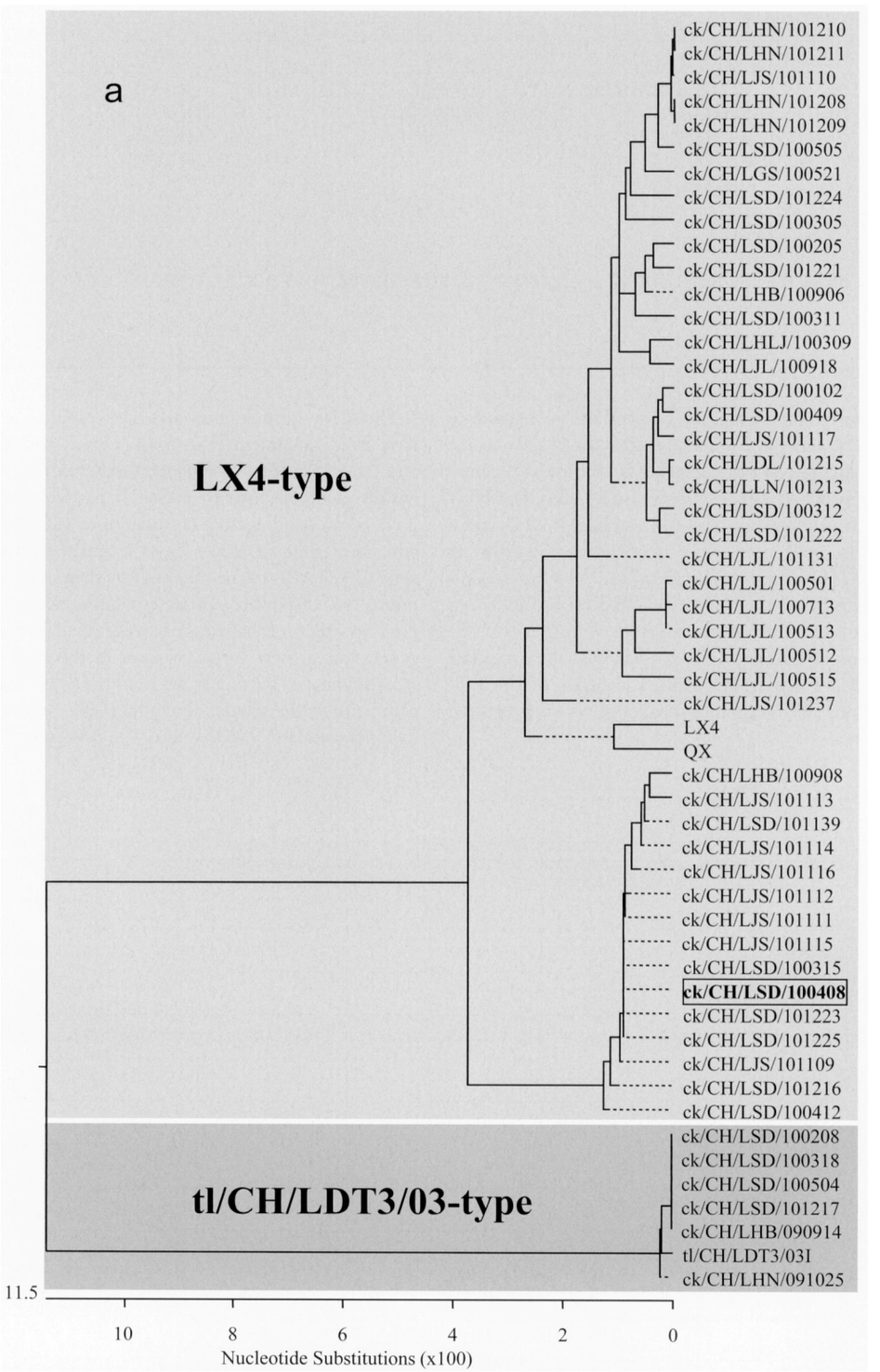


Fig. 5. Phylogenetic relationships investigated in both S1 regions separately. (a) Tree of the 5′ region (first 1398 nucleotides). The ck/CH/LSD/100408 is clustered with the LX4-type strains. (b) Tree of the 3′ region (nucleotides 1413–1649). The ck/CH/LSD/100408 is clustered with the tl/CH/LDT3/03-type strains.

