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Genetic diversity of avian infectious bronchitis coronavirus strains isolated in China between 1995 and 2004

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Summary. Twenty-six avian infectious bronchitis (IB) viruses (IBV) were isolated from outbreaks in chickens in China between 1995 and 2004. They were characterized by comparison with twenty-six Chinese reference strains and five other IBV strains. Chinese IBVs, which were mainly nephropathogenic, were placed into seven genotypes. Fourteen Chinese IBV isolates were placed in genotype I, having small evolutionary distances from each other. Genotype II included 6 strains that were isolated in the 1990s in China. Genotype III consisted of eight Chinese isolates that showed close relationship with Korean IBV isolates. Another eight IBV isolates clustered in genotype IV and showed larger evolutionary distances. The Massachusetts serotype was present in China in 1990s and was in a separate genotype. Two isolates, HN99 and CK/CH/LHN/00I, which might be a reisolation of vaccine strains, clustered into genotype VI. Four Chinese IBV isolates formed another genotype and showed larger evolutionary distances from other Chinese IBV genotypes (genotype VII). IBVs in same genotypes showed more than 90% amino acid sequence similarities, whereas most of the viruses in different genotypes showed less than 90%. The results showed that IBVs in China came from genetic changes both in IBV populations that existed before the advent of vaccination and in the viruses that were introduced through live vaccines. IBVs showing various genetic differences are cocirculating in China.

Introduction

Infectious bronchitis virus (IBV) is a highly infectious and contagious pathogen of chickens worldwide [11]. The primary tissue of IBV infection is the respiratory tract, though some isolates replicate in the kidney and oviduct, resulting

in nephritis and reduced egg production. Generally, infectious bronchitis (IB) has been controlled with serotype-specific vaccines, but outbreaks of IB still occur, because vaccines offer little cross-protection between serologically distinct viruses [19]. A high mutation frequency and RNA recombination leads to the emergence of new viruses capable of causing disease in vaccinated chickens [31, 41, 35]. Although many countries share some common antigenic types, IBV strains within a geographic region are unique and distinct [1, 4, 15–17, 21, 47]. The identification of the circulating IBV field strains is extremely important for the selection of vaccine strains for the corresponding geographical region.

IBV is the type species of the genus *Coronavirus* in the family *Coronaviridae*, order *Nidovirales* [11]. It is a pleomorphic enveloped virus and has a single-stranded RNA genome, approximately 27 kb in length, of positive polarity that specifies the production of three major structural proteins: the phosphorylated nucleocapsid (N) protein, the membrane (M) glycoprotein, and the spike (S) glycoprotein. The S glycoprotein of IBV, located on the outside of all virions, is responsible for fusion (virus envelope to cell membrane and cell membrane to cell membrane) and is translated as a precursor protein (S_0), then cleaved into a carboxy-terminal S2 subunit (approximately 625 amino acid residues), which anchors S in the virus envelope, and an amino-terminal S1 subunit (approximately 520 residues), believed to largely form the distal bulbous part of S [3, 7].

The S1 subunit of spike glycoprotein of IBV is responsible for inducing neutralizing and serotype-specific antibodies in chickens, and mutations in the antigenically important spike glycoprotein S1 subunit leads to the emergence and proliferation of variant serotypes [34] associated with disease outbreaks. Serotypic evolution in IBV is associated primarily with the sequences of the S1 glycoprotein, and the genetic diversity of IBV is mainly monitored by analysis of the S1 gene [2, 9, 10, 23, 27, 32, 42].

IBV strains have been isolated and identified since 1982 in China. The outbreaks of IB have been ongoing, and IB continues to be an economically important disease to the poultry industry, although vaccines based on Massachusetts (Mass) strains such as H120 and H52 have been used for many years. However, the epidemiological analysis of IBV isolates in China has not been thorough except for with a few strains [31, 28, 46]. The relationships between Chinese IBV isolates and foreign IBV isolates, especially Korean, Taiwanese and Japanese IBV isolates, are not known. The focus of this study was to determine the molecular typing of the spike glycoprotein S1 subunit of IBV isolated between the years 1995 and 2004 in China. This will determine the IBV type(s) which are necessary for understanding the epidemiology and evolution of IBVs, as well as isolation of the virus, which is important for improved vaccination.

Materials and methods

Virus isolation and propagation

Twenty-six field IBVs were isolated from kidney, preventriculus, or oviduct of IB-suspected broilers or layers using specified pathogen-free (SPF) embryonated eggs between 1995

