Genomic Characteristics and Changes of Avian Infectious Bronchitis Virus Strain CK/CH/LDL/97I after Serial Passages in Chicken Embryos

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Abstract

Background: We previously attenuated the infectious bronchitis virus (IBV) strain CK/CH/LDL/97I and found that it can convey protection against the homologous pathogenic virus. ***Objective:*** To compare the full-length genome sequences of the Chinese IBV strain CK/CH/LDL/97I and its embryo-passaged, attenuated level to identify sequence substitutions responsible for the attenuation and define markers of attenuation. ***Methods:*** The full-length genomes of CK/CH/LDL/97I P5 and P115 were amplified and sequenced. The sequences were assembled and compared using the MEGALIGN program (DNAStar) and a phylogenetic tree was constructed using MEGA4 software. ***Results:*** The CK/CH/LDL/97I virus population contained subpopulations with a mixture of genetic mutants. Changes were observed in nsp4, nsp9, nsp11/12, nsp14, nsp15, nsp16, and ORF3a, but these did not result in amino acid substitutions or did not show functional variations. Amino acid substitutions occurred in the remaining genes between P5 and P115; most were found in the S region, and some of the nucleotide mutations resulted in amino acid substitutions. Among the 9 nsps in the ORF1 region, nsp3 contained the most nucleotide substitutions. ***Conclusions:*** Sequence variations in different genes, especially the S gene and nsp3, in the genomes of CK/CH/LDL/97I viruses might contribute to differences in viral replication, pathogenicity, antigenicity, immunogenicity, and tissue tropism.

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Introduction

Coronaviruses (CoVs) infect a variety of animals and can cause respiratory, enteric, hepatic and neurological diseases of varying severities. CoVs were recently divided into four genera based on genotypic and serological characterizations: alpha, beta, gamma, and delta. Infectious bronchitis virus (IBV) is a *Gammacoronavirus* of the subfamily *Coronavirinae*, family *Coronaviridae*, order *Nidovirales* [1]. IBV is distributed worldwide, it is highly infectious, and extremely difficult to control because of its extensive genetic diversity, short generation time, and high mutation rate. Although live-attenuated infectious bronchitis (IB) vaccines have been used worldwide since the 1950s, IBV continues to cause disease in chickens, even in vaccinated birds, because the virus is constantly changing

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and evolving to avoid the host immune response. IBV can cause respiratory disease in chickens of all ages [2]. Some strains are nephropathogenic, resulting in renal-induced mortality of 25–80% in susceptible flocks [3]. Other strains can replicate in the oviduct and cause permanent damage in immature females or pullets resulting in limited egg production in mature hens [2].

CoVs are enveloped viruses that replicate in the cell cytoplasm and contain an unsegmented, single-stranded, positive-sense RNA genome of 28–32 kb, with a 5′ cap and a 3′ polyA tail [4]. Like all CoVs, the proximal two thirds of the IBV genome encode two overlapping open reading frames (ORFs), 1a and 1b, which are proteolytically cleaved by two virus-encoded replicase proteins, the papain-like and 3C-like proteinases, into at least 15 nonstructural proteins (nsp2–16) [4]. Replication of the CoV RNA genome and transcription of a nested set of 5–8 subgenomic RNA species are carried out by these proteinases. However, the exact functions of individual replicases remain largely unknown. The remaining third of the IBV genome encodes four major structural proteins: the spike (S) glycoprotein, the small envelope (E) protein, the membrane (M) glycoprotein, and the nucleocapsid (N) protein. Two small accessory genes, genes 3 and 5, have been described in IBV, which express accessory proteins 3a, 3b and 5a, 5b, respectively [3, 5, 6]. In addition, the 5′ and 3′ untranslated regions (UTRs) in the IBV genome, like those of other CoVs, usually harbor important structural elements involved in replication and/or translation [7–11].

IB is mainly controlled by the use of live-attenuated vaccines derived from virulent viruses prevalent in a particular country or region by multiple serial passages in 9- to 11-day-old embryonated chicken eggs [12–16]. As a consequence of this process, the virus becomes more adapted and more highly pathogenic to the embryo, with concomitant attenuation in mature chickens. The IBV strain Beaudette was attenuated after several hundred passages in embryonated hen's eggs [17] resulting in loss of virulence and also potential loss of immunogenicity. A similar result was observed in IBV strain CK/CH/LHLJ/04V, the immunogenicity of which was substantially decreased after 110 passages in embryonated hen's eggs, resulting in loss of virulence to chickens [16]. However, most attenuated IB vaccine strains, such as H120, maintain their immunogenicity after adaptation to embryonated chicken eggs [12–15], though the genomic mutations in passaged strains associated with attenuation of pathogenicity, immunogenicity, tissue tropism, and replication capacity in chicken tissues are unknown and

variable, leading to differing efficacies associated with different vaccines.

A novel type of IBV, designated as CK/CH/LDL/97I, emerged in the mid-1990s and circulated among both vaccinated and non-vaccinated chicken flocks in China, causing 100% morbidity and about 10% mortality in young infected chicks. In recent years, this type of IBV has also been found in Taiwan [18] and regions of the Middle East, resulting in severe losses to both the layer and broiler industries [19]. Both experimental and field studies suggested that vaccination using available commercial vaccines provided poor protection against this type of virus, emphasizing the need for the development of an efficacious, live-attenuated vaccine against CK/CH/LDL/97I to prevent and control this type of IBV [20]. We previously attempted to develop an IB vaccine by serial passage of the IBV strain CK/CH/LDL/97I in embryonated eggs [21]. Although the vaccination challenge test showed that the attenuated P115 virus could provide complete protection against the homologous pathogenic parent virus P5, in terms of clinical response and virus recovery in the trachea and kidney [20, 21], the genomic changes in the pathogenic parental CK/CH/LDL/97I strain and its attenuated virus, and the genomic changes associated with attenuation of pathogenicity, immunogenicity, tissue tropism, and replication capacity after serial passages remain unknown.