and some Chinese and Korean strains (26,32,37,40,60). In this study, we isolated and identified an IBV isolate, ck/CH/LSD/100408, that may have been generated naturally by recombination as a result of cocirculation of LX4- and tl/CH/LDT3/03-like field strains. Surprisingly, unlike other naturally recombinant viruses, isolate ck/CH/LSD/100408 had a completely consistent sequence in the 5′ region of the S1 gene with ck/CH/LSD/101223 and in the 3′ region with ck/CH/LSD/100504. The gene sequences are unlikely to have resulted from experimental contamination. In this study, the three IBV strains were isolated in different months from different chicken flocks in 2010. Therefore, isolate ck/CH/LSD/100408 was probably derived from a recent recombinant event and had not been

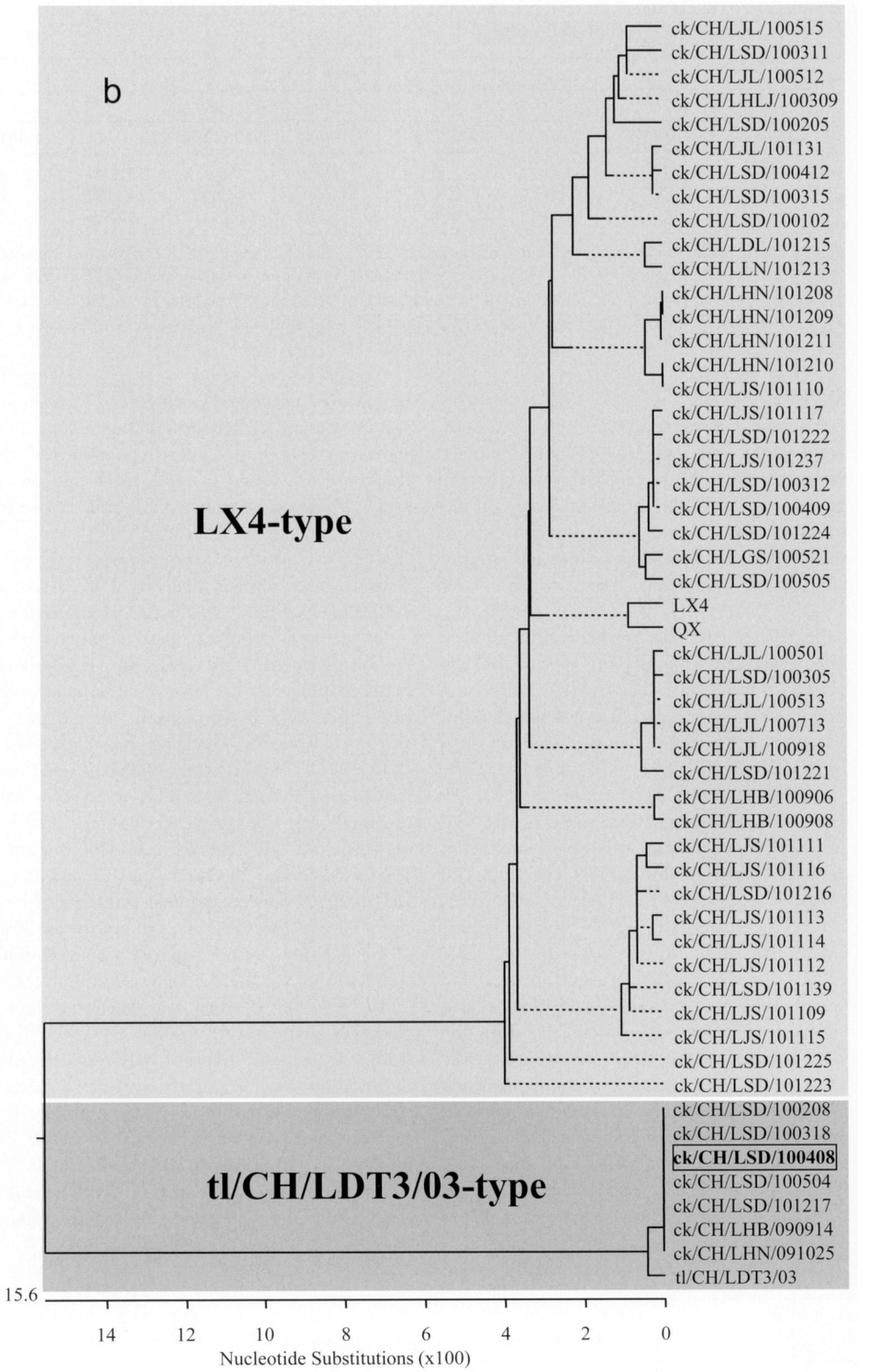


Fig. 5. Continued.

passaged in chickens; consequently, no point mutations, deletions, or insertions had occurred in the S1 gene between ck/CH/LSD/100408 and its parent strains. Taken together, these observations suggest that possible recombination at positions 1399 to 1436 may have contributed to the emergence of isolate ck/CH/LSD/100408. To our knowledge, this is the first report of natural recombination among LX4- and tl/CH/LDT3/03-like field strains.

Given the vast scope of commercial broiler operations in Shandong province, China, that most of the chickens are maintained at a high density, and that multiple types of IBV cocirculate in this district (45) during the breeding period, recombination events seem likely. Although the generation of many recombinant IBV strains can occur naturally, each recombinant strain displays different level of fitness for the host environment (17). For instance, the

