Kafkas Univ Vet Fak Derg
21 (2): 179-186, 2015
DOI: 10.9775/kvfd.2014.11999

Kafkas Universitesi Veteriner Fakultesi Dergisi
Journal Home-Page: http://vetdergi.kafkas.edu.tr
Online Submission: http://vetdergikafkas.org

Research Article

Epidemiology and Evolutionary Characteristics of Avian Infectious Bronchitis Virus in China

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KVFD-2014-11999 Received: 17.07.2014 Accepted: 08.01.2015 Published Online: 09.01.2015

Abstract

To examine the epidemiology and evolutionary characteristics of avian infectious bronchitis virus (IBV) in mainland China, the S1 gene of 63 IBVs isolated after 1993 was amplified by reverse transcription (RT) –polymerase chain reaction (PCR) and sequenced. Our results showed that the sequence length of 63 IBV strains ranged from 1.608 to 1.635 nucleotide (nt). Compared to the published representative strains, the homology of S1 nt sequences and their deduced amino acid (aa) residues between these isolates and reference strains ranged from 74.6% to 99.9% and 71.7% to 100%, respectively. Analysis of S proteins demonstrated that 63 isolates had eight kinds of cleavage sites (RRFRR, RRIRR, RRSKR, RRTGR, RRHRR, HRRRR, RRSRR and RRLRR), and the amount of RRFRR and HRRRR reached 46. Furthermore, the study showed that there was no direct relation between cleavage sites and genotypes, and cleavage sites were unable to decide the pathology types. Compared to vaccine strain H120, S1 nt sequences of 63 isolates had four sites deleted or inserted frequently, and the S1 proteins had three hypervariable regions. A comprehensive study was also carried out to study the S1 nt sequences of 63 isolates, 210 published reference strains and 10 vaccine strains; the investigation showed that all these stains could be divided into two types including 11 kinds of genotypes by the phylogenetic analysis. However, 63 isolates belonged to Mass, American, LX4, LHLJ/95 I, LDT3/03, J, BJ and LDL/97 I genotypes, and the LX4 genotype was co-circulated dominantly in chicken flocks over an 18-year period. It indicated that there were several IBV genotypes with new changes circulating in China, emphasizing the importance of continued IBV surveillance.

Keywords: *IBV, S1 sequence, Genetic variation, Phylogenetic analysis*

Çin'de Avian Enfeksiyöz Bronşitis Virüsünün Epidemiyolojisi ve Evrimsel Özellikleri

Özet

Avian Enfeksiyöz Bronşitis Virüsünün (IBV) epidemiyolojisini ve evrimsel ezelliklerini araştırmak amacıyla Çin'de 1993 yılından sonra izole edilen 63 IBV S1 geni ters transkriptaz (RT)-polimeraz zincir reaksiyonu (PCR) ile amlifiye edilerek sekansı yapıldı. Çalışma sonuçları 63 IBV suşunun sekans boyutunun 1.608 ile 1.635 nükleotide (nt) arasında değiştiğini gösterdi. Yayınlanmış benzer suşlar ile karşılaştırıldığında bu izolatlar ile referans suşları arasında S1 nt sekanslarının homolojisi ve açığa çıkan amino asit (aa) rezidüleri sırasıyla %74.6 ile %99.9 ve %71.7 ile %100 arasında değişti. S proteinlerinin analizi 63 izolatın 8 çeşit bölünme bölgesine (RRFRR, RRIRR, RRSKR, RRTGR, RRHRR, HRRRR, RRSRR ve RRLRR) sahip olduğunu ve RRFRR ve HRRRR miktarının 46'ya ulaştığını ortaya koydu. Ayrıca, bölünme bölgesi ile genotipler arasında doğrudan bir ilişki olmadığı ve bölünme bölgelerinin patoloji tiplerini belirlemediği tespit edildi. Aşı suşu H120 ile karşılaştırıldığında 63 izolatın S1 nt sekanslarının sıklıkla çıkarılmış veya eklenmiş 4 bölgeye sahip olduğu belirlendi. S1 proteinleri üç adet oldukça değişken bölgeye sahip olduğu gözlemlendi. 63 izolatın S1 nt sekansları, 210 yayınlanmış referans suşu ve 10 aşı suşunu araştırmak amacıyla ayrıca geniş çaplı bir çalışma yürütüldü. Tüm bu suşlar filogenetik analiz ile 11 çeşit genotipi de içeren iki tipe ayrılabilir. Ancak, 63 izolat Mass, American, LX4, LHLJ/95 I, LDT3/03, J, BJ ve LDL/97 I genotiplerine aitti ve LX4 genotipinin 18 yıldan daha uzun süredir tavuk sürülerinde baskın olarak bulunduğu belirlendi. Bu çalışma ile Çin'de yeni değişimlerle birlikte birkaç IBV genotipinin bulunduğu ve sürekli takibin önemi ortaya konuldu.