Table 1. IBV strains isolated from flocks in different provinces of China

IBV isolates	Province[a]	Years of isolation	Organs[b] used for virus isolation	Production type	Chicken embryo passage[c]
CK/CH/LTJ/95I	Tianjin	1995	kidney	layer hen	3
CK/CH/LHLJ/95I	Heilongjiang	1995	kidney	layer hen	5
CK/CH/LSC/95I	Sichuan	1995	kidney	layer hen	5
CK/CH/LHB/96I	Hebei	1996	kidney	broiler	3
CK/CH/LGD/96I	Guangdong	1996	kidney	layer hen	6
CK/CH/LDL/97I	Liaoning	1997	preventriculus	layer hen	5
CK/CH/LLN/98I	Liaoning	1998	kidney	broiler	6
CK/CH/LDL/98I	Liaoning	1998	preventriculus	layer hen	3
CK/CH/LHLJ/99I	Heilongjiang	1999	preventriculus	layer hen	3
CK/CH/LSC/99I	Sichuan	1999	preventriculus	layer hen	3
CK/CH/LAH/99I	Anhui	1999	kidney	layer hen	7
CK/CH/LHN/00I	Henan	2000	preventriculus	broiler	3
CK/CH/LDL/01I	Liaoning	2001	oviduct	layer hen	7
CK/CH/LXJ/02I	Xinjiang	2002	kidney	layer hen	4
CK/CH/LHLJ/02I	Heilongjiang	2002	kidney	layer hen	2
CK/CH/LSHH/03I	Shanghai	2003	kidney	broiler	3
CK/CH/LSHH/03II	Shanghai	2003	kidney	broiler	3
CK/CH/LGD/03I	Guangdong	2003	kidney	layer hen	5
CK/CH/LAH/03I	Anhui	2003	kidney	layer hen	7
CK/CH/LSD/03I	Shandong	2003	kidney	layer hen	4
CK/CH/LJL/04I	Jilin	2004	preventriculus	layer hen	5
CK/CH/LHLJ/04V	Heilongjiang	2004	kidney	broiler	3
CK/CH/LDL/04II	Liaoning	2004	kidney	broiler	4
CK/CH/LGD/04II	Guangdong	2004	kidney	layer hen	5
CK/CH/LGD/04III	Guangdong	2004	kidney	layer hen	5
CK/CH/LHLJ/04XI	Heilongjiang	2004	kidney	layer hen	3

[a]Province where the viruses were isolated

[b]Kidney = Swollen kidney, Preventriculus = Swollen preventriculus, Oviduct = Atrophic oviduct

[c]Different passages were performed until the dwarfing and death of embryos were observed between 2 and 7 days after inoculation

and 2004 in different parts of China (Table 1 and Fig. 1). For virus isolation, samples of kidney, preventriculus, or oviduct (Table 1) were pooled and 10% w/v tissue suspensions were made in 0.1% phosphate-buffered saline containing 100 u penicillin and 100 μg streptomycin/ml. After 12 h at 4 °C, 200 μl supernatant from the suspensions was inoculated into the allantonic cavity of 9- to 11-day-old embryos. Three to 5 eggs were used for each sample. The inoculated eggs were incubated at 37 °C and candled daily. Two to 7 blind passages were performed until the characteristic embryo changes such as the dwarfing, stunting, or curling of embryos were observed between 2 and 7 days after inoculation [12]. All allantoic fluids were harvested and tested for the presence of IBV using electron microscopy.

Fig. 1. Location of provinces (shaded) where the IBV strains were isolated in China

Electron microscopy

Samples of allantoic fluids were submitted for electron microscopy. Briefly, after low-speed centrifugation at 1500 g for 30 min (Allegra^{TM} 21R centrifuge; Beckman), the supernatant of the 1.5 ml allantoic fluids 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 microscope (JEM-1200, EX).

Viral RNA extraction, RT-PCR amplification, and sequencing

Genomic RNA was extracted from virus-inoculated allantoic fluid with TRIzol reagent (Invitrogen) following the manufacturer's instructions. The first-strand cDNA was synthesized according the procedures of a previous report [31] using S1Oligo3′ [25] and genomic antisense IBV-212 oligonucleotide, 5′-ATACAAAATCTGCCATAA-3′. IBV-212 was designed based on a comparison and alignment of the GenBank sequences of several known Chinese IBV strains and situated in the downstream of S1Oligo3′ which had 5 nt overlapped between them. The PCR profiles involved an initial denaturation for 5 min at 95 °C followed by 30 cycles of denaturation at 94 °C for 1 min, annealing at 50 °C for 1 min, and polymerization at 72 °C for 2 min. The final polymerization step was performed at 72 °C for 10 min. Owing to genetic variations among IBV isolates, it is difficult to design PCR primers that can be used to detect all IBV isolates. Therefore, three genome-sense oligonucleotides, S1Oligo5′ [25], S1Uni2 [1], or IBV-87, 5′-TATTGATTAGAGATGTTGGG-3′, which was selected from conserved areas by aligning several known Chinese IBV sequences from GenBank, were used with S1Oligo3′ [25] or IBV-212 as antisense primer (Table 2). The PCR products were analyzed on a 1.0% agarose gel and were sequenced directly. In addition, PCR products were also sequenced after cloning into the pMD18-T vector (TaKaRa). Each region was sequenced at least three times from two PCR products from different RT reactions.

Sequence analysis of the S1 protein genes

The nucleotide and amino acid sequences of the S1 protein gene of the twenty-six IBV isolates were assembled, aligned, and compared with reference IBV strains using the MEGALIGN program in DNAStar. Phylogenetic analysis of the nucleotide sequences and the deduced amino acid sequences of the S1 protein gene was performed by the Clustal V method using DNAStar software [18]. Thirty-one reference strains were selected for molecular

Table 2. Spike glycoprotein cleavage recognition sites and accession number of IBV isolates in China

