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Identification of the avian infectious bronchitis coronaviruses with mutations in gene 3

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Abstract

The sequence of a 6.0-kb fragment was compared in the 3′-encoding region of the genome in 27 infectious bronchitis virus (IBV) strains. All these strains have the same S-3-M-5-N gene order, as is the case for other IBVs. However, the sizes of the corresponding open reading frames (ORFs) of some genes varied among the virus strains. Phylogenetic analysis and sequence alignments demonstrated that recombination events had occurred in the origin and evolution of the strains CK/CH/LSD/03I and CK/CH/LLN/98I and the possible recombinant junction sites might be located at the 3c and M genes, respectively. The normal product of ORF 3a is 57 amino acids long, whereas a 43-bp deletion at the 3′-end of the CK/CH/LSD/03I 3a gene was detected, resulting in a frameshift event and C-terminally truncated protein with 47 amino acids. Comparison of the growth ability in embryos and replication and pathogenicity in chickens with IBV carrying the normal 3a gene indicated that this deleted sequence in the 3a gene of CK/CH/LSD/03I was not necessary for viral pathogenesis and replication either *in vitro* or *in vivo*. Occurrence of a mutation at the corresponding position of the CK/CH/LLN/98I start codon in the 3a gene led to the absence of ORF 3a in this virus, resulting in a novel genomic organization at the 3′-encoding regions: S-3b, 3c-M-5a, 5b-N. Comparison with other viruses carrying the normal 3a gene revealed that CK/CH/LLN/98I had replication and pathogenicity abilities *in vivo* similar to those of other IBVs; however, its growth ability in embryos was lower, although the relationship between the lower growth ability and the ORF 3a defect requires further confirmation.

Keywords: Infectious bronchitis coronavirus; Gene order; Mutation; Replication; Pathogenicity

1. Introduction

Coronaviruses belong to the family *Coronaviridae*, a member of the order *Nidovirales*, and are classified into 3 groups based on the lack of genetic and antigenic relationships between the species of different groups (González et al., 2003; Masters, 2006). They have been known to cause upper and lower respiratory diseases, gastroenteritis, and central nervous system infection in a number of avian and mammalian hosts, including humans (Weiss and Navas-Martin, 2005). The etiological importance of coronaviruses has received much attention since the discovery of the newly emerged severe acute respiratory syndrome-associated coronavirus (SARS-CoV) in 2003. In particular, how coronaviruses break the host species barrier, cause interspecies infection, and become zoonotic are questions of interest to the public. The infectious bronchitis virus (IBV), an avian coronavirus, together with the genetically closely related turkey coronavirus (Cavanagh et al., 2003; Guy, 2000), pheasant coronavirus (Cavanagh et al., 2002), and

Abbreviations: IB, infectious bronchitis; IBV, infectious bronchitis virus; ORF, open reading frame; SARS-CoV, severe acute respiratory syndrome-associated coronavirus; ns, nonstructural; S, spike; M, membrane; E, envelop; N, nucleocapsid; RT, Reverse transcription; PCR, polymerase chain reaction; EID_{50}, 50%; (median) embryo infectious doses; TRS, transcription regulatory sequence; TOC, tracheal organ cultures.

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viruses recently detected in 3 species of wild birds (Cavanagh et al., 2001), forms group 3 coronaviruses. IBV primarily causes respiratory disease in domestic fowl, although it also replicates at many epithelial surfaces of the alimentary tract, oviduct, and kidney (Cavanagh, 2003), and it is one of the most economically important pathogens in the poultry industry (Cavanagh et al., 2003).

Coronaviruses have the largest RNA viral genomes ranging from 26 to 32 kilobases (kb) in length (Masters, 2006). Genetic diversity among coronaviruses is generated by point mutations, insertions, and deletions introduced into the viral genome by viral RNA-dependent RNA polymerase, which lacks proofreading capabilities, and by genetic recombination, which occurs by a genomic template-switching mechanism (Masters, 2006). Two-thirds of the viral genome encodes replicase activity and the remaining one-third, structural proteins and small nonstructural (ns) accessory proteins (Masters, 2006). IBV has 4 essential structural proteins, the 3 membrane proteins being the spike (S), integral membrane (M), and small envelope (E) proteins, and a phosphorylated nucleocapsid (N) protein. Although the S1 subunit of the S protein carries virus-neutralizing and serotype-specific determinants, the S2 subunit may also induce neutralizing antibodies, and the IBV serotypes can be grouped based on the S2 gene sequence (Bosch et al., 2003). The N gene and N-terminus of the IBV M protein also vary between strains (Cavanagh, 2007). Furthermore, mutations and recombination events have been observed in multiple structural genes of IBV recovered from naturally occurring infections.