Anahtar sözcükler: IBV, S1 sekansı, Genetik varyasyon, Filogenetik analiz

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INTRODUCTION

Avian infectious bronchitis (IB) is caused by IBV, which is a member of the *Coronaviridae* family, is recognized as one of the significant diseases of poultry, and leads to great economic losses to the poultry industry all over the world [1,2]. As a highly contagious pathogen, IBV not only brings chicken high pathogenicity rate with upper respiratory disease, nephritis or enteritis, but also causes fertility problems accompanied by low egg production and poor quality. Furthermore, the disease is a risk for increasing susceptibility to infections with other pathogens, resulting in an even higher morbidity and mortality rate [3,4].

IBV is an enveloped, non-segmented and single-stranded positive RNA virus with a genome around 27.6-kb in length [5-7]. The genome encodes four structural proteins, including spike glycoprotein (S), integral membrane glycoprotein (M), small membrane protein (E) and phosphorylated nucleocapsid (N) protein. Additionally, the genome also encodes the replicase complex, which carries out the unique discontinuous transcription process, leading to a nested set of 3′ coterminal subgenomic mRNAs [8-10]. The S glycoprotein is proteolytically processed into two non-covalently bound peptide chains, S1 and S2. The S1 subunit located on the outside of virion is responsible for the fusion between the virus envelope and the cell membrane of the host, and, it contains epitopes and determinants for virus neutralizing antibodies, protective immunity, cell attachment and serotype specificity [11-13]. Classification of genotype has been done on the base of the antigenic site especially the hypervariable regions (HVR) of S1 nt sequence. The difference of several aa residues in S1 sequence can produce a new serotype strain and result in poor cross-protection between the strains. So, analysis of the S1 nt sequences has been usually used for differentiating IBV genotypes and serotypes [14,15].

More than 20 serotypes of IBV have been found around the world, and many IBV variant strains have been identified in various regions, leading to clinical symptom of birds infected by IBV varies greatly. The complex epidemiology characteristics of IB raised the difficulty in controlling this disease. These IBVs have no or low degree cross-protection for their serotype's diversity and virus genome varied frequently [16,17]. There are at least two reasons for explaining IBV continuous evolution. Firstly, it is thought that nucleotide deletions, insertions or point mutations within IBV genome result in errors made by viral RNA-dependent RNA polymerase [18,19]. Secondly, it can be explained by the IBV genome recombination in field strains or even between field strains and vaccine strains by the extensive and continuing use of live vaccine on chicken flock. Therefore, it is important to choose an appropriate vaccine with a serotype/genotype that is consistent with the epidemic virus to prevent and control the disease in each geographical region or country [5,11].

In China, after the first IBV has been identified by virus isolation since the early 1980s, more and more strains have been isolated in different regions. It becomes more difficult to prevent and control IB when novel IBV variants are circulating in chicken flocks. A vaccine programme with live-attenuated and inactivated vaccines of Massachusetts (Mass) serotype has been carried out widely and is partially successful in China. But in recent years, the phenomenon of IBV outbreaks occurred frequently in different vaccinated and non-vaccinated chicken flocks showed that the vaccines had poor or no protection against field virus [20-24]. Bing et al.[25] found that the proportion of nephro-pathogenic type IBV is higher than any other types by analysis of the strains isolated from China in these years. How to avoid enormous economic loss caused by nephro-pathogenic IB through selecting appropriate vaccine comes to be very important.

In this study, to reveal the epidemiology and evolutionary characteristics of recent IBV field isolates and to demonstrates which IBV genotypes are circulating in China, we investigated 63 IBVs isolated from 1993 to 2010 in mainland China, analyzed the sequences of whole S1 nt and took sequence alignment and phylogenetic analysis compared with other reference strains available in the GenBank database.

MATERIAL and METHODS

Virus

IBV was isolated from kidney, proventriculus and trachea from chicken flocks suspected to be infected with IBV during 1993-2010, covering 16 provinces, which occupied most of the chicken-raising regions of China *(Table 1)*.