IBV isolates	Oligonucleotides[a]	Spike glycoprotein cleavage recognition sites[b]	Accession number
CK/CH/LTJ/95I	S1Oligo5′ + IBV-212	Arg-Arg-Phe-Arg-Arg	DQ167151
CK/CH/LHLJ/95I	S1Oligo5′ + IBV-212	His-Arg-Arg-Arg-Arg	DQ167141
CK/CH/LSC/95I	S1Oligo5′ + IBV-212	Arg-Arg-Phe-Arg-Arg	DQ167146
CK/CH/LHB/96I	S1Oligo5′ + IBV-212	His-Arg-Arg-Arg-Arg	DQ167137
CK/CH/LGD/96I	S1Oligo5′ + IBV-212	His-Arg-Arg-Arg-Arg	DQ167136
CK/CH/LDL/97I	IBV–87 + S1Oligo3′	Arg-Arg-Thr-Gly-Arg	DQ068701
CK/CH/LLN/98I	S1Oligo5′ + IBV-212	His-Arg-Arg-Arg-Arg	DQ167145
CK/CH/LDL/98I	IBV–87 + S1Oligo3′	Arg-Arg-Thr-Gly-Arg	DQ167132
CK/CH/LHLJ/99I	S1Oligo5′ + IBV-212	His-Arg-Arg-Arg-Arg	DQ167142
CK/CH/LSC/99I	S1Oligo5′ + IBV-212	Arg-Arg-Phe-Arg-Arg	DQ167147
CK/CH/LAH/99I	S1Oligo5′ + IBV-212	Arg-Arg-His-Arg-Arg	DQ167129
CK/CH/LHN/00I	S1Oligo5′ + IBV-212	Arg-Arg-Ser-Arg-Arg	DQ167143
CK/CH/LDL/01I	IBV–87 + S1Oligo3′	Arg-Arg-Thr-Gly-Arg	DQ167130
CK/CH/LXJ/02I	S1Oligo5′ + IBV-212	His-Arg-Arg-Arg-Arg	DQ167152
CK/CH/LHLJ/02I	S1Oligo5′ + S1Oligo3′	His-Arg-Arg-Arg-Arg	DQ167138
CK/CH/LSHH/03I	S1Uni2 + S1Oligo3′	His-Arg-His-Arg-Arg	DQ167149
CK/CH/LSHH/03II	S1Uni2 + S1Oligo3′	His-Arg-His-Arg-Arg	DQ167150
CK/CH/LGD/03I	S1Oligo5′ + IBV-212	Arg-Arg-Phe-Arg-Arg	DQ167133
CK/CH/LAH/03I	S1Oligo5′ + IBV-212	Arg-Arg-His-Ser-Arg	DQ167128
CK/CH/LSD/03I	S1Oligo5′ + IBV-212	His-Arg-Arg-Arg-Arg	DQ167148
CK/CH/LJL/04I	S1Oligo5′ + IBV-212	His-Arg-Arg-Arg-Arg	DQ167144
CK/CH/LHLJ/04V	S1Oligo5′ + IBV-212	His-Arg-Arg-Arg-Arg	DQ167139
CK/CH/LDL/04II	S1Oligo5′ + IBV-212	Arg-Arg-Tyr-Arg-Arg	DQ167131
CK/CH/LGD/04II	S1Oligo5′ + IBV-212	Arg-Arg-Phe-Arg-Arg	DQ167134
CK/CH/LGD/04III	S1Oligo5′ + IBV-212	Arg-Arg-Leu-Arg-Arg	DQ167135
CK/CH/LHLJ/04XI	S1Oligo5′ + IBV-212	His-Arg-Arg-Arg-Arg	DQ167140

[a]Oligonucleotides used for amplifying S1 protein gene

[b]*Arg* arginine, *Phe* phenylalanine, *His* histidine, *Thr* threonine, *Gly* glycine, *Ser* serine, *Tyr* tyrosine, *Leu* leucine

analysis. Of these, twenty-six were Chinese IBV strains from the GenBank database, and they represented most of the Chinese IBV field isolates available through GenBank or other publications.

A total of fifty-two Chinese IBV field isolates, including our twenty-six isolates, were chosen to give a representation based on geographic distribution, year of isolation, and phylogenetic position. In addition, two IBV strains, 3051/02 and T07/02, representing TW I and TW II IBV isolates in Taiwan [20], were selected. A Korean IBV isolate, K069-01, was also selected. This IBV strain belonged to genotype III of Korean IBV strains, and this genotype was a major type of IBV in Korea. JP8127, a Japanese IBV strain, was also selected, and its S1 protein gene was compared with Chinese IBV isolates. IBV strains from the above 3 geographically different areas were selected because we were interested in knowing whether the IBV isolates in China were introduced from neighboring countries and continents or whether they arose by mutations of circulating Chinese IBV strains. Furthermore, the S1

protein gene of the H120 vaccine strain was selected and compared in this study because the vaccine was widely used for many years on poultry farms in China. The entire coding region of the S1 protein gene of these strains was chosen for analysis.