Although it is possible to study the evolution of viruses and the subsequent impact on viral characteristics by comparing genomic sequences between heterologous strains, analysis of homologous strains provides a unique opportunity to elucidate the specific genes or gene sites involved in viral characteristics, such as pathogenicity, immunogenicity, and tissue tropism. To identify specific sequence changes responsible for adaptation of the field IBV isolate to chicken embryonic tissue and subsequent attenuation, the whole viral genome of the IBV P5 pathogenic parental CK/CH/LDL/97I strain was sequenced and compared with the attenuated, P115 level virus, to provide a better understanding of the relationship between the genomic differences and related characteristics of the pathogenic and attenuated viruses.

Materials and Methods

Eggs and Viruses
Fertile White Leghorn specific pathogen-free (SPF) chicken eggs were obtained from the Laboratory Animal Center, Harbin Veterinary Research Institute, the Chinese Academy of Agricultural Sciences, Harbin, China.

The pathogenic IBV strain CK/CH/LDL/97I (P5), previously characterized as belonging to the CK/CH/LDL/97I type [21], and its embryo-passaged, attenuated virus (P115) [20] were used in this study. CK/CH/LDL/97I was isolated in 1997 from the swollen proventriculi of broiler chickens and propagated following standard procedures at 37° in the chorioallantoic cavities of 9- to 11-day-old SPF embryonated eggs. This attenuated virus can provide complete protection against challenge of the parental CK/CH/LDL/97I strain according to clinical response and virus recovery in tracheas and kidneys [20, 21].

The virus stocks of CK/CH/LDL/97I P5 and P115 were produced by inoculating embryonated SPF chicken eggs via the allantoic cavity with the virus, and by collecting the infectious allantoic fluid 72 h after inoculation, respectively, as described previously [21]. Each virus was identified by electron microscopy, reverse transcriptase-polymerase chain reaction (RT-PCR), and sequencing of the entire S1 protein gene, as described previously [21], before aliquoting and storage as virus stocks at –80°. The infectious virus titer was determined by inoculation of SPF embryonated eggs with 10-fold serial dilutions and measured as embryo infectious doses of 50% (EID_{50}) following the Reed and Muench method [22].

Cloning and Sequencing of CK/CH/LDL/97I P5 and P115 Genomes

Viral RNA was extracted from 200 μl of CK/CH/LDL/97I P5 and P115 virus stocks, respectively, using TRIzol reagent (Invitrogen, Grand Island, N.Y., USA) according to the manufacturer's instructions. RNA was air-dried for 2–10 min, re-dissolved in 30 μl of RNase-free water, and stored at –70° for further use. The initial results (data not shown) revealed that primers N (–) contained sequences consistent with those of CK/CH/LDL/97I, and reverse transcription (RT) was therefore performed with M-MLV reverse transcriptase (Invitrogen) using the primer N (–). RT procedures were performed using 40 μl of RNA in an 80-μl reaction volume, as previously described [16]. Each cDNA fragment was PCR-amplified from the RT products, as described previously [16]. PCR products were purified from agarose gels using a DNA extraction kit (Boehringer Mannheim GmbH, Mannheim, Germany) and cloned into the pMD18-T vector (TaKaRa Bio, Inc., Dalian, China), following the manufacturer's instructions.

Fourteen pairs of overlapping primers were used for PCR amplification. The sequences and locations of the primers used in this study are listed in online supplementary table 1 (for all online suppl. material, see www.karger.com/doi/10.1159/000365193). RNA extraction, cDNA generation, PCR amplification, and gene-fragment cloning and sequencing were conducted independently for each of the two passages [16]. The 3′ and 5′ ends of the viral genomes were confirmed by rapid amplification of cDNA ends (RACE) using a 3′/5′ RACE kit (TaKaRa Bio, Inc.), according to the manufacturer's instructions. PCR products were purified from agarose gels using a DNA extraction kit (Boehringer Mannheim GmbH) and cloned into the pMD18-T vector (TaKaRa Bio, Inc.), following the manufacturer's instructions. A total of five clones of each gene fragment were selected and sequenced for each of the passages to exclude errors due to RT and PCR reactions. Sequences were assembled using the MEGALIGN program (DNAStar, Madison, Wis., USA) and edited manually to produce final sequences of the viral genomes.

In addition, another 15 overlapping pairs of primers covering the full-length genome of IBV were designed (online suppl. table 1) based on the conserved genomic nucleotide consensus sequences of most published IBVs, and were used to facilitate the sequence validation of CK/CH/LDL/97I P5 and P115 by conventional one-step RT-PCR using the One Step PrimeScript™ RT-PCR kit (TaKaRa Bio, Inc.), according to the manufacturer's instructions. The viral stocks used in each RT-PCR assay were from independently inoculated embryos. PCR products were also purified from agarose gels using a DNA extraction kit (Boehringer Mannheim GmbH) and cloned into the pMD18-T vector (TaKaRa Bio, Inc.), following the manufacturer's instructions. Five clones of each gene fragment were selected and sequenced for P5 and P115.

Determination and Sequence Analysis of the Genome Sequence

The nucleotide and amino acid sequences of the entire IBV CK/CH/LDL/97I P5 and P115 genomes were assembled, aligned, and compared with those of other reference IBV strains using the MEGALIGN program. ORFs 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, Wis., USA). A total of 55 IBV reference strains for which the entire genomic sequences were available in the GenBank database (www.ncbi.nlm.nih.gov/genbank) were selected for phylogenetic analysis using the neighboring-joining method in MEGA4 software (www.megasoftware.net) at a bootstrap value of 1,000 replicates. The selected avian CoV reference strains and their accession numbers are shown in online supplementary table 2.