Primer Design

S1 oligonucleotide primers were designed using eight IBV reference strains (Beaudette, Mass41, Cal99, BJ, KQ6, GD/S14/2003, SAIBK, LX4)logged in GenBank, for amplifying the entire S1 region (including leading sequence) and S1/S2 cleavage sites (1750 bp). The sense primer was S1-F (5′-TTGAAAACTGAA CAAAAGACCG-3′), and the sequence S1-R (5′-TACAAAACCTGCCATAACTAACAT-3′) was used as antisense primer. The PCR conditions included 5 min incubation at 95°C followed by 30 cycles at 94°C for 1 min, annealing at 52°C for 1min, and extension at 72°C for 2 min. A final extension step was performed at 72°C for 10 min.

RT-PCR Amplification and Sequencing

Sixty-three IBVs were propagated in 9-day-old specific pathogen-free chicken embryos. Then the allantoic fluids of the infected embryos were harvested and total RNA was extracted with TRIzol reagent (Invitrogen, Grand Island, USA). RT-PCR products were analyzed on 1% agarose gel and sequenced after cloning into the pMD18-T (Takara,

Table 1. *63 IBV Strains isolated during 1993-2010 in China*
Tablo 1. *Çin'de 1993-2010 yılları arasında izole edilen 63 IBV suşu*