Table 3. Data for reference IBV strains and sequences

IBV strains (origin)	Years of isolation	Genotype/Pathogenicity type	Spike glycoprotein cleavage recognition sites	Accession number
HBN (China)	Between 1996 and 1998	nepphropathogenicity	His-Arg-Arg-Arg-Arg[a]	DQ070837
QXIBV (China)	1997	proventriculus	His-Arg-Arg-Arg-Arg	AF193423
A2 (China)	After 2000	nd[b]	His-Arg-Arg-Arg-Arg	AY043312
LX4 (China)	1999	nepphropathogenicity	His-Arg-Arg-Arg-Arg	AY189157
BJ (China)	1998	nd	Arg-Arg-Thr-Arg-Arg	AY319651
BJY (China)	Between 1996 and 1998	nepphropathogenicity	Arg-Arg-Thr-Arg-Arg	DQ070836
BJS (China)	Between 1996 and 1998	nepphropathogenicity	His-Arg-Thr-Lys-Arg	DQ070838
tl/CH/LDT3/03 (China)	2003	nepphropathogenicity	Arg-Arg-Phe-Arg-Arg	AY702975
JX/99/01 (China)	1999	nd	Arg-Arg-His-Arg-Arg	AF210735
BJQ (China)	Between 1996 and 1998	nepphropathogenicity	Arg-Arg-Phe-Arg-Arg	DQ070839
TJ/96/02 (China)	1996	nd	Arg-Arg-Phe-Arg-Arg	AF257075
SH2 (China)	2005	nepphropathogenicity	Arg-Arg-Phe-Arg-Arg	DQ075324
J (China)	1998	nepphropathogenicity	Arg-Arg-Phe-Arg-Arg	AF352312
SC021202 (China)	2002	nepphropathogenicity	Arg-Arg-His-Arg-Arg	AY237817
HaN1-95 (China)	1995	nd	Arg-Arg-Phe-Arg-Arg	AY251817
W93 (China)	1993	nepphropathogenicity	Arg-Arg-Phe-Arg-Arg	AY427818
D41 (China)	1987	nepphropathogenicity	Arg-Arg-Phe-Arg-Arg	AF036937
SD/97/01 (China)	1997	nd	Arg-Arg-Phe-Arg-Arg	AF208240
2/97 (China)	1997	proventriculus	Arg-Arg-Phe-Arg-Arg	AY043218
ZJ971 (China)	1997	proventriculus	Arg-Arg-Phe-Arg-Arg	AF352313
1/98 (China)	1998	proventriculus	Arg-Arg-Phe-Arg-Arg	AY043220
JL/97/01 (China)	1997	nd	Arg-Arg-Phe-Arg-Arg	AF258780
JS/95/03 (China)	1995	nd	Arg-Arg-Phe-Arg-Arg	AF208239
SDA (China)	After 2000	nd	Arg-Arg-Phe-Arg-Arg	AY043313
HN99 (China)	1999	nepphropathogenicity	Arg-Arg-Ser-Arg-Arg	AY775551
J2 (China)	Between 1996 and 1998	proventriculus	Arg-Arg-Thr-Gly-Arg	AF286303
K069-01 (Korea)	2001	Korean Genotype III	Arg-Arg-Phe-Arg-Arg	AY257061
JP8127 (Japan)	1993	Closely related to Australia classical strains	Arg-Arg-Phe-Lys-Arg	AY296744
3051/02 (Taiwan)	2002	TW I	Arg-Arg-Phe-Arg-Arg	AY606318
T07/02 (Taiwan)	2002	TW II	Arg-Arg-Phe-Arg-Arg	AY606322
H120	Vaccine strain	Mass serotype	Arg-Arg-Phe-Arg-Arg	M21970

[a] *His* histidine, *Arg* arginine, *Thr* threonine, *Lys* lysine, *Phe* phenylalanine, *Ser* serine, *Gly* glycine
[b] *nd* not documented

Strains and accession numbers used for molecular analysis

The fifty-seven IBV strains, including our twenty-six isolates, were molecularly analyzed. The twenty-six IBV isolates in this study and their accession numbers are listed in Table 2. The IBV reference strains and their accession numbers are listed in Table 3.

Results

Detection of IBV

Twenty-six IBV strains were isolated from flocks that were suspected of IBV infection. The isolates were from flocks in different parts of China (Fig. 1) that showed clinical IB and had 5 to 60% mortality. The nephritis observed in all flocks was characterized by enlarged and pale kidneys, frequently with urate deposits in the tubules, and severe dehydration and weight loss. Typical signs, including dwarfing and death of the embryo, were observed in different passages when each isolate was inoculated into embryos (Table 1). Diagnoses based on electron microscopy examination showed all isolates had typical coronavirus morphology and were free of other agents such as Newcastle disease virus (NDV) (results not shown).

Phylogenetic analysis

To assess the genetic relatedness among the IBV strains, a phylogenetic tree was performed with S1 protein genes. The results are shown in Fig. 2. The fifty-seven IBV strains were separated into seven genotypes (I to VII) by phylogenetic analysis of the S1 protein genes (Fig. 2). Genotype I consisted of fourteen Chinese strains having small evolutionary distances from each other as shown in the rooted tree (Fig. 2). Genotype II included 6 strains that were isolated in the 1990s in China. Most of the Chinese IBV isolates included in genotype III were also isolated in the 1990s, except tl/CH/LDT3/03 and CK/CH/LGD/03I, which were both isolated in Guangdong province in 2003 from teal [29] and layer hens, respectively. The Korean IBV isolate, K069-01, which belonged to genotype III of Korean IBV strains [27], was closely related to those isolates in genotype III. Six of eight IBV isolates displayed in genotype IV were isolated after 2000, and most of them came from southern China. Furthermore, isolates included in genotype IV showed larger evolutionary distances (Fig. 2). Ten Chinese IBV isolates formed the genotype V in which H120 was included, and none of our twenty-six isolates were grouped under this genotype. The isolates HN99 and CK/CH/LHN/00I, both isolated in Henan province in 1999 and 2000, respectively, together with a Japanese isolate, JP8127, were grouped into genotype VI. Our three IBV isolates recovered in Dalian, China, between 1997 and 2001, were grouped into genotype VII. A Chinese IBV isolate, J2, which was isolated from the proventricular tissues of infected chickens [46], was also placed in genotype VII. Two IBV isolates, 3051/02 and T07/02, belonging to TW I and TW II, formed a unique genotype.