Table 3. Pairwise comparison of nucleotides and amino acids in the 3′ region of the ck/CH/LHB/100801 genome with those of the Taiwan TW II–type TW2296/95 and Chinese ck/CH/LJL/090608 strains.

	Gene 2 (S)								
	S1								
IBV strains	127 (43)[A]	139 (47)	166 (56)	187 (63)	190 (64)	349 (117)	391 (131)	481 (161)	1641 (547)
TW2296/95	C(H)	A(N)	C(L)	T(S)	G(V)	T(S)	C(H)	A(S)	T(C)
ck/CH/LHB/100801	C(H)	T(Y)	T(F)	C(P)	T(F)	G(A)	T(Y)	T(C)	T(C)
ck/CH/LJL/090608	T(Y)	T(Y)	C(L)	T(S)	G(V)	G(A)	T(Y)	T(C)	C(C)

[A]The nucleotides (deduced amino acids) in each target gene of isolate ck/CH/LHB/100801, from the AUG translation start codon to the stop codons, were compared. Of the five genes (gene 2 to gene 6) in the 3′ region of LHB/100801, mutations in four genes (gene 2 to gene 5) that resulted in amino acid substitutions were found; most of them were in the S1 subunit of the spike protein. Amino acid abbreviations: A, alanine; H, histidine; Y, tyrosine; N, asparagine; F, phenylalanine; L, leucine; P, proline, S, serine; V, valine; C, cysteine; E, glutamic acid; G, glycine; I, isoleucine; T, threonine; K, lysine.
[B]NA, Not available.

recombinant strain EJ95 has disappeared from Korea, probably because of its unfitness for the host environment (54), while the GA98 strain has persisted in the field long enough to maintain an epidemic in North America (38). The recombination event may have provided replication benefits, enabling the offspring of this founder to become dominant (36). Hence, monitoring the spread and persistence of ck/CH/LSD/100408-like and other IBV strains, especially in heavily populated commercial poultry areas, is of importance.