IBV Isolates	Year of Isolation	Province	Organs Used for Virus Isolation	Cleavage Recognition Motifs	Genotypes	IBV Isolates	Year of Isolation	Province	Organs Used for Virus Isolation	Cleavage Recognition Motifs	Genotypes
CK/CH/HIBV97	1997	Shandong	kidney	RRFRR	LDT3/03-type (VI)	CK/CH/GX-98 I	1998	Guangxi	trachea	RRSRR	American group (II)
CK/CH/SWIBV97	1997	Shandong	kidney	RRFRR	LDT3/03-type (VI)	CK/CH/HeN-93 II	1993	Henan	kidney	HRRRR	LX4-type (IV)
CK/CH/JNIBV98	1998	Shandong	kidney	RRIRR	BJ-type (VIII)	CK/CH/SC-93 II	1993	Sichuan	kidney	RRFRR	Mass-type (I)
CK/CH/QXIBV2	1998	Shandong	proventriculus	RRFRR	Mass-type (I)	CK/CH/GD-98 VI	1998	Guangdong	kidney	RRHRR	J-type (VII)
CK/CH/GNIBV98	1998	Shandong	kidney	RRFRR	J-type (VII)	CK/CH/GX-09 I	2009	Guangxi	kidney	HRRRR	LHLJ/95 I-type (V)
CK/CH/HeBIBV98	1998	Hebei	kidney	RRIRR	BJ-type (VIII)	CK/CH/GX-08 I	2008	Guangxi	kidney	HRRRR	LHLJ/95 I-type (V)
CK/CH/HeNIBV98	1998	Henan	kidney	RRFRR	LDT3/03-type (VI)	CK/CH/GX-08 II	2008	Guangxi	kidney	HRRRR	J-type (VII)
CK/CH/JSNJ97	1997	Jiangsu	kidney	RRSKR	LHLJ/95 I-type (V)	CK/CH/HaN-09 I	2009	Hainan	kidney	RRFRR	J-type (VII)
CK/CH/SDPL-01 I	2001	Shandong	kidney	RRTGR	LDL/97 I-type (IX)	CK/CH/GX-08 III	2008	Guangxi	kidney	HRRRR	J-type (VII)
CK/CH/SDZC-01 I	2001	Shandong	proventriculus	RRFRR	Mass-type (I)	CK/CH/GD-09 I	2009	Guangdong	kidney	HRRRR	LX4-type (IV)
CK/CH/SDTA-01 I	2001	Shandong	kidney	RRTGR	LDL/97 I-type (IX)	CK/CH/GX-07 I	2007	Guangxi	trachea	RRFRR	Mass-type (I)
CK/CH/SDTA-01 II	2001	Shandong	kidney	RRFRR	Mass-type (I)	CK/CH/GX-08 IV	2008	Guangxi	kidney	RRTGR	LDL/97 I-type (IX)
CK/CH/JX-99I	1999	Jiangxi	kidney	RRHRR	LDT3/03-type (VI)	CK/CH/HaN-09 III	2009	Hainan	kidney	RRFRR	J-type (VII)
CK/CH/JL-97 I	1997	Jilin	trachea	RRFRR	Mass-type (I)	CK/CH/GX-09 IV	2009	Guangxi	kidney	HRRRR	LX4-type (IV)
CK/CH/JS-97 I	1997	Jiangsu	kidney	RRSKR	LHLJ/95 I-type (V)	CK/CH/HaN-09 IV	2009	Hainan	kidney	HRRRR	LHLJ/95 I-type (V)
CK/CH/JS-95 III	1995	Jiangsu	kidney	RRFRR	Mass-type (I)	CK/CH/GX-09 V	2009	Guangxi	kidney	HRRRR	LX4-type (IV)
CK/CH/TJ-96 II	1996	Tianjin	kidney	RRFRR	LDT3/03-type (VI)	CK/CH/CQ-09 I	2009	Chongqing	kidney	RRTGR	LDL/97 I-type (IX)
CK/CH/SD-97 I	1997	Shandong	trachea	RRFRR	Mass-type (I)	CK/CH/GX-08 VII	2008	Guangxi	kidney	RRLRR	LDT3/03-type (VI)
CK/CH/SD-97 II	1997	Shandong	kidney	HRRRR	LX4-type (IV)	CK/CH/GX-08 VIII	2008	Guangxi	kidney	HRRRR	J-type (VII)
CK/CH/HeN-96 IV	1996	Henan	kidney	RRFRR	Mass-type (I)	CK/CH/HaN-09 VI	2009	Hainan	kidney	HRRRR	LHLJ/95 I-type (V)
CK/CH/GDGZ-97 I	1997	Guangdong	trachea	RRFRR	J-type (VII)	CK/CH/GX-08 IX	2008	Guangxi	proventriculus	RRFRR	Mass-type (I)
CK/CH/SD-97 IV	1997	Shandong	kidney	RRFRR	LDT3/03-type (VI)	CK/CH/GX-08 X	2009	Guangxi	proventriculus	RRFRR	Mass-type (I)
CK/CH/HLJ-98 I	1998	Heilongjiang	kidney	HRRRR	LHLJ/95 I-type (V)	CK/CH/GD-09 II	2009	Guangdong	trachea	RRFRR	Mass-type (I)
CK/CH/SD-96 III	1996	Shandong	kidney	HRRRR	LX4-type (IV)	CK/CH/GD-09 III	2009	Guangdong	kidney	RRFRR	J-type (VII)
CK/CH/SX-96 I	1996	Shanxi	trachea	RRFRR	Mass-type (I)	CK/CH/GX-09 VI	2009	Guangxi	proventriculus	RRFRR	Mass-type (I)
CK/CH/HeB-96 I	1996	Hebei	trachea	RRFRR	Mass-type (I)	CK/CH/HaN-09 VIII	2009	Hainan	kidney	RRIRR	BJ-type (VIII)
CK/CH/SD-96 VI	1996	Shandong	trachea+muscle	RRSRR	American group (II)	CK/CH/GD-09 IV	2009	Guangdong	kidney	RRFRR	J-type (VII)
CK/CH/AH-97 I	1997	Anhui	kidney	RRSKR	LHLJ/95 I-type (V)	CK/CH/GD-10 I	2010	Guangdong	kidney	HRRRR	LX4-type (IV)
CK/CH/HeN-99 III	1999	Henan	kidney	RRFRR	LDT3/03-type (VI)	CK/CH/GD-10 II	2010	Guangdong	kidney	HRRRR	LX4-type (IV)
CK/CH/XJ-99 II	1999	Xinjiang	trachea+muscle	RRSRR	American group (II)	CK/CH/GD-10 III	2010	Guangdong	kidney	HRRRR	LX4-type (IV)
CK/CH/HaN-95 I	1995	Hainan	trachea	RRSRR	American group (II)	CK/CH/GD-10 IV	2010	Guangdong	kidney	RRFRR	LDT3/03-type (VI)
CK/CH/HaN-95 II	1995	Hainan	trachea	RRFRR	Mass-type (I)						

RRFR: *Arg-Arg-Phe-Arg-Arg,* ***RRIRR:*** *Arg-Arg-Ile-Arg-Arg,* ***RRSKR:*** *Arg-Arg-Ser-Lys-Arg,* ***RRTGR:*** *Arg-Arg-Thr-Gly-Arg,* ***RRHRR:*** *Arg-Arg-His-Arg-Arg,* ***HRRRR:*** *His-Arg-Arg-Arg-Arg,* ***RRSRR:*** *Arg-Arg-Ser-Arg-Arg,* ***RRLRR:*** *Arg-Arg-Leu-Arg-Arg*

Dalian, China). Each region of the S1 nt sequence in each IBV isolate was sequenced in the forward and reverse directions at least five times and the consensus sequence was determined.