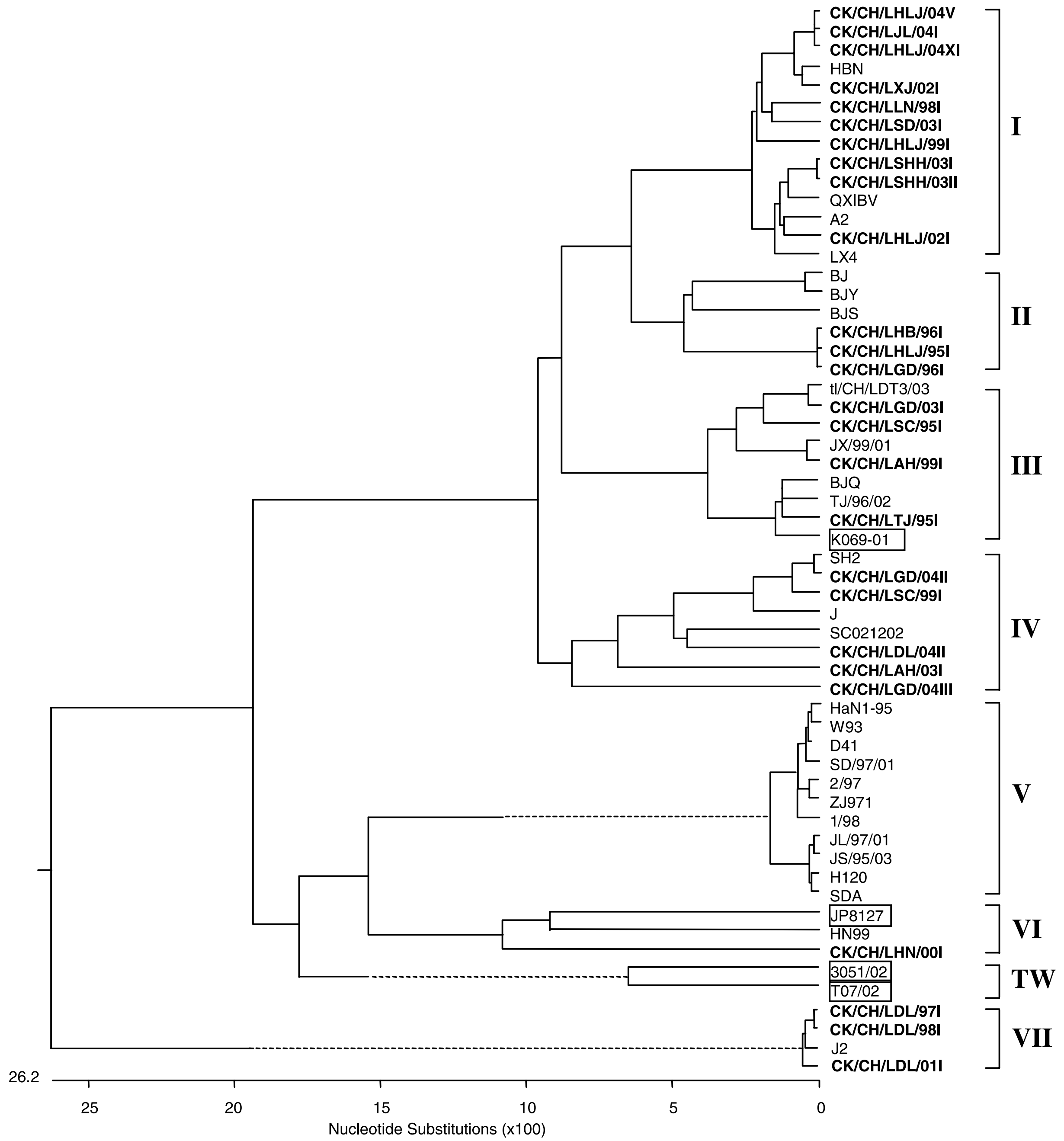

Fig. 2. Phylogenetic relationships, based on the sequence of the S1 subunit of the S protein gene, of our twenty-six isolates and thirty-one reference strains (the first 1669 nt, starting at the AUG translation initiation codon, of the S protein genes) using the MEGALIGN program DNAStar with the Clustal V method [18]. Our IBV isolates are in bold type

The spike glycoprotein cleavage recognition site

The spike glycoprotein of IBV is translated as a precursor protein (S0) and then cleaved into two subunits S1 and S2 [9, 24]. Cleavage site motifs of the fifty-seven

IBV strains are listed in Table 2 and Table 3, and twelve different cleavage site sequences were observed. The most common cleavage recognition site observed (24 of 57 viruses) was Arg-Arg-Phe-Arg-Arg. Viruses with this cleavage recognition site are the H120 vaccine strain, one Korean strain, K069-01, Taiwan isolates 3015/02 and T07/02, ten Chinese Mass-type isolates, and ten other Chinese isolates included in genotype III (six strains) and IV (four strains). The second most common site was His-Arg-Arg-Arg-Arg. Viruses with this cleavage recognition site include twelve isolates in genotype I and three in genotype II. This recognition site was unique for virus isolates in China. The third most common site was Arg-Arg-Thr-Gly-Arg. Viruses with this cleavage recognition site were placed in genotype VII, which included our three isolates (CK/CH/LDL/97I, CK/CH/LDL/98I, and CK/CH/LDL/01I) and isolate J2. This cleavage recognition site was also unique to viruses in China. The JX/99/01, CK/CH/LAH/99I, and SC021202 viruses had a cleavage recognition site, Arg-Arg-His-Arg-Arg, as did D1466 [22]. Chinese IBV isolates HN99 and CK/CH/LHN/00I, which were grouped in genotype VI, had a cleavage recognition site, Arg-Arg-Ser-Arg-Arg, which was the most common site reported by Jackwood [22], who had compared the cleavage recognition sites of fifty-five IBV isolates to determine if the site sequence correlates with host cell range, serotype, geographic origin, and pathogenicity. The CK/CH/LSHH/03I and CK/CH/LSHH/03II viruses had a unique cleavage recognition site, His-Arg-His-Arg-Arg, as did isolates BJ and BJY, Arg-Arg-Thr-Arg-Arg, CK/CH/LAH/03I, Arg-Arg-His-Ser-Arg, CK/CH/LDL/04II, Arg-Arg-Tyr-Arg-Arg, CK/CH/LGD/04III, Arg-Arg-Leu-Arg-Arg, BJS, His-Arg-Thr-Lys-Arg, and Japanese Strain, JP8127, Arg-Arg-Phe-Lys-Arg.