In addition, accurate estimation and comparison of the nucleotide sequences of the entire genomes of IBV CK/CH/LDL/97I P5 and P115 were conducted using the ClustalW algorithm (www.clustal.org). All sequences were edited manually and adjusted for errors. Mutations and insertions were determined according to the results of pairwise comparisons.

Accession Numbers

The genomic sequences of IBV strains CK/CH/LDL/97I P5 and P115 were submitted to the GenBank database and assigned the accession numbers JX195177 (CK/CH/LDL/97I P5) and JX195178 (P115).

Results

IBV CK/CH/LDL/97I Showed Close Relationship with Delaware-Type IBV Strains

To investigate substitutions in the nucleotide and/or amino acid sequences during passage, P5 and P115 were subjected to entire genomic amplification and sequence analysis by RT-PCR. All RT-PCR products were analyzed and a single band of the expected size was visualized after ethidium bromide staining of the products on a 1.0% agarose gel. Five independent clones of each fragment from two independent RT-PCRs (in total, the entire genomes of each passage were sequenced 10 times) were selected and sequenced, thus the sequence profiles represented all

Table 1. Nucleotide and amino acid substitutions identified in IBV CK/CH/LDL/97I P5 compared to the embryo-passaged, attenuated P115 strain

IBV gene		Genome position[a]	Position in gene[b]	Nucleotide change	Codon change	Amino acid substitution
5′-UTR		53	–	T→C	–	–
		93	–	C→T	–	–
ORF1	nsp1/2	837	309	T→C	GTT→GTC	Val→Val
		1160	632	C→T	GCA→GTA	Ala→Val
		1700	1172	T→C	GTT→GCT	Val→Ala
		2263	1735	C→T	CTT→TTC	Leu→Phe
	nsp3	2685	138	A→A(7)/G(3)	GAA→GAA(7)/GAG(3)	Glu→Glu
		5073	2526	T→T(6)/G(4)	CAT→CAT(6)/CAG(4)	His→His(6)/Gln(4)
		5261	2714	G→A	GGA→GAA	Gly→Glu
		5331	2787	C→T(6)/C(4)	GAC→GAT(6)/GAC(4)	Asp→Asp
		6480	3936	T→T(7)/C(3)	CCT→CCT(7)/CCC(3)	Pro→Pro
		6503	3959	A→A(7)/G(3)	GAA→GAA(7)/GGA(3)	Glu→Gly
		7204	4660	T→T(7)/C(3)	TGT→TGT(7)/CGT(3)	Cys→Cys(7)/Arg(3)
	nsp4	8331	1005	G(7)/T(3)→T	TGG(7)/TGT(3)/TGT	Try(7)/Cys(3)→Cys(3)
	nsp5	–	–	–	–	–
	nsp6	–	–	–	–	–
	nsp7	–	–	–	–	–
	nsp8	–	–	–	–	–
	nsp9	11684	137	C(7)/T(3)→C	TCA(7)/TTA(3)→TCA	Ser(7)/Leu(3)→Leu
	nsp10	–	–	–	–	–
	nsp11/ 12	12669	354	G(7)/A(3)→G(7)/A(3)	ATG(7)/ATA(3)→ATG(7)/ATA(3)	Met(7)/Ile(3)→Met(7)/Ile(3)
		13143	828	C→C(7)/T(3)	CTC→CTC(7)/CTT(3)	Leu→Leu
		13201	886	T(7)/C(3)→T	TTA(7)/CTA(3)→TTA	Leu→Leu
		13312	997	C(7)/T(3)→C	CTT(7)/TTT(3)→CTT	Leu(7)/Phe(3)→Leu
		13581	1266	C→A(7)/C(3)	GTC→GTA(7)/GTC(3)	Val→Val
		13875	1560	T→G(7)/T(3)	AAT→AAG(7)/AAT(3)	Asn→Lys(7)/Asn(3)
		14025	1710	C(7)/T(3)→T(7)/C(3)	TCC(7)/TCT(3)→TCT(7)/TCC(3)	Ser→Ser
	nsp13	15713	579	G→T(7)/G(3)	GTG→GTT(7)/CTG(3)	Val→Val
		15824	690	C(7)/T(3)→C	CAC(7)/CAT(3)→CAC	His→His
		15859	725	T→C	CTA→CCA	Leu→Pro
		16255	1121	A(7)/G(3)→A	GAT(7)/GGT(3)→GAT	Asp(7)/Gly(3)→Asp
		16306	1172	C(7)/T(3)→C	ACA(7)/ATA(3)→ACA	Thr(7)/Ile(3)→Thr
		16879	1745	G(7)/A(3)→A	AGT(7)/AAT(3)→AAT	Ser(7)/Asn(3)→Asn
		16919	1785	T(7)/A(3)→T	AGT(7)/AGA(3)→AGT	Ser(7)/Arg(3)→Ser
	nsp14	18086	1152	G(7)/A(3)→G	GTG(7)/GTA(3)→GTG	Val→Val
		18200	1266	C→C(7)/T(3)	CAC→CAC(7)/CAT(3)	His→His
		18318	1384	C→T(7)/C(3)	CTA→TTA(7)/CTA(3)	Leu→Leu
	nsp15	18713	216	G(7)/A(3)→G	CTG(7)/CTA(3)→CTG	Leu→Leu
		18772	275	C(7)/T(3)→T	ACA(7)/ATA(3)→ACA	Thr(7)/Ile(3)→Thr
		18881	384	A(7)/C(3)→A(5)/C(5)	GCA(7)/GCC(3)→GCA(5)/GCC(5)	Ala→Ala
		19294	797	A→A(7)/G(3)	CAA→CAA(7)→CGA	Gln→Gln(7)/Arg(3)
		19356	859	C→C(7)/T(3)	CTG→CTG(7)/TTG(3)	Leu→Leu
	nsp16	20309	798	G→A	AAG→AAA	Lys→Lys
		20351	840	C→C(7)/T(3)	AAC→AAC(7)/AAT(3)	Asn→Asn
		20415	904	A→G(7)/A(3)	ATG→GTG(7)/ATG(3)	Met→Val(7)/Met(3)