Genetic Variability Analysis

Length and homology of S1 nt sequences and their deduced aa residues were analyzed by using MEGALIGN program in DNAStar. Furthermore, the homology, variation and cleavage sites of S1 nt and their aa sequences were determined between 36 reference strains (including 10 vaccine strains and 26 mainly genotypes derived from China) and 63 isolates in this study. Simultaneously, sequence variation characteristics of glycoprotein of 63 isolates were analyzed by comparing to vaccine strain H120.

Phylogenetic Analysis of IBV S1 nt

Comparative analysis of S1 nt sequences between 63 isolates and 233 references strains (including 210 main epidemic strains in mainland China, 10 vaccine strains and 13 IBV representative strains from other countries) was performed, and the phylogenetic tree was obtained by using the neighbour-joining method with 1000 boot-strapping replicates integrated in the MEGA software version 4.1.

RESULTS

Length and Homology Analysis

Length and homology of S1 nt and aa sequence were analyzed in this study. The results showed that the length of S1 nt sequence (from initiation codon ATG to S gene precursor protein cleavage sites) included 1608, 1611, 1617, 1620, 1623, 1626, 1629, 1632 and 1635 nt, and the length of their deduced amino acid residues accordingly were 536, 537, 539, 540, 541, 542, 543, 544 and 545 aa.

Homology of the S1 nt sequences and their deduced aa residues between 63 isolates ranged from 75.5% to 99.9% and 72.5% to 100%, respectively, the similarity of S1 nt sequences and their deduced aa residues between 99 strains (including 63 isolates in this study plus the 36 reference strains) were from 74.6% to 99.9% and 71.7% to 100%, respectively. It indicated low homology and high sequence variation due to nt point mutations, insertions and deletions on S1 sequences.

Cleavage Sites and Mutation Analysis

Analysis of S1 nt and their deduced aa sequences between 63 isolates and 36 reference strains indicated that the variant regions focused mainly on S1 nt 5' tail end, which were identical to the published data. Ten kinds of S1/S2 cleavage sites could be found in these 99 IBV strains, and the cleavage site sequences were RRFRR (29/44), RRIRR (3/4), RRSKR (3/3), RRTGR (4/6), RRHRR (2/2), HRRRR (17/22), RRSRR (4/14), RRLRR (1/1), HRFRR (0/1) and HRSRR (0/2), respectively (numerator, number of cleavage sites in IBV isolates; denominator, total number of cleavage sites in IBV isolates and reference strains). Most of the isolates had S1/S2 cleavage sites, RRFRR, HRRRR (46/63). The cleavage site RRFRR was common for Mass genotype (24/25), RRIRR for BJ genotype strains (4/4), RRTGR for LDL/97 I genotype strains (6/6), and HRRRR for LX4 genotype strains (13/13). Six cleavage sites RRIRR, RRSKR, RRTGR, RRHRR, HRRRR and RRLRR were found only in mainland China *(Table 1)*. Some strains with high gene similarity had the same cleavage sites while some strains lied in same genotype had different cleavage sites, indicating that cleavage sites played a limited role in the IBV S1 nt sequence typing.

Compared to vaccine strain H120, S1 nt sequences of 63 isolates had four regions, which were inserted or deleted frequently and located between nt 64-75, 210-214, 355-358 and 417-418 (numbered according to the S1 sequence of H120 strain), respectively. S1 protein of 63 isolates had three hypervariable regions and had 7 aa deletion or insertion mainly among residues 3-25, 52-154 and 266-294. Behind the residue 24, aspartic acid (N) was inserted in 12 isolates and serine (S) was deleted in four isolates. Amino acids (NYTNGNSD) were inserted between residues 71 and 72 in 18 isolates and 5-6 aa were inserted in the site of residue 141 in four isolates. Amino acid (KKSVVGPSD) were inserted behind the residue 138 and SD was inserted behind the residue 141 in IBV isolate GX-98 I, which was consistent with international reference strain Holte with classical amino acid sequence FKKKSVVGPSD after residue 138.