Interestingly, we found IBV isolates that are genetically close to strains of the TW II type in this study. Initially, this type of IBV was isolated in mainland China in 2009 (55). Taiwan IBVs have been characterized molecularly and can be grouped into two populations, TW 1 and TW II, which are considered to exist as distinct virus populations globally (27,28,58). Recent work involving the investigation of the 7.3 kb 3′ genome of eight strains isolated from 1992 to 2007 in Taiwan, China, reported that some of the IBV isolates had chimeric genome arrangements, which originated from recombinant events between parental IBV strains circulating in Taiwan and other provinces of China (12). However, this seems unlikely to be the case for the emergence of IBV isolate ck/CH/LHB/100801, obtained in this study. It is noteworthy that IBV isolate ck/CH/LHB/100801 showed a closer relationship with IBV strains isolated in Taiwan by both phylogenetic analysis (Fig. 1) and pairwise nucleotide and deduced amino acid comparisons using the entire genomic sequence (Table 3). These observations support the possibility that IBV TW II–type strains isolated in mainland China have the same origin as those isolated in Taiwan. At this point an intriguing question emerges regarding how this type of virus was introduced into mainland China, given the earlier isolation of the TW II–type viruses in Taiwan in 1964 (58). The trading of chickens and chicken products, legal or illegal; the use of approved or unapproved attenuated vaccines; and the involvement of wild birds as possible natural carriers of IBV might contribute to the explanation. However, we do not exclude the possibility of other causes. Nucleotide deletions were found in nsp2, nsp4, and the 3′ UTR regions, and synonymous and nonsynonymous mutations were also observed in the genome of isolate ck/CH/LHB/100801, though mainly in the S1 gene (Table 3), when it was compared with those of IBV strains isolated in Taiwan. This implies that the TW II–like isolate ck/CH/LHB/100801 maybe have experienced a long-term evolution.

It has been reported that, although IBV strains of different pathogenicity are cocirculating in chicken flocks in China (45,55), nephropathogenity is the predominant type of pathogenicity (3,39,44,59,64,65). In contrast, we did not observe clinical signs and gross lesions in chickens challenged with IBV isolate ck/CH/LHB/100801 in this study, which implies that this virus has low virulence in SPF chickens. This observation differed from the results obtained with TW I–type 2575/98 and TW II–type 2296/95, both of which can cause mortality in SPF chickens (28). However, the seroconversion of the birds after inoculation with isolate ck/CH/LHB/100801 was comparable with that of birds given the virulent ck/CH/LDL/091022 strain, but not the H120 strain. Interestingly, a 49-bp deletion, located eight nucleotides downstream of the stop codon of the N protein gene, was found in the 3′-UTR of isolate ck/CH/LHB/100801, which was consistent with that of the embryo-passaged, attenuated IBV strain TW2595/96 (29). In our previous study, we proposed that not only this deletion, but also the size of the deletion, may be correlated with IBV attenuation (46). In this study, comparison of the sequences of more IBV strains leads us to speculate that deletion in this region of the IBV genome could be involved in pathogenicity and attenuation.

China has large populations of commercial chickens maintained at a high density. The increasing poultry population and greater use

Table 4. Results of challenge test using the IBV ck/CH/LHB/100801 strain.

Group	Dose, median embryo infectious dose (log 10)[A]	Morbidity (%)	Mortality (%)	Antibody response, no. challenged (%)[B]				
				5[C]	10	15	20	25
ck/CH/LHB/100801	4.8	0/10 (0)	0/10 (0)	0/10 (0)	9/10 (90)	10/10 (100)	10/10 (100)	10/10 (100)
ck/CH/LDL/091022	5.0	7/10 (70)	10/10 (100)	0/9 (90)	3/3 (100)	3/3 (100)	3/3 (100)	3/3 (100)
H120	4.8	0/10 (0)	0/10 (0)	0/10 (0)	5/10 (50)	10/10 (100)	10/10 (100)	10/10 (100)
Negative Control	—	0/10 (0)	0/10 (0)	0/10 (0)	0/10 (0)	0/10 (0)	0/10 (0)	0/10 (0)

[A]Dose per chick, 100 μl.
[B]Number that seroconversed/number inoculated.
[C]Days after challenge.

Table 3. Extended.