Table 1 (continued)

IBV gene		Genome position[a]	Position in gene[b]	Nucleotide change	Codon change	Amino acid substitution
ORF2 (S gene)	S1	20415	45	A→G(7)/A(3)	CTA→CTG(7)/CTA(3)	Leu→Leu
		20717	347	A→ G(7)/A(3)	AAA→AGA(7)/AAA(3)	Lys→Arg(7)/Lys(3)
		20725	355	G→A(7)/G(3)	GAC→AAC(7)/GAC(3)	Asp→Asn(7)/Asp(3)
		20954	584	C→A(7)/C(3)	ACA→AAA(7)/ACA(3)	Thr→Lys(7)/Thr(3)
		21014	644	T→C(7)/T(3)	GTC→GCC(7)/GTC(3)	Val→Ala(7)/Val(3)
		21112	742	C→T	CCT→TCT	Pro→Ser
		21415	1045	C(7)/T(3)→T(7)/C(3)	CTT(7)/TTT(3)→TTT(7)/CTT(3)	Leu(7)/Phe(3)→Phe(7)/Leu(3)
		21544	1174	G(7)/C(3)→G(7)/C(3)	GAG(7)/CAG(3)→GAG(7)/CAG(3)	Glu(7)/Gln(3)→Glu(7)/Gln(3)
		21548	1178	C→T	TCA→TTA	Ser→Leu
		21584	1214	T(7)/G(3)→T	GTG(7)/GGG(3)→GTG	Val(7)/Gly(3)→Val
		21852	1482	C→C(7)/T(3)	CCC→CCC(7)/CCT(3)	Pro→Pro
	S2	22008	1638[c]	G(7)/T(3)→T(7)/G(3)	AAG(7)/AAT(3)→AAT(7)/AAG(3)	Lys(7)/Asn(3)→Asn(7)/Lys(3)
		22045	1675	T→C	TGT→CGT	Cys→Arg
		22104	1734	G(7)/A(3)→G	GTG(7)/GTA(3)→GTG	Val→Val
		22178	1808	T→C	ATA→ACA	Ile→Thr
		22181	1811	A(7)/G(3)→A	CAA(7)/CGA(3)→CAA	Gln(7)/Arg(3)→Gln
		22238	1868	T(7)/C(3)→T	ATT(7)/ACT(3)→ATT	Ile(7)/Thr(3)→Ile
		22316	1946	G(7)/A(3)→G	AGG(7)/AAG(3)→AGG	Arg(7)/Lys(3)→Arg
		22470	2100	T(7)/G(3)→T	ATT(7)/ATG(3)→ATT	Ile(7)/Tyr(3)→Ile
		22510	2140	G(7)/C(3)→G	GCT(7)/CCT(3)→GCT	Ala(7)/Pro(3)→Ala
		22640	2270	C(5)/T(5)→C	TCT(7)/TTT(5)→TCT	Ser(5)/Phe(5)→Ser
		22868	2498	G(7)/A(3)→G	AGT(7)/AAT(3)→AGT	Ser(7)/Asn(3)→Ser
		22922	2552	T→C	TTA→TCA	Leu→Ser
		23396	3026	T(5)/C(5)→T	TTT(5)/TCT(5)→TTT	Phe(5)/Ser(5)→Phe
		23526	3156	T(7)/C(3)→T	GAT(7)/GAC(3)→GAT	Asp→Asp
		23742	3372	C→T	TGC→TGT	Cys→Cys
ORF3	3a	24006	136	T(7)/C(3)→T	TTT(7)/TTC(3)→TTT	Phe→Phe
	3b	–	–	–	–	–
	3c (E gene)	24402	184	C→T	GCC→TCC	Ala→Ser
ORF4 (M gene)		24563	44	C(7)/T(3)→C	GCG(7)/GTG(3)→GCG	Ala(7)/Val(3)→Ala
		24747	228	C→C(7)/T(3)	GAC→GAC(7)/GAT(3)	Asp→Asp
		24951	432	C→C(7)/T(3)	TCC→TCC(7)/TCT(3)	Ser→Ser
		25186	667	G→A	GGT→AGT	Gly→Ser
M-ORF5		25470→25471[c]		→TA(Insertion)	–	–
		25547→25549		AGA→AGA(5)/CTT(5)	–	–
ORF5	5a	–	–	–	–	–
	5b	25901	146	A→G	CAA→CGA	Gln→Arg
		25927	172	T→G	TAT→GAT	Tyr→Asp
ORF6 (N gene)		26344	398	A→T	GAT→GCT	Asp→Ala
		26866	920	C(7)/A(3)→C	CCA(7)/CAA(3)→CCA	Pro(7)/Gln(3)→Pro
3′-UTR		27406	–	A→G(7)/A(3)	–	–
		27482	–	G→T	–	–
		27506	–	G→T	–	–

[a] Based on the sequence of the IBV CK/CH/LDL/97I P5 genome, GenBank accession No. JX195177.
[b] Position of nucleotides in each gene.
[c] A 2-bp sequence, TA, was inserted in CK/CH/LDL/97I P115, compared to that of the P5 sequence, in the position between the M gene and ORF5. The position of the 2-bp sequence in CK/CH/LDL/97I P115 is shown.