Phylogenetic Analysis of S1 nt between 63 IBV Isolates and 233 Reference Strains

Sixty-three IBV isolates and 233 reference strains were divided into two groups by analyzing S1 nt sequences. One group had 49 strains including 20 IBV isolates, 10 vaccine strains, seven representative strains isolated from other countries and 12 reference strains in China. The second group had 247 strains including 43 isolates, six international represent strains isolated from other countries and 198 reference strains in China *(Fig. 1)*.

In this research, all IBV strains could be divided into 11 genotypes by homology analysis of S1 nt sequences. Sixty-three isolates were clustered into eight genotypes, including genotype I (Mass-type), genotype II (American type), genotype IV (LX4 type), genotype V (LHLJ/95 I type), genotype VI (LDT3/03 type), genotype VII (J type), genotype VIII (BJ type) and genotype IX (LDL/97 I type) respectively while they did not appeared in genotype III (4/91 type), genotype X (LGD04/III type) and genotype XI (Taiwan group). Sixteen isolates belonged to genotype I, 10 isolates were genotype VII, 9 isolates lied in genotypes VI, respectively, and eight isolates existed in genotype V? The proportion of the five kinds of genotypes to all isolates was 82.5%, which was a little close to mainland China

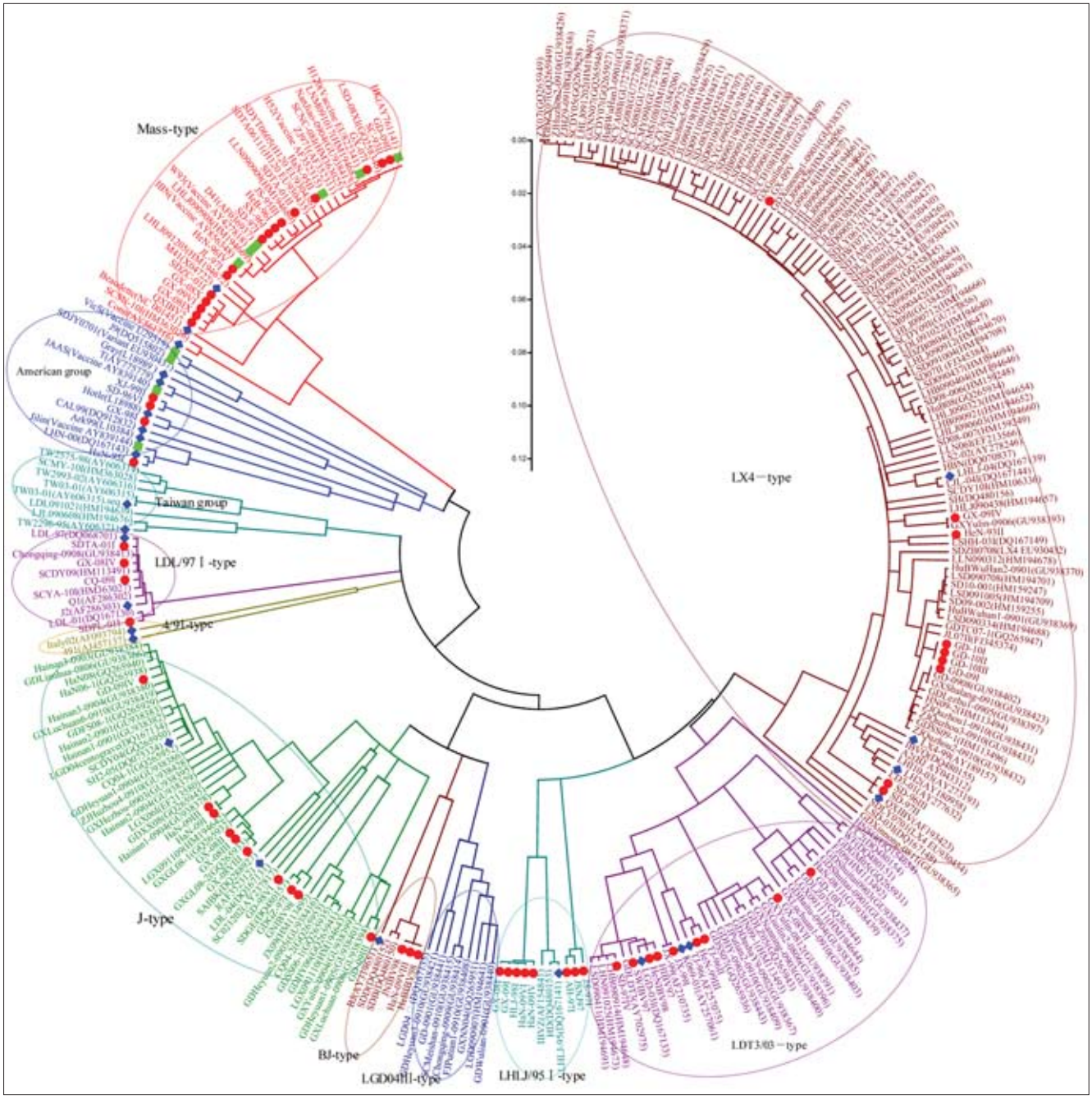

Fig 1. The phylogenetic tree constructed based on IBV S1 nucleotide sequences by N-J method
"●" IBV isolate strains, "■" vaccine strains "◆" international represent strains

Sekil 1. N-J metodu kullanılarak IBV S1 nükleotid sekanslarına dayanarak oluşturulan filogenetik ağaç
"●" IBV izolat suşlar, "■" aşı suşlar "◆" uluslararası tanımlı suşlar

reference strains (87.6%) *(Table 1, Fig. 2)*. It was noteworthy that the proportion of genotype V (12.7%) was much higher than that of the reference strains (1.4%) in mainland China. It all suggested that the genotype of the virus had new changes.

DISCUSSION