Gene 2 (S)					Gene3				Gene 5
S1	S2				3a	E (3c)	Gene 4 (M)		5a
1644 (548)	2651 (884)	3098 (1033)	3222 (1074)	3400 (1134)	93 (31)	172 (58)	377 (126)	562 (188)	94 (32)
A(P)	T(V)	A(E)	C(L)	A(I)	T(L)	G(A)	C(T)	A(T)	G(E)
A(P)	C(A)	G(G)	T(L)	T(L)	G(L)	T(S)	T(I)	G(A)	A(K)
T(P)	NA[B]	NA	NA	NA	NA	NA	NA	NA	NA

of vaccines will encourage the more rapid generation of new types of virus (35). The predominant LX4-type IBVs are not only evolving into different clusters but also, as parents, providing genes for recombination that may account for the emergence of some of the new strains of IBV. Given that this type of IBV is also circulating widely in Europe and in other Asian countries, and that it poses a serious threat to the poultry industry (22), the results of this study are of significance for IBV control in China as well as other Asian and European countries. In addition, the emergence of new types of IBVs that are endemic in other regions, such as TW II–type IBVs, in mainland China is another challenging aspect of the epidemiology of IBV in China and underscores the importance of dynamic surveillance of IBV.

REFERENCES

1. Adzhar, A., R. E. Gough, D. Haydon, K. Shaw, P. Britton, and D. Cavanagh. Molecular analysis of the 793/B serotype of infectious bronchitis virus in Great Britain. Avian Pathol. 26:625–640. 1997.

2. Armesto, M., D. Cavanagh, and P. Britton. The replicase gene of avian coronavirus infectious bronchitis virus is a determinant of pathogenicity. PLoS One 4:e7384. 2009.

3. Bing, G. X., X. Liu, J. Pu, Q. F. Liu, Q. M. Wu, and J. H. Liu. Different genotypes of nephropathogenic infectious bronchitis viruses cocirculating in chicken population in China. Virus Genes 35:333–337. 2007.

4. Cao, J., C. C. Wu, and T. L. Lin. Complete nucleotide sequence of polyprotein gene 1 and genome organization of turkey coronavirus. Virus Res. 136:43–49. 2008.

5. Casais, R., M. Davies, D. Cavanagh, and P. Britton. Gene 5 of the avian coronavirus infectious bronchitis virus is not essential for replication. J. Virol. 79:8065–8078. 2005.

6. Cavanagh, D. Coronavirus avian infectious bronchitis virus. Vet. Res. 38:281–297. 2007.

7. Cavanagh, D., P. J. Davis, J. K. Cook, D. Li, A. Kant, and G. Koch. 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. 1992.

8. Cavanagh, D., P. J. Davis, and A. P. A. Mockett. Amino acids within hypervariable region 1 of avian coronavirus IBV (Massachusetts serotype) spike glycoprotein are associated with neutralization epitopes. Virus Res. 11:141–150. 1988.

9. Cavanagh, D., and J. Gelb. Infectious bronchitis. In: Diseases of poultry, 12th ed. Y. M. Saif, A. M. Fadly, J. R. Glisson, L. R. McDougald, L. K. Nolan, and D. E. Swayne, eds. Wiley-Blackwell Publishing, Ames, IA. pp. 117–135. 2008.

10. Cavanagh, D., K. Mawditt, M. Sharma, S. E. Drury, and H. L. Ainsworth. Detection of a coronavirus from turkey poults in Europe genetically related to infectious bronchitis virus of chickens. Avian Pathol. 30:355–368. 2001.

11. Cavanagh, D., K. Mawditt, D. B. Welchman, P. Britton, and R. E. Gough. Coronaviruses from pheasants (*Phasianus colchicus*) are genetically closely related to coronaviruses of domestic fowl (infectious bronchitis virus) and turkeys. Avian Pathol. 31:81–93. 2002.

12. Chen, H. W., Y. P. Huang, and C. H. Wang. Identification of Taiwan and China-like recombinant avian infectious bronchitis viruses in Taiwan. Virus Res. 140:121–129. 2009.

13. de Groot, R. J., J. Ziebuhr, L. L. Poon, P. C. Woo, P. Talbot, P. J. M. Rottier, K. V. Holmes, R. Baric, S. Perlman, L. Enjuanes, and A. E. Gorbalenya. Revision of the family Coronaviridae. Taxonomic proposal to the ICTV Executive Committee. 1–37. 2008.

14. de Wit, J. J., M. C. M. de Jong, A. Pijpers, and J. H. M. Verheijden. Transmission of infectious bronchitis virus within vaccinated and unvaccinated groups of chickens. Avian Pathol. 27:464–471. 1998.