Fig. 1. Sequence alignment of ORFs 3a and 3b of 4 IBV strains. Deletion in gene 3 of IBV CK/CH/LSD/03I results in a 3′-end truncated 3a gene and the mutation in gene 3 of CK/CH/LLN/98I results in the absence of ORF 3a in this virus. Sequences of the pf/CH/LKQ3/03 and CK/CH/LJL/04I strains as representatives of the normal 3a gene were compared. The putative transcription regulatory sequence (TRS), CTGAACAA, of gene 3 is indicated in gray. The ATGs with a single underline in boldface are the start codons of genes 3a, 3b, and 3c. The single mutation at the first site of the start codon of gene 3 in the CK/CH/LLN/98I strain (ATG→ATT) results in the absence of ORF 3a in this virus. The TAAs/TGAs in boxes are the termination codons of genes S, 3a, and 3b, respectively. A 43-bp nucleotide sequence is deleted in the CK/CH/LSD/03I strain (represented as –).

Ns proteins associated with viral RNA replication and transcription are encoded by gene 1. Interspersed among the structural protein genes are small ns protein genes that vary in number, position, and sequence among the coronaviruses (Cavanagh, 2007; Masters, 2006). IBV has 2 such genes, genes 3 and 5 (Boursnell et al., 1987). Gene 3 is functionally tricistronic (Liu et al., 1991) with 3 ORFs, 3a, 3b, and 3c. ORFs 3a and 3b of IBV encode 2 small ns proteins of unknown function, 3a and 3b, respectively, and structural protein E is encoded by ORF 3c (Fig. 1; Liu and Inglis, 1991; Smith et al., 1990). It has been recently reported that neither the RNA nor the proteins of ORFs 3a and 3b of IBV are essential for replication (Hodgson et al., 2006; Shen et al., 2003). However, in most cases, the reasons that these ORFs have been maintained throughout evolution in natural conditions and the effects of these gene products on animal hosts are yet to be understood.

The primary aim of this work was to compare and analyze the 3′ 6.0-kb encoding regions that bore the 4 structural protein genes S2, M, E, and N and the 4 ns protein genes 3a, 3b, 5a, and 5b, in 27 IBV strains. The second objective was to examine the replication and pathogenicity of the IBV strains with abnormal genes in gene 3 either *in vitro* or *in vivo*.

2. Materials and methods

2.1. IBV field and vaccine strains

As described in Table 1, 19 IBV field strains and 8 commercially available vaccine strains were used in the present study. All the field strains were isolated as previously described (Liu et al., 2006b; Liu and Kong, 2004) and the vaccine strains were produced by different manufacturers in China (Liu et al., 2006b). These IBV strains were representatives of different types based on S1 gene analysis and comparison with the prototype (Liu et al., 2006a,b, 2007, in press). All the IBV field and vaccine strains were propagated once in 9 to 11-day-old embryonated chicken-specific pathogen-free (SPF) eggs (Harbin Veterinary Research Institute, China) and confirmed using negative contrast electronic microscopy (JEM-1200, EX) in the allantoic fluids of inoculated eggs as described previously (Liu and Kong, 2004), before being used in sequence analysis.

2.2. Reverse transcriptase (RT)-polymerase chain reaction (PCR), cloning, and sequencing

First, 5 fragments spanning the IBV 3′ 6.0-kb encoding regions of genome were obtained by RT-PCR from each of the 27 IBV strains. Secondly, the other 2 sets of primers, IBV-168 and IBV-281 and IBV-280 and N (−), were subsequently used to amplify, sequence, and confirm the entire 6.0-kb fragment. The primers IBV-280 and IBV-281 were designed based on the consensus nucleotide sequences obtained above. The sequences and locations of the primers used in this study are presented in Table 2.