Infectious bronchitis (IB) has become one of the most common diseases to outbreaks frequently and persistently in commercial chicken farms of China. The disease is difficult to be controlled by IBV vaccines, which have been used widely in different chicken farms, because of the rapid circulation and complicated evolution of IBV strains. The chicken flocks have potential danger of IB outbreak when the serotype/genotype of vaccine strains are inconsistent to infective strains [20-24]. Therefore, it is important to choose a suitable IBV candidate vaccine strain to prevent the disease based on profound understanding of IBV epidemiology in China. In this work, we examined the epidemiology and

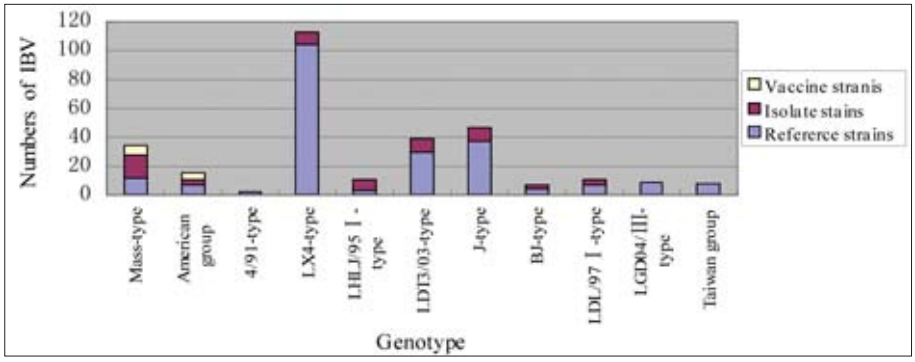

Fig 2. The gene quantitative statistics of isolates, vaccine and reference strains genotypes based on IBV S1 nucleotides

Şekil 2. IBV S1 nükleotidlerine dayanarak oluşturulan izolatlar, aşı ve referans genotiplerinin gen kuantitatif istatistikleri

evolutionary characteristics of IBV by analyzing S1 nt of 63 IBVs isolated from 16 geographic provinces in China over an 18-year period from 1993 to 2010. Our results showed that the S1 nt and their aa had significant characteristics with low homology and high variation between the isolates and reference strains, indicating that new changes have taken place in IBV genotypes circulating in China.

The previous studies suggested that site mutation, insertion and deletion in S1 nt sequence were very important factors resulting in poor immune effect of conventional IBV vaccine [11,26]. In order to learn S1 nt of IBV isolated from China, we analyzed the differences in S1 nt sequences of 63 isolates and vaccine strain H120, which belonged to Mass serotype. Our study found that, compared to vaccine strain H120, S1 nt of 63 isolates had four regions, which were inserted or deleted frequently between nt 64-75, 210-214, 355-358 and 417-418, respectively. In addition, the S1 protein of 63 isolates had three hypervariable regions between aa 3-25, 52-154 and 266-294, which was consistent with the published data [13,26-28]. Differences on S1 nt sequences suggest that vaccine contained H120 strain has limited protection on chickens.