Amino acid sequence comparison

The complete nucleotide and predicted amino acid sequences of the S1 protein of the fifty-seven IBV strains were determined and compared. Except for isolates in genotype V, which included the Mass-type strains, none of the Chinese IBV isolates examined in this study shared more than 83% amino acid similarity in the S1 protein with the H120 vaccine strain. The S1 protein genes, which varied from 0.2 to 26.7% among the strains examined, indicated that point mutations, deletions, and insertions contribute to the evolution of IBV. IBVs in same genotypes showed more than 90% amino acid sequence similarities, whereas most of the viruses in different genotypes showed less than 90%, with the exceptions of isolate BJS (genotype II) and isolates in genotype IV, BJQ (genotype III) and isolates in genotype V, isolates between genotypes I and III, which showed amino acid similarities of 91–94.5%, 90.5–92%, and 93.2–95.6%, respectively. The overall predicted amino acid sequence comparisons of the entire S1 protein of fifty-seven IBV strains reflected that most of the sequence variations were concentrated in three regions. The first included residues 50–87, corresponding to the S1 protein of the H120 vaccine strain, in which the hypervariable region 1 (HVR1) is located [8, 37, 42]. The second contained amino acid sequences between residues 114–140, which encompasses the hypervariable region 2 (HVR2) [8, 37, 42]. The last included residues 273–293, in which the hypervariable region 3 (HVR3)

Table 4. Deletions and insertions of the predicted amino acids of the S1 protein of Chinese IBV isolates compared with the H120 vaccine strain[a]

Strains	Deletions or substitutions[b]						Insertions[c]				Genotype
	24	25	58–60	117–118	25–26	72–73	116–117	137–138	140–141	283–284	
H120	S	S	NNA	HV	–	--------	--	–	----	–	V
CK/CH/LHLJ/04V	D	N	SNA	AG	N	--------	SG	–	----	–	I
CK/CH/LJL/04I	D	N	NNA	AG	N	--------	SG	–	----	–	I
CK/CH/LHLJ04XI	D	N	NNA	AG	N	--------	SG	–	----	–	I
HBN	D	N	NNA	AG	N	--------	SG	–	----	–	I
CK/CH/LXJ/02I	D	N	NNA	AG	N	--------	SG	–	----	–	I
CK/CH/LLN/98I	D	N	NNA	SG	N	--------	SG	–	----	–	I
CK/CH/LSD/03I	D	N	NNA	SG	N	--------	SG	–	----	–	I
CK/CH/LHLJ/99I	G	S	NNA	AG	–	--------	SG	–	----	–	I
CK/CH/LSHH/03I	A	N	---	SG	N	--------	SG	–	----	–	I
CK/CH/LSHH/03II	A	N	---	SG	N	--------	SG	–	----	–	I
QXIBV	A	N	NNA	SG	N	--------	SG	–	----	–	I
A2	A	N	NNA	TG	N	--------	SG	–	----	–	I
CK/CH/LHLJ/02I	G	N	SNA	PT	–	--------	SG	–	----	–	I
LX4	A	N	NNA	SG	–	--------	SG	–	----	–	I
BJ	G	N	NNA	--	–	YTNGNSDV	N-	–	----	–	II
BJY	G	N	NNA	--	–	YANGNSDV	N-	–	----	–	II
BJS	G	S	NNA	--	–	YSNG-IDV	N-	–	----	–	II
CK/CH/LHB/96I	D	-[d]	NNA	--	–	YNNGNSDV	K-	–	----	–	II
CK/CH/LHLJ/95I	D	–	NNA	--	–	YNNGNSDV	K-	–	----	–	II
CK/CH/LGD/96I	D	–	NNA	--	–	YNNGNSDV	K-	–	----	–	II
tl/CH/LDT3/03	A	N	NNA	SG	N	--------	SG	–	----	–	III
CK/CH/LGD/03I	A	N	NNA	SG	N	--------	SG	–	----	–	III
CK/CH/LSC/95I	D	N	NNA	SG	N	--------	SG	–	----	–	III
JX/99/01	–	H	NNA	SG	N	--------	SG	–	----	–	III
CK/CH/LAH/99I	–	H	NNA	SG	N	--------	SG	–	----	–	III
BJQ	–	G	NNA	SG	N	--------	SG	–	----	–	III
TJ/96/02	–	N	NNA	SG	S	--------	SG	–	----	–	III
CK/CH/LTJ/95I	–	S	NNA	SG	N	--------	SG	–	----	–	III
K069-01	–	N	NNA	SG	N	--------	RG	–	----	–	III
SH2	D	–	NNA	--	–	YTNG-NDV	N-	–	----	–	IV
CK/CH/LGD/04II	D	–	NNA	--	–	YTNG-NDV	N-	–	----	–	IV
CK/CH/LSC/99I	D	–	NNA	--	–	YTNG-NDV	N-	–	----	–	IV
J	D	N	NNA	--	–	YTNG-KDV	N-	–	----	–	IV
SC021202	D	N	NNA	--	–	YTNG-NDV	N-	–	----	–	IV
CK/CH/LDL/04II	G	N	NNA	--	N	YSNG-NDV	P-	–	----	–	IV
CK/CH/LAH/03I	N	–	NNA	--	–	YANG-NHA	N-	–	----	L	IV
CK/CH/LGD/04III	H	D	NNA	--	–	YSNG-NDV	N-	–	----	–	IV
HaN1-95	S	S	NNA	QG	–	--------	--	–	----	–	V
W93	S	S	NNA	QG	–	--------	--	–	----	–	V
D41	S	S	NNA	QG	–	--------	--	–	----	–	V
SD/97/01	S	S	NNA	HV	–	--------	--	–	----	–	V
2/97	S	S	NNA	HV	–	--------	--	–	----	–	V
ZJ971	S	S	NNA	HV	–	--------	--	–	----	–	V
1/98	S	S	NNA	HV	–	--------	--	–	----	–	V
JL/97/01	S	S	NNA	YD	–	--------	--	–	----	–	V
JS/95/03	S	S	NNA	YD	–	--------	--	–	----	–	V
SDA	S	S	NNA	YD	–	--------	--	–	----	–	V
JP8127	D	T	NNA	NN	–	--------	SG	Q	GPAD	T	VI
HN99	G	N	SNA	AG	–	--------	SG	Q	GPSD	–	VI
CK/CH/LHN/00I	E	S	NNA	SN	–	--------	SG	H	MPGH	–	VI
3051/02	D	T	NNA	SG	–	--------	SG	–	----	–	TW
T07/02	D	T	ANA	QG	–	--------	ST	–	----	–	TW
CK/CH/LDL/97I	N	E	NNA	DG	–	--------	HG	K	----	–	VII
CK/CH/LDL/98I	N	E	NNA	NG	–	--------	HG	K	----	–	VII
J2	N	E	NNA	NG	–	--------	HG	K	----	–	VII
CK/CH/LDL/01I	N	E	NNA	NG	–	--------	HG	K	----	–	VII