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CK/CH/LDL/97I P5    ATGGTGTGTGT GTAGAGAATA TTTAAAATTA TTCTTTGACA GTGCCTCTGT  50
CK/CH/LDL/97I P115   ATGGTGTGTGT GTAGAGAATA TTTAAAATTA TTCTTTGACA GTGCCTCTGT  50

CK/CH/LDL/97I P5    TTTAAGAGCG CGGAAGAGTA TTATTTTTGA GGATATTAAT ATAAATCCTC  100
CK/CH/LDL/97I P115   TTTAAGAGCG CGGAAGAGTA TTATTTTTGA GGATATTAAT ATAAATCCTC  100

CK/CH/LDL/97I P5    TCTGTTTTAT ATTATCTTTT CAAGAGCTAT TATTTAAAAA ACAGTTTTTC  150
CK/CH/LDL/97I P115   TCTGTTTTAT ATTATCTTTT CAAGAGCTAT TATTTAAAAA ACAGTTTTTC  150

CK/CH/LDL/97I P5    CACCCTTTTG TGCCAAAAAC TATTGTTGTT AATGGTGTAA CCTTTCAAGT  200
CK/CH/LDL/97I P115   CACCCTTTTG TGCCAAAAAC TATTGTTGTT AATGGTGTAA CCTTTCAAGT  200

CK/CH/LDL/97I P5    AGATAATGGA AAAGTCTACT ATGAAGGAAA ACCCATTTTT CAGAAAGGTT  250
CK/CH/LDL/97I P115   AGATAATGGA AAAGTCTACT ATGAAGGAAA ACCCATTTTT CAGAAAGGTT  250

CK/CH/LDL/97I P5    GTTGTAGGTT GTGGTCCCAT --TAAGAGGG ATTAGAAATAG TTAAACCACC  298
CK/CH/LDL/97I P115   GTTGTAGGTT GTGGTCCCAT TATAAGAGGG ATTAGAAATAG TTAAACCACC  300

CK/CH/LDL/97I P5    AACAACAATC TCTTGTGTTA GAGGTGTTTG GGTTTACAAG CGCTTAAAAG  348
CK/CH/LDL/97I P115   AACAACAATC TCTTGTGTTA GAGGTGTTTG GGTTTACAAG CGCTTAAAAG  350

CK/CH/LDL/97I P5    AAACAAATAC GGA                                          361
CK/CH/LDL/97I P115   AAACAAATAC GGA                                          363
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a

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CK/CH/LDL/97I P5    MCVCREYLKLFFDSASVLRARKSIIFEDININPLC

CK/CH/LDL/97I P115   MCVCREYLKLFFDSASVLRARKSIIFEDININPLC

CK/CH/LDL/97I P5    FILSFQELLFKKQFFHPFVPKTIVVNGVTFQVDNG

CK/CH/LDL/97I P115   FILSFQELLFKKQFFHPFVPKTIVVNGVTFQVDNG

CK/CH/LDL/97I P5    KVYYEGKPIFQKGCCRLWSH#

CK/CH/LDL/97I P115   KVYYEGKPIFQKGCCRLWSHYKRD@
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b

Fig. 1. Multiple nucleotide (**a**) and amino acid (**b**) sequence alignments of the intergenic UTRs of IBV CK/CH/LDL/97I P5 and P115. The lengths of the intergenic UTR from P5 and P115 were 361 and 363 bp in length, respectively. The initiation and stop codons are underlined. The two nucleotide insertions in P115 are boxed.

the genetic diversity within the populations of the viral RNAs from a given passage.

The dominant sequences of both CK/CH/LDL/97I P5 and P115 were assembled into one contiguous sequence to represent the entire viral genome, irrespective of the subpopulations. Sequences of 27,680 and 27,682 nucleotides were obtained from CK/CH/LDL/97I P5 and P115, respectively, excluding the polyadenylation tail at the 3′ end. P115 had two nucleotide insertions in the intergenic UTR between M and gene 5, compared to P5 (table 1; fig. 1a, b). The genomes of both viruses were similar in terms of overall coding capacity and genomic organization to those of other IBVs. The genome organization of CK/CH/LDL/97I was as follows: 5′-UTR-gene 1 (ORF1a, 1b)-S-gene 3 (ORF3a, 3b, E)-M-gene 5 (ORF5a, 5b)-N-UTR-3′.

Phylogenetic reconstruction of the whole genomes of 57 IBVs using maximum likelihood and maximum parsimony produced well-supported trees (fig. 2) that placed CK/CH/LDL/97I P5/P115 with the Delaware-type and other IBV types isolated in the USA, which demonstrated a close genetic relationship between CK/CH/LDL/97I

and the US strains. However, other Chinese IBV strains, the Massachusetts type, and West Africa IBV isolates were classified in separate groups.