15. Dolz, R., J. Pujols, G. Ordóñez, R. Porta, and N. Majó. Antigenic and molecular characterization of isolates of the Italy 02 infectious bronchitis virus genotype. Avian Pathol. 35:77–85. 2006.

16. Dolz, R., J. Pujols, G. Ordóñez, R. Porta, and N. Majó. Molecular epidemiology and evolution of avian infectious bronchitis virus in Spain over a fourteen-year period. Virology 374:50–59. 2008.

17. Domingo, E., E. Martinez-Salas, F. Sobrino, J. C. de la Torre, A. Portela, J. Ortin, C. Lopez-Galindez, P. Perez-Brena, N. Villanueva, R. Najera, S. Vandepol, D. Steinhauer, N. Depolo, and J. J. Holland. The quasispecies (extremely heterogeneous) nature of viral RNA genome populations: biological relevance—a review. Gene 40:1–8. 1985.

18. Dong, B. Q., W. Liu, X. H. Fan, D. Vijaykrishna, and X. C. Tang. Detection of a novel and highly divergent coronavirus from Asian leopard cats and Chinese ferret badgers in Southern China. J. Virol. 81:6920–6926. 2007.

19. Farsang, A., C. Ros, L. H. M. Renstrom, C. Baule, T. Soós, and S. Belák. Molecular epizootiology of infectious bronchitis virus in Sweden indicating the involvement of a vaccine strain. Avian Pathol. 31:229–236. 2002.

20. Fu, K., and R. S. Baric. Evidence for variable rates of recombination in the MHV genome. Virology 189:88–102. 1992.

21. Fu, K., and R. S. Baric. Map locations of mouse hepatitis virus temperature sensitive mutants: confirmation of variable rates of recombination. J. Virol. 68:7458–7466. 1994.

22. Geerligs, H. J., G.-J. Boelm, C. A. M. Meinders, B. G. E. Stuurman, J. Symous, J. Tarres-Call, T. Bru, R. Vila, M. Mombarg, K. Karaca, W. Wijmenga, and M. Kumar. Efficacy and safety of an attenuated live QX-like infectious bronchitis virus strain as a vaccine for chickens. Avian Pathol. 40:93–102. 2011.

23. Gelb, J., Jr., J. B. Wolff, and C. A. Moran. Variant serotypes of infectious bronchitis virus isolated from commercial layer and broiler chickens. Avian Dis. 35:82–87. 1991.

24. Gomaa, M. H., J. R. Barta, D. Ojkic, and D. Yoo. Complete genomic sequence of turkey coronavirus. Virus Res. 135:237–246. 2008.

25. Guy, J. S. Turkey coronavirus is more closely related to avian infectious bronchitis virus than to mammalian coronaviruses: a review. Avian Pathol. 29:207–212. 2000.

26. Han, Z., C. Sun, B. Yan, X. Zhang, Y. Wang, C. Li, Q. Zhang, Y. Ma, Y. Shao, Q. Liu, X. Kong, and S. Liu. A 15-year analysis of molecular epidemiology of avian infectious bronchitis coronavirus in China. Infect. Genet. Evol. 11:190–200. 2011.

27. Huang, Y. P., H. C. Lee, M. C. Cheng, and C. H. Wang. S1 and N gene analysis of avian infectious bronchitis viruses in Taiwan. Avian Dis. 48:581–589. 2004.

28. Huang, Y. P., and C. H. Wang. Development of attenuated vaccines from Taiwanese infectious bronchitis virus strains. Vaccine 24:785–791. 2006.

29. Huang, Y. P., and C. H. Wang. Sequence changes of infectious bronchitis virus isolates in the 3′ 7.3 kb of the genome after attenuating passage in embryonated eggs. Avian Pathol. 36:59–67. 2007.

30. Irvine, R. M., W. J. Cox, V. Ceeraz, S. M. Reid, R. J. Ellis, R. M. Jones, J. Errington, A. M. Wood, C. McVicar, and M. I. Clark. Detection of IBV QX in commercial broiler flocks in the UK. Vet. Rec. 167:877–879. 2011.

31. Janies, D., F. Habib, B. Alexandrov, A. Hill, and D. Pol. Evolution of genomes, host shifts and the geographic spread of SARS-CoV and related coronaviruses. Cladistics 24:111–130. 2008.