Viral RNA was extracted from 200 μl of infectious allantoic fluid by TRIzol reagents (Invitrogen, Grand Island, USA) following the manufacturer's protocol. RT was performed with M-MLV Reverse Transcriptase (Invitrogen, Grand Island, USA) using the reverse primer N (−). The RT procedures were

Table 1
IBV strains used in the present study

Virus	Field/vaccine strain	Virus type[a]	GenBank accession numbers
D41	Vaccine	Mass-type	EF602437
IBN	Vaccine	Mass-type	EF602439
W93	Vaccine	Mass-type	EF602443
H94	Vaccine	Mass-type	EF602438
HK	Vaccine	Mass-type	AY761141
J9	Vaccine	an Australian associated strain	EF602440
Jilin	Vaccine	an Australian associated strain	EF602442
JAAS	Vaccine	an Australian associated strain	EF602441
CK/CH/LHN/00I	Field strain	an Australian associated strain	EF602456
LX4	Field strain	LX4-type	AY338732
CK/CH/LHLJ/04XI	Field strain	LX4-type	EF602461
CK/CH/LJL/04I	Field strain	LX4-type	EF602452
CK/CH/LSHH/03I	Field strain	LX4-type	EF602449
CK/CH/LLN/98I	Field strain	LX4-type	EF602451
CK/CH/LSD/03I	Field strain	LX4-type	EF602457
CK/CH/LHLJ/99I	Field strain	LX4-type	EF602453
CK/CH/LHLJ/02I	Field strain	LX4-type	EF602455
CK/CH/LHLJ/95I	Field strain	LX4-type	EF602454
CK/CH/LXJ/02I	Field strain	LX4-type	EF602458
CK/CH/LHB/96I	Field strain	LX4-type	EF602460
CK/CH/LGD/96I	Field strain	LX4-type	EF602459
CK/CH/LSC/99I	Field strain	CK/CH/LSC/99I-type	EF602450
CK/CH/LGD/04II	Field strain	CK/CH/LSC/99I-type	EF602444
CK/CH/LGD/04III	Field strain	CK/CH/LSC/99I-type	EF602447
CK/CH/LTJ/95I	Field strain	tl/CH/LDT3/03I-type	EF602448
CK/CH/LDL/97I	Field strain	CK/CH/LDL/97I-type	EF602445
CK/CH/LDL/98I	Field strain	CK/CH/LDL/97I-type	EF602446

[a] The types of virus are based on S1 gene comparison and protectotype analysis between different types of IBV (Liu and Kong, 2004; Liu et al., 2006a,b, 2007, in press).

Table 2
Sequence and position of the primers used in RT-PCRs

Primers	Sense[a]	Sequence (5′→3′)	Gene	Position in genome[b]
IBV-167	+	GCTTCTTGAGAA(T/C)CAGTTTTA	Partial S2 (5′)	21921–21941
IBV-168	−	AGACGATCAACTTGTGCATCTG	Partial S2 (5′)	22952–22973
IBV-182	+	GACATTTAC(C/G)(A/C)GCAACTTGA	Partial S2 (3′) and 3a	22921–22940
IBV-183	−	AACATTAGTCTAGGCTGTGC	Partial S2 (3′) and 3a	24014–24033
IBV-199	+	CAAGTTTTATTCCAAATTAT	Partial 3a (3′), 3b, 3c and partial M (5′)	23961–23980
IBV-200	−	TCGCCAGTGTCTACTGACTG	Partial 3a (3′), 3b, 3c and partial M (5′)	25120–25139
IBV-170	+	CCAAAGCGGAAATAAGAAAA	Partial M (3′), gene 5 and partial N (5′)	25074–25093
IBV-171	−	AACCAAGATGCATTTCCAGA	Partial M (3′), gene 5 and partial N (5′)	25960–25979
N(+)	+	GACGCCCCAGCGCCAGTCATTAAA	N and 3′-UTR	25903–25926
N(−)	−	ACGCGGAGTACGATCGAGGGTACA	N and 3′-UTR	27484–27507
IBV-280	+	CCC(C/A)GAATCTAATGCCGTAGG	S2, gene 3 and partial M (5′)	24846–24866
IBV-281	−	GCCACTGACC(C/A)TCACAATAAAG	Partial M (3′), gene 5, N and 3′-UTR	24955–24976

[a] Negative-sense (−) or positive-sense (+) primer.
[b] The nucleotide positions correspond to those in the sequence of the IBV Beaudette genome, GenBank accession number M95169.

performed using 20 μl of RNA in a 40-μl reaction volume as previously described (Liu et al., 2005). Each cDNA fragment was amplified from RT products by PCR as previously described (Liu et al., 2005). The PCR products were purified from agarose gels by using a DNA extraction kit (Boehringer Mannheim, Germany) and sequenced directly or cloned into pMD-18T (TaKaRa, Dalian, China) vector by following the manufacturer's instructions. Each region of the fragment of the 27 IBV strains was sequenced 4 to 5 times from PCR products or independent clones.