Substitutions in Genomic Sequence of CK/CH/LDL/97I after Serial Passage

Subpopulations were identified in both CK/CH/LDL/97I P5 and P115, evidenced by the variations in genomic sequences of the viruses (table 1). As summarized in table 1, no sequence substitutions were observed in nsp5, nsp6, nsp7, nsp8, nsp10, ORF3b, or ORF5a after 115 passages. Substitutions were observed in nsp4, nsp9, nsp11/12, nsp14, nsp15, nsp16, and ORF3a, but these did not result in amino acid substitutions and/or variations. Amino acid substitutions occurred in the remaining genes between P5 and P115. Two and three nucleotide substitutions were observed, respectively, in the 5′- and 3′-UTRs of CK/CH/LDL/97I P115, compared to the parental strain P5. Most nucleotide mutations were found in the S region between P5 and P115, and some of the nucleotide mutations resulted in amino acid substitutions. More mutations occurred in the S2 gene than in the

Fig. 2. A phylogenetic tree constructed based on the full-length genomic sequences of IBVs using the neighbor-joining method. IBV CK/CH/LDL/97I P5 and P115 are boxed. Each of the reference strains are indicated and the countries where the IBV strains were isolated are shown in parentheses.
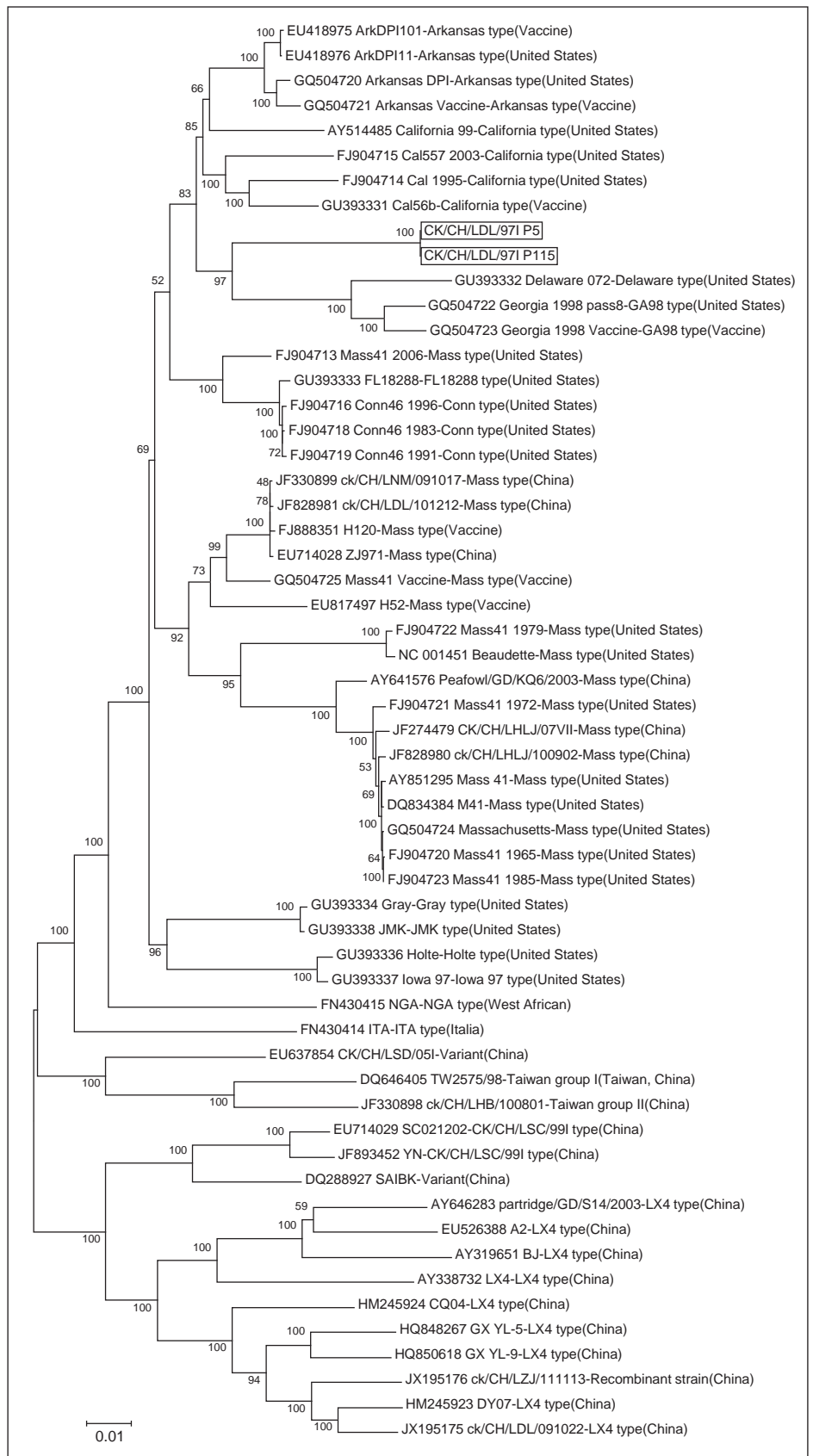
S1 gene. Among the 9 nsps in the ORF1 region, which showed nucleotide alterations between the pathogenic P5 and attenuated P115 viruses, nsp3 had the most.

Discussion

In this study, CK/CH/LDL/97I was unexpectedly grouped together with the US IBV strains, but was distinct from all other IBV strains reported so far in China. CK/CH/LDL/97I emerged in the mid-1990s as a novel IBV type in China [21, 23] and was associated with IB outbreaks on the Liaotung Peninsula between 1997 and 2001. This isolate seems to have high affinity for the proventriculus in chickens and is implicated in a novel pathogenicity associated with transmissible proventriculitis [23], though few experimental proventriculitis models have been generated using this type of IBVs. The origin of this virus type remains unknown and no complete genomic sequence for it is available in the GenBank database. The lack of sufficient information limits the comprehensive tracing of the origin and evolution of the CK/CH/LDL/97I strain, but it is noteworthy that this type of IBV was also found in Taiwan [18] and regions of the Middle East in recent years [19]. We identified a close genetic relationship between CK/CH/LDL/97I and the Delaware-type IBVs, which was isolated in the US, by entire genome analysis, suggesting a common origin or epidemiological link between the CK/CH/LDL/97I and Delaware-type IBV strains.