[a]Amino acid abbreviations: *S* serine, *D* aspartic acid, *G* glycine, *A* alanine, *N* asparagine, *H* histidine, *E* glutamic acid, *T* threonine, *Y* tyrosine, *P* proline, *Q* glutamine, *V* valine, *I* isoleucine, *K* lysine, *R* arginine, *M* methionine; [b]positions of residues in deduced amino acid sequences of the S1 protein of the H120 vaccine strain; [c]positions of residues in deduced amino acid sequences of the S1 protein of the H120 vaccine strain between which the residue(s) of other IBVs was (were) inserted; [d]missing amino acid residues

is present [39]. Furthermore, almost all of the Chinese IBV isolates contained deletions and insertions except for those of the Mass-type IBV, which were included in genotype V in this study, and had amino acid sequences similar to those of the H120 strain (Table 4). The deletions and insertions, which occurred in the predicted amino acid sequences of the S1 proteins of fifty-seven IBV strains in this study, were correlated with the genotypes of S1 protein genes, as shown in Table 4. In addition, the Korean strain, K069-01, shared most of the motifs of deletions and insertions with Chinese IBV field isolates in genotype III.

Discussion

In 1962, Winterfield and Hitchner reported a nephrosis condition associated with IB in the United States, and Cumming reported an IB outbreak causing severe kidney lesion in chickens in Australia [13, 43]. Since this time, various nephropathogenic strains of IBV have been identified throughout the world [33]. In China, IB with nephritis was first reported in 1982 and several nephropathogenic IBV strains have been isolated in different parts of China since then [31, 32, 44]. Of the twenty-six IBV isolates in this study, one was isolated from atrophic oviduct of a diseased layer hen, six from swollen proventricular tissues of infected chickens, and the rest from swollen kidneys of IB-suspected chickens. Although seven IBV strains were isolated from tissues other than kidney, the gross lesions of kidney in these diseased chickens were also obvious. Based on the fact that these IBV strains were isolated from 1995 to 2004 in China, we considered that IB was prevalent all the while in China, although vaccines based on Mass-type strains such as H120 and H52 have been used for many years on poultry farms, and nephropathogenic IBV was the major type of IBV circulating in China.

Although the genetic basis of IBV pathogenicity is not known, the S1 protein gene of IBV has serotype-specific and neutralization-specific epitopes, and serotypic evolution and the genetic diversity of IBV is mainly monitored by analysis of the S1 gene [2, 9, 10, 23, 27, 32, 42]. In the present study, phylogenetic analysis of S1 genes showed that Chinese IBV isolates were grouped into seven genotypes (Fig. 2). IBVs isolated ten years ago were included in the same genotype with the strains isolated recently (for example, CK/CH/LSC/95I and CK/CH/LGD/03I in genotype III), indicating that this genotype may be indigenous and has been prevalent in China for at least ten years. Serotype differences among the genetically distinct IBVs generally correlated with variations in the HVR of the S1 protein gene [4, 8] and differences of as little as 5% between S1 sequences of IBV could result in poor cross-protection offered by currently used vaccines [19]. The low identities (<83%) of amino acid sequences between Chinese IBV isolates and H120, except for those of the Mass-type IBV, which were included in genotype V in this study, may account for the prevalence of the viruses during the past ten years in spite of the extensive use of Mass-type vaccines in the field in China. Hence, developing vaccines from local strains is necessary for IBV control in China.