32. Jia, W., K. Karaca, C. R. Parrish, and S. A. Naqi. A novel variant of avian infectious bronchitis virus resulting from recombination among three different strains. Arch. Virol. 140:259–271. 1995.

33. Johannesen, J., W. Wickler, U. Seibt, and F. Moritz. Population history in social spiders repeated: colony structure and lineage evolution in *Stegodyphus mimosarum* (Eresidae). Mol. Ecol. 18:2812–2818. 2009.

34. Jonassen, C. M., T. Kofstad, I. L. Larsen, A. Lovland, and K. Handeland. Molecular identification and characterization of novel coronaviruses infecting graylag geese (*Anser anser*), feral pigeons (*Columbia livia*) and mallards (*Anas platyrhynchos*). J. Gen. Virol. 86:1597–1607. 2005.

35. Jones, R. C. Viral respiratory diseases (ILT, aMPV infections, IB): are they ever under control? British Poultry Science 51:1–11. 2010.

36. Kuo, S. M., C. H. Wang, M. H. Hou, Y. P. Huang, H. W. Kao, and H. L. Su. Evolution of infectious bronchitis virus in Taiwan: characterisation of RNA recombination in the nucleocapsid gene. Vet. Microbiol. 144:293–302. 2011.

37. Kusters, J. G., E. J. Jager, H. G. Niesters, and B. A. van der Zeijst. Sequence evidence for RNA recombination in field isolates of avian infectious bronchitis virus. Vaccine 8:605–608. 1990.

38. Lee, C. W. Evolution of avian infectious bronchitis virus: genetic drift and recombination. Korean J. Vet. Serv. 25:97–103. 2002.

39. Li, H., and H. C. Yang. Sequence analysis of nephropathogenic infectious bronchitis virus strains of the Massachusetts genotype in Beijing. Avian Pathol. 30:535–541. 2001.

40. Lim, T.-H., H.-J. Lee, D.-H. Lee, Y.-N. Lee, J.-K. Park, H-N. Youn, M.-S. Kim, J.-B. Lee, S.-Y. Park, I.-S. Choi, and C.-S. Song. An emerging recombinant cluster of nephropathogenic strains of avian infectious bronchitis virus in Korea. Infect. Genet. Evol. 11:678–685. 2011.

41. Lin, T. L., C. C. Loa, and C. C. Wu. Complete sequences of 3′ end coding region for structural protein genes of turkey coronavirus. Virus Res. 106:61–70. 2004.

42. Liu, S., J. Chen, J. Chen, X. Kong, Y. Shao, Z. Han, and G. Tong. Isolation of avian infectious bronchitis coronavirus from domestic peafowl (*Pavo cristatus*) and teal (*Anas*). J. Gen. Virol. 86:719–725. 2005.

43. Liu, S., J. Chen, Z. Han, Q. Zhang, Y. Shao, and X. Kong. Infectious bronchitis virus: S1 gene characteristics of vaccines used in China and efficacy of vaccination against heterologous strains from China. Avian Pathol. 35:394–399. 2006.

44. Liu, S., and X. Kong. A new genotype of nephropathogenic infectious bronchitis virus circulating in vaccinated and non-vaccinated flocks in China. Avian Pathol. 33:321–327. 2004.

45. Liu, S., Y. Wang, Y. Ma, Z. Han, Q. Zhang, Y. Shao, J. Chen, and X. Kong. Identification of a newly isolated avian infectious bronchitis coronavirus variant in China exhibiting affinity for the respiratory tract. Avian Dis. 52:306–314. 2008.

46. Liu, S., X. Zhang, L. Gong, B. Yan, C. Li, Z. Han, Y. Shao, H. Li, and X. Kong. 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. 2009.

47. Liu, S., X. Zhang, Y. Wang, C. Li, Q. Liu, Z. Han, Q. Zhang, X. Kong, and G. Tong. Evaluation of the protection conferred by commercial vaccines and attenuated heterologous isolates in China against the CK/CH/LDL/97I strain of infectious bronchitis coronavirus. Vet. J. 179:130–136. 2009.