It has been suggested that minor differences near the receptor-binding domain of the S1 subunit of the spike protein could modify the affinity and virulence of CoVs for different cell surface glycoproteins [24–26]. Consequently, the S protein has been shown to be correlated to the virulence attenuation and broadening of host or cell-type ranges [27, 28]. This protein has also been reported as a determinant of cell tropism in IBV, as some amino acids contained in S protein contribute to attenuation of IBV [29]. Variations in the S1 gene may reduce the ability of CK/CH/LDL/97I P115 to infect host cells after serial passage in eggs, but not its ability to infect the chorioallantoic membrane, where IBV replication initially occurs in embryonated chicken eggs. However, such conclusions should be drawn with caution, as the results of this and other studies found no identical substitutions, insertions, or deletions in S proteins between different pathogenic IBV strains and their embryo-passaged, attenuated derivatives, even though the attenuated IBV strains showed distinctly different biological features, including lack of pathogenicity to 1-day-old SPF chicks compared to the pathogenic parental strains [26, 30, 31]. Some findings suggested that the replicase gene, rather than the S1 or S gene, may be responsible for viral pathogenicity [32, 33].

The S2 subunit of the spike protein of CoVs associates non-covalently with the S1 subunit and contains the transmembrane and C-terminal cytoplasmic tail domains. The ectodomain region [33] and two heptad repeat regions [34] of subunit S2 are involved in oligomerization of the S protein and required for entry into susceptible cells [35–37]. Changes in the S2 gene may affect viral entry [32]. In this study, multiple changes in the S2 gene occurred during passage of CK/CH/LDL/97I in embryonated eggs, which may have had an effect on viral entry into chicken host cells in vivo, indirectly decreasing the replication capacity. However, introduction of an S protein from a virulent IBV isolate did not confer virulence to the apathogenic strain Beau-R [29]. A further study showed that replacing all the Beaudette structural and accessory proteins with those from a virulent IBV isolate did not restore virulence [45]. These results suggest that IBV attenuation may not be caused by changes in the S2 gene. However, it has been speculated that changes in the S2 protein may contribute to adaptation of the field virus to chick embryonic tissue [32].

As in other CoVs, the IBV E protein is a small structural protein containing a single hydrophobic domain with multiple functions [38–42]. Although information on the IBV E protein is limited [41, 43], those from different CoVs perform similar functions during viral infection [44]. In the current study, we identified a mutation of nucleotide 184 (C-T) in P115, compared to P5, resulting in an amino acid substitution (Ala→Ser). However, this substitution may have only a minor effect on viral replication after 115 passages in eggs because only the hydrophobic domain (amino acids 12–32) of IBV E is important for efficient viral replication [41]. The cytoplasmic tail of IBV E is responsible for its interaction with IBV M [4], and mutations in the cytoplasmic tail of mouse hepatitis virus (MHV) E by targeted RNA recombination resulted in the production of elongated virions [45]. Therefore, substitutions in the cytoplasmic tail of the IBV P115 E protein caused by serial passages warrant further investigations.

No previous studies to date have investigated the function of the IBV intergenic UTR between M and gene 5, though some sequence diversities and mutations have been found in this region among IBV strains [32, 46]. This region contains a potential ORF with an initiation

codon immediately downstream of the M gene stop codon in some IBV strains. This study identified a similar 273-nucleotide ORF that potentially encodes a 90-amino acid product in CK/CH/LDL/97I P5 at a similar position; however, this ORF was extended in P115 by two nucleotide insertions, resulting in an ORF of 285 nucleotides that potentially encodes a 11-kD, 94-amino acid product. We could not predict the functions of the ORF in CK/CH/LDL/97I P5 or the potential effect of the insertions. The lack of subgenomic mRNA for this potential ORF, the long distance between the initiation codon and potential transcription regulatory sequence, and the loss of the potential ORF in some IBV strains indicate that this ORF is probably a pseudogene [32]. Furthermore, removal of the intergenic UTR from an apathogenic IBV had no effect on the functionality of the virus [32].

This study detected a substitution (Tyr→The) in the N protein due to the mutation at nucleotide position 398, between the P5 and P115 viruses. The N protein binds to the 3′ end of the UTR, which is essential for the synthesis of negative-strand viral RNA. The change in the N gene suggests that it may have an impact on viral replication and thus on viral pathogenesis, although the sequence-specific interaction between the N gene and 3′-UTR remains unclear. The amino acid substitution at residue 188 (Thr→Ile/Ala) in the M protein, which was related to antigenicity and/or virulence in IBV strains H52/H120, TW2296/95, and Arkansas [30], was not found in our CK/CH/LDL/97I strain; however, a nucleotide substitution between P5 and P115 was detected that resulted in an amino acid substitution at residue 223 (Gly→Ser). This substitution may affect the interaction between M and the cytoplasmic tail of IBV E [4], and thereby viral budding.

The IBV replicase gene encodes 15 nsps, some with known enzymatic functions [47]. However, the functions of these proteins in the context of pathogenesis remain poorly understood, although some nsps of other CoVs have been linked to the loss of virulence [32]. Like its homologs in other CoVs, 3CLpro of IBV is encoded by ORF1a and resides in nsp5. This proteinase specifically cleaves polyproteins 1a and 1ab at 11 sites to produce 12 mature products (nsp5–nsp16). A cluster of small nsps, including nsp6, nsp7, nsp8, nsp9, and nsp10, is located at the C-terminal region of the 1a polyprotein. Although the functional roles of these proteins in IBV are unknown, roles for these nsps in RNA replication and transcription of other CoVs are emerging from biochemical and structural analyses [48]. In the current study, we found no differences in nucleotide sequences between CK/CH/LDL/97I P5 and P115 in nsp5, nsp6, nsp7, nsp8, or nsp10.