Although the number of basic residues around the spike glycoprotein cleavage recognition site of IBV does not appear to correlate with increased cleavability,

host cell range, and increased virulence as it does with the envelope glycoproteins of orthomyxoviruses and paramyxoviruses, the sequences of cleavage recognition sites was correlated with geographic distribution of the viruses [22]. Nine spike glycoprotein cleavage recognition site sequences were found in viruses of genotypes I to IV, in which six were unique to isolates in China, indicating genetically distinct evolution from viruses in other countries by cleavage recognition site analysis. However, a Korean IBV strain, K069-01, shared the same cleavage recognition site sequence, Arg-Arg-Phe-Arg-Arg, with ten Chinese isolates included in subgenotypes III (six strains) and IV (four strains), ten Chinese Mass-type isolates (genotype V), and two Taiwan isolates, 3015/02 and T07/02. Furthermore, K069-01 and Chinese isolates in genotype III shared more than 90% amino acid identities and they were also grouped into the same genotype (Fig. 2). K069-01 was clustered into genotype III of Korean IBV strains, which was the major type of IBV circulating in Korea, and isolates in this genotype induced 50% mortality in 1-day-old chicks as well as severe renal urate deposition on the kidneys [40]. This was similar to Chinese isolates in genotype I [29, 31]. Based on these facts, IBVs between Chinese genotype III and Korean genotype III had a close relationship, as did NDV [26], owing to the increased trade of agricultural products including poultry between two countries. Unlike K069-01, isolates 3051/02 and T07/02, which represented TW I and TW II strains, respectively [20], were clustered into a separate branch that was separated from the Chinese genotypes, indicating that they had different origins.

Ten Chinese IBV strains were classified into the Mass serotype (genotype V by phylogenetic analysis). As in China, Mass-type IBVs were also present in other Asian countries, such as Korea [27, 40], Japan [32, 38], and Taiwan [20, 30], although Mass-type vaccines were commonly used in these countries or continents. However, Chinese Mass-type strains were all isolated in the 1990s and were not the major IBV type circulating in recent years in China. Molecular studies have shown that a new serotype or variant can emerge as a result of only a few changes in the amino acid composition in the S1 part of the virus spike protein, with the majority of the virus genome remaining unchanged [6]. This could be due to immunologic pressure caused by the widespread use of vaccines, to recombination as a consequence of mixed infections, or to the decrease of dominant serotypes as a result of vaccination, allowing other field strains to emerge. To this study, the Mass-type viruses may have come from the vaccine strains by point mutation, although the possibility that some of them were reisolations of vaccine strains cannot be excluded.

Two strains, HN99 and CK/CH/LHN/00I, both isolated in Henan province in China, together with a Japanese strain, JP8127, constituted a "novel" genotype VI (Fig. 2). These two Chinese isolates did not show close similarity to any of the S1 protein sequences of other Chinese IBV isolates available through GenBank or publications. Interestingly, BLAST searches revealed significant similarity (99%) of S1 protein genes between isolate HN99 and a vaccine strain, JAAS (AY839140), which was from Australia and used in China to control IBV. The isolate CK/CH/LHN/00I shared 99% similarity in the S1 protein gene with another

IBV vaccine strain, Jilin (AY839144), which was also used in China. When CK/CH/LHN/00I was inoculated experimentally into 15-day-old SPF chickens, no disease signs were apparent (S. Liu et al., unpub. data). The spreading of a virus from one area or country to another could be due, at least in part, to its improper introduction by the trading of birds or by the use of attenuated vaccines. To our knowledge, no other IBV strains related to HN99 or CK/CH/LHN00I were isolated in recent years in China, and considering the pathogenicity and genetically close relationship between the two isolates and the corresponding vaccine strains, we speculated that the two isolates would be reisolations of vaccine strains.

Isolate J2, which was very similar to Q1 and T3, was genetically distinct from most of the Chinese IBV isolates [45]. In this study, our three isolates (CK/CH/LDL/97I, CK/CH/LDL/98I, and CK/CH/LDL/01I) were clustered into the same group (genotype VII) with J2. Similar to J2, CK/CH/LDL/97I and CK/CH/LDL/98I were isolated in swollen proventriculars tissues of infected chickens, whereas CK/CH/LDL/01I was isolated from atrophic oviduct of a diseased layer hen. It was found that the gross lesions of the kidney in these diseased chickens were also obvious, as with isolate 2992/02 [20]. 2992/02 was isolated in Taiwan and was very similar to the J2 strain by comparison of S1 protein genes [20]. The diversity of the pathogenicity of IBV strains was expected; although the primary tissue of IBV infection is the respiratory tract, some isolates can grow in nonrespiratory organs such as the kidney, the female reproductive tract, intestine, and spleen of chickens [2, 14, 33, 36].

With the exception of the Massachusetts strain, a very interesting aspect of IBV epidemiology, as far as it is possible to know, is the presence and the spreading of the various IBV serotypes in different continents. About 20 emergent serotypes in North America did not spread to other continents. Similarly, the European, Australian, and Asiatic serotypes apparently did not spread elsewhere. In China, IBV epidemiology is more complicated. Besides genotypes I to IV and VII, the Mass-type IBV and IBV closely related to Australian classical strains were also present, indicating IBVs showing various genetic differences were cocirculating in China.

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