IBV S protein has a proteinase cleavage site; it can be cleaved into S1 and S2 subunits when the virus particles are replicating [11,13]. Our investigations showed that S protein of 63 isolates and 36 reference strains had 10 kinds of different cleavage sites, among which RRFRR and HRRRR were prominent, and the cleavage sites HRRRR, RRIRR, RRHRR, RRLRR, RRSKR and RRTGR only existed in strains coming from China. This result revealed that the IBV lied in continuing change status in China, but the role of these cleavage sites to virus pathogenicity and vaccine immunity was still uncertain. Highly similar IBV S1 proteins often had the same cleavage sites [11,13], but we found that some IBVs possessed different cleavage sites though they were laid in the same genotype, implying that genotyping of IBV by S1 cleavage sites has limited application. Just as Jackwood et al.[29] reported, IBV genotype can not only be decided by cleavage sites and pathogenicity and tissue tropism.

The 63 Chinese IBV isolates and 233 reference strains had been divided into two groups by analysis of the homology of S1 nt. We found that one group had 49 strains including 20 IBV isolates, 10 vaccine strains, seven representative strains isolated from other countries and 12 Chinese reference strains, and the other group had 247 strains including 43 IBV isolates, six international representative strains isolated from other countries and 198 Chinese reference strains. All the strains were divided into 11 genotypes, and the 63 isolates came from eight different genotypes. Han et al.[20] reported that they found 13 IBV variant strains, making the IBV epidemiology more intricate. Moreover, we could not find IBV variant strains by analyzing isolates sequence. The reason most probably was that we used different reference strains.

At the present time, only the Mass type vaccine has been permitted to be used in the market in China, which had controlled the disease effectively [11], but IB still broke out in some chicken flocks though they had been immunized with Mass type IBV vaccine, causing chicken death or deterioration in the quality of eggs. The investigations in IBV isolated from immunized chickens discovered that immunity effect declined partly owing to the genetic variation in the process of IBV evolution [7,22,23,25,30]. We found that more than 40% IBVs belonged to LX4 type, which was circulating prevalently in chicken flocks in China, the result corresponded with the reported literature [20,22,30]. The study also indicated that IBV S1 gene of LX4 type had point mutation, insertion and deletion, probably because virus evolved in different regions. Compared to Mass type vaccine strains, the homology of nt and aa sequences of the field strain of LX4 type derived from China were less than 78.8% and 78.3%, respectively, which helps to explain the failure of the vaccine. As the second and third major types of IBV circulating in chicken flocks in China, the homology of aa sequences of J and LDT3/03 types were less than 82% compared to Mass type vaccine strains.

It should be noted that Mass type IBV can be isolated frequently and be considered as the fourth type to appear in chicken flock even though the Mass type IBV vaccine has been used for several years in China. A similar situation also appeared in other countries [31]. The vaccine virus was isolated frequently from chicken flocks, The reason maybe the wide use of attenuated vaccine in farms. This study discovered that the homology of nt and aa

sequences of isolates and vaccine strains were more than 94.6%, and S1 nt of most isolates had point mutations and deletions compared to vaccine strain. It was reported by Cavanagh et al that a small change of IBV S1 glycoprotein could result in a change in virus serotype [11]. Amino acid substitutions might be caused by immune pressure due to comprehensive use of vaccines. Therefore, it can partially explain why IBV still broke out and circulated in chicken flocks immunized or not with Mass type vaccine. Other IBV genotypes such as BJ, LDL/97 I? and LGD04/III? spread widely around the world except for four genotypes LX4, J, LDT3/03 and Mass described above. It indicated that new variant strains might be generated by gene recombination from different IBV genotypes, bringing enormous pressures for IBV control and prevention.

As serological tests (virus neutralization test and immune poisoning test) serve as effective tools for determining the exact protective effect of the vaccine strains to field virus, deep sequence analysis of IBV S1 becomes necessary to predict the efficiency of vaccine. However, due to the diversity of IBV serotypes and genotypes, it is particularly important to re-screen immune effects of Mass type vaccine strains and re-assess and analyze IBV isolates on the basis of laboratory and field tests.

Acknowledgments

This work was supported by a grant from the National Natural Science Foundation of China (31072319, 31372412) and the Specialized Research Fund for the Doctoral Program of Higher Education of China (20124404110016).

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