Other groups have attenuated the virulent Ark DPI 11 strain of IBV following 101 passages in embryonated eggs (Ark DPI 101) and compared the complete genomes of both viruses. They identified two and one amino acid substitutions in nsp6 and nsp10, respectively, but none in nsp5, nsp7, or nsp8 [47], as in the present study. In addition, although substitutions were found in nsp4, nsp9, nsp11/12, nsp14, nsp15, and nsp16 in our study, these substitutions did not produce amino acid substitutions due to viral subpopulation selection, other than several mutations.

In contrast, apart from alterations due to viral subpopulations, three, two, and one amino acid substitutions were found in nsp1/2, nsp3, and nsp13 proteins, respectively, between P5 and P115. Of the three amino acid substitutions, one similar substitution was also reported in this coding region between challenge and vaccine M41 strain, and Ark DPI 11 and attenuated DPI 101 [47]. A recent report showed that nsp2 plays a role in facilitating de novo IBV protein synthesis by blocking protein kinase R phosphorylation of eukaryotic initiation factor 2 (eIF-2α), which shuts down protein synthesis. In addition, nsp2 induces expression of GADD34, which dephosphorylates eIF-2α [49]. The nsp3 of CoV contains multiple domains including an acidic domain, an ADP-ribose-1″-phosphatase, a papain-like proteinase (PL2pro), a Y domain, and a transmembrane domain [50]. Inactivation of ADP-ribose-1″-phosphatase activity in MHV nsp3 reduced viral replication in the livers of infected mice, but did not induce liver disease [51]. Two amino acid substitutions in this study were located in the viral proteinase PL2pro and Y domains, respectively, which were similar to the positions altered between Ark DPI 11 and 101 [47]. The substitution in PL2pro may restrict viral maturation or replication. However, we were unable to predict the effects of the Y-domain change because the role of this domain in IBV is currently unknown. nsp13 contains an RNA helicase and has an important function in viral replication. An amino acid substitution between CK/CH/LDL/97I P5 and P115 was found in the helicase domain at nucleotide position 15859, resulting in an amino acid substitution (Leu→Pro). This substitution was similar to that in the helicase domain of Ark PDI, located at nt 15763. This substitution in the attenuated P115 strain might alter viral replication [47].

The IBV replicase gene is not usually associated with antigenicity, immunogenicity, or tissue tropism. The amino acid substitutions in the ORF1a/1b proteins offer insight into putative residues that may be involved in the adaptation of chick embryonic tissue and subsequent at-

tenuation of the virus. It is possible that very few nucleotide substitutions within the replicase gene may cause attenuation following serial passage in embryonated eggs [32, 47], although the role of the replicase gene in pathogenicity is not well understand.

The 5′- and 3′-UTRs in the genome of CoVs, like most RNA viruses, usually harbor important structural elements involved in replication and/or translation [7–11]. In the present study, we found two and three variations in the 5′- and 3′-UTRs of the attenuated CK/CH/LDL/97I P115 virus, respectively, compared to the pathogenic P5 parental strain. These nucleotide variations may play a role in pathogenicity by affecting the secondary structures of *cis*-acting elements involved in viral replication [52].

Selection of a more fit virus subpopulation and mutations in the S1 gene have reportedly been associated with the replication of modified live IBV vaccine viruses in chickens [53]. S1 gene sequence analysis demonstrated that distinct virus subpopulations were selected when chickens were vaccinated with live-attenuated IBV strains [21]. Further analysis confirmed that the virus subpopulations differed between microenvironments in distinct host tissues [54]. This investigation did not compare the replication capacities of CK/CH/LDL/97I before and after serial embryo passage in the oviduct of female chickens, or assess damage to the reproductive tract, egg production, or egg quality in laying chickens. However, IBV replication in the epithelium of the oviduct has been shown to result in decreased egg production and quality [55]. The damage is more severe and permanent if young chicks are affected [56], although repeated embryo passage was shown to reduce the virulence of some IBVs in the chicken oviduct [57]. Limited information is currently available regarding the pathogenicity of CK/CH/LDL/97I-type IBV strains and embryo-passaged, attenuated P115 in the chicken oviduct. Future studies will therefore focus on the effects of these IBV strains on the chicken oviduct.

Compared with the large genetic differences between cell-adapted CoVs and their parental viruses [58, 59], few genetic changes were found between the embryo-passaged, attenuated CoVs and their pathogenic parental viruses [15, 16, 47]. The result is still very interesting to understanding of molecular markers of IBV attenuation, tissue tropism, and replication capacity changes, as in the case of the transmissible gastroenteritis virus, which showed that two amino acid substitutions at the N-terminus of spike protein resulted in loss of enteric tropism [60]. The current sequence analyses were unable to identify specific amino acid residues responsible for these changes because the attenuated strain differed from the parental strain at multiple positions. Further investigations using reverse genetics and animal studies are therefore necessary to verify the exact functions of these changes. In addition, none of the sequence substitutions were shared by all pathogenic IBV strains or their attenuated derivatives [26, 30, 31, 47], indicating changes in viral replication, pathogenicity, antigenicity, immunogenicity, or tissue tropism might be attributable to different genes in the viral genome. However, some of the sequence substitutions identified in this study could be used as the potential molecular markers, at least between the CK/CH/LDL/97I-type pathogenic and attenuated strains, providing important information for the development of specific diagnostics of differentiating CK/CH/LDL/97I-type IBV infections from vaccination because the attenuated P115 can be used as a potential vaccine candidate [20] to control the circulation of CK/CH/LDL/97I-type IBVs in China and regions of the Middle East in recent years [18, 19, 21, 23].

Acknowledgements

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