48. Makino, S., J. G. Keck, S. A. Stohlman, and M. M. Lai. High-frequency RNA recombination of murine coronaviruses. J. Virol. 57: 729–737. 1986.

49. Masters, P. S. The molecular biology of coronaviruses. Adv. Virus Res. 66:193–292. 2006.

50. Mihindukulasuriya, K. A., G. Wu, J. St Leger, R. W. Nordhausen, and D. Wang. Identification of a novel coronavirus from a beluga whale by using a panviral microarray. J. Virol. 82:5084–5088. 2008.

51. Reed, L. J., and H. Muench. A simple method of estimating fifty percent endpoints. Am. J. Hyg. 27:493–497. 1938.

52. Rimondi, A., M. I. Craig, A. Vagnozzi, G. Konig, M. Delamer, and A. Pereda. Molecular characterization of avian infectious bronchitis virus strains from outbreaks in Argentina (2001–2008). Avian Pathol. 38: 149–153. 2009.

53. Roussan, D. A., G. Y. Khawaldeh, and I. A. Shaheen. Infectious bronchitis virus in Jordanian chickens: seroprevalence and detection. Can. Vet. J. 50:77–80. 2009.

54. Song, C. S., and Y. J. Lee. Molecular and epidemiological characteristics of infectious bronchitis virus isolated in Korea. Korean J. Poultry Sci. 2:91–98. 2000.

55. Sun, C., Z. Han, H. Ma, Q. Zhang, B. Yan, Y. Shao, J. Xu, X. Kong, and S. Liu. 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. 2011.

56. Swofford, D. L. PAUP: phylogenetic analysis using parsimony (*and other methods), version 4.0. Sinauer Associates, Sunderland, MA. 1999.

57. Wang, C. H., and Y. C. Huang. Relationship between serotypes and genotypes based on the hypervariable region of the S1 gene of infectious bronchitis virus. Arch. Virol. 145:291–300. 2000.

58. Wang, C. H., and C. T. Tsai. Genetic grouping for the isolates of avian infectious bronchitis virus in Taiwan. Arch. Virol. 141:1677–1688. 1996.

59. Wang, H. N., Q. Z. Wu, Y. Huang, and P. Liu. Isolation and identification of infectious bronchitis virus from chickens in Sichuan, China. Avian Dis. 41:279–282. 1997.

60. Wang, L., D. Junker, and E. V. Collisson. Evidence of natural recombination within the S1 gene of infectious bronchitis virus. Virology 192:710–716. 1993.

61. Wang, Y. D., Y. L. Wang, Z. Zhang, G. Fan, Y. Jiang, X. Liu, J. Ding, and S. Wang. Isolation and identification of glandular stomach type IBV (QX IBV) in chickens. Chin. J. Anim. Quarantine 15:1–3. 1998.

62. Woo, P. C., S. K. Lau, Y. Huang, and K. Y. Yuen. Coronavirus diversity, phylogeney and interspecies jumping. Exp. Biol. Med. (Maywood) 234:1117–1127. 2009.

63. Woo, P. C., S. K. Lau, C. S. Lam, K. K. Lai, and Y. Huang. Comparative analysis of complete genome sequences of three avian coronaviruses reveals a novel group 3c coronavirus. J. Virol. 83:908–917. 2009.

64. Xu, C., J. Zhao, X. Hu, and G. Zhang. Isolation and identification of four infectious bronchitis virus strains in China and analyses of their S1 glycoprotein gene. Vet. Microbiol. 122:61–71. 2007.

65. Yu, L., Z. Wang, Y. Jiang, S. Low, and J. Kwang. Molecular epidemiology of infectious bronchitis virus isolates from China and Southeast Asia. Avian Dis. 45:201–209. 2001.

66. Zou, N. L., F. F. Zhao, Y. P. Wang, P. Liu, S. J. Cao, X. T. Wen, and Y. Huang. Genetic analysis revealed LX4 genotype strains of avian infectious bronchitis virus became predominant in recent years in Sichuan area, China. Virus Genes 41:202–209. 2010.

ACKNOWLEDGMENT

This work was funded by a grant from the earmarked fund for Modern Agro-industry Technology Research System.