2.3. ORF determination and sequence analysis

Sequences were compiled and ORFs were determined using the Gene Runner program version 3.00 (http://www.generunner.com) (Liu et al., 2005). Nucleotide and amino acid sequences of different genes of different IBVs in the 3′ 6-kb fragment were assembled, aligned, and compared with the IBV pf/CH/LKQ3/03 strain (Liu et al., 2005) as the reference strain by using the MEGALIGN program (DNAStar). Phylogenetic analysis of the nucleotide sequences of different genes in different IBV strains was performed by the Clustal V method using DNAStar software (Liu et al., 2006b). The sequences of the 27 IBV strains have been submitted to the GenBank database, and their accession numbers are listed in Table 1. The sequence of the IBV pf/CH/LKQ3/03 strain used as a reference strain for comparison was from the GenBank database with the GenBank accession number AY702085.

2.4. Growth ability of the IBV strains in 9-day-old chicken embryos

Each of the nine 9-day-old SPF chicken embryos was inoculated with the isolates CK/CH/LLN/98, CK/CH/LSD/03I, CK/CH/LJL/04I, CK/CH/LDL/97I, and CK/CH/LGD/04II passage level 3 (the latter 3 virus strains, each representing different IBV serotypes in China, were used as controls) with 10^2 EID_{50} per embryo in 0.1 ml inoculum into the allantoic cavity. The embryos were inoculated at 37 °C in an egg incubator (Heraeus, Germany), and the allantoic fluid from the 3 embryos of each virus was harvested at 24, 48, and 72 h, after the embryos were chilled at 4 °C. The allantoic fluid was stored at −70 °C individually and subsequently used for virus titration.

Virus titrations were performed in 9-day-old embryonated chicken SPF eggs via the allantoic cavity route of inoculation (Yachida et al., 1979), and the titers were expressed as 50% (median) embryo infectious doses (EID_{50}). Serial l0-fold dilutions were used for titrations. At each dilution, 5 embryos received 0.1 ml inoculum. The embryos were candled daily and examined for 1 week; those showing characteristic IBV lesions, such as dwarfing, stunting, or curling of embryos, were recorded as infected by IBV.

2.5. Experimental infection of birds

We housed 6 groups of 10 White Leghorn SPF chickens (Harbin Veterinary Research Institute, China) each in separate isolators under negative pressure. They were provided with food and water *ad libitum*. As listed in Table 4, 5 IBV strains were used for experimental infection at day 15 by oculonasal application. Groups 1 to 5 were inoculated with each of the 5 IBV field strains, and group 6 was mock inoculated with sterile allantoic fluid and served as the control (Table 4). The actual inoculation dose per bird, as determined by egg titration of the inocula, is summarized in Table 4. The chicks were examined daily for signs of infection up to 30 days after inoculation.

Cloacal swabs were taken on days 4, 8, 12, and 16 post-inoculation. Directly after sampling, the swabs were stored individually in 200 μl of virus isolation medium (50% glycerol; 50% phosphate-buffered saline [PBS]) at −70 °C until virus isolation. Blood samples were also collected on these days and stored at −70 °C.

2.6. Virus recovery and RT-PCR detection

Swab samples that were taken at the same time point in each group post-inoculation were pooled for virus isolation. The pooled sample containing 10 000 U penicillin and 10 000 μg streptomycin was inoculated into at least 4 SPF embryos via the allantoic cavity (0.2 ml per egg). The eggs were candled daily, and allantoic fluids from 2 of the inoculated embryos were

collected 72 h post-inoculation for RT-PCR amplification, and the remaining embryos were examined 1 week later for characteristic IBV lesions such as dwarfing, stunting, or curling of embryos. For RT-PCR negative samples, another 2 passages were performed and RT-PCR was carried out.

RT-PCR detection was performed as previously described (Liu et al., 2006a). Briefly, RT was conducted using IBV primer N (−) and PCR was carried out using primers N (−) and N (+) to generate a product of approximately 1600 bp from the allantoic fluids inoculated with samples collected post-inoculation. The PCR products were analyzed on 1.0% agarose gel.

2.7. Amplification and sequencing of the 3a gene in strains CK/CH/LSD/03I and CK/CH/LLN/98I from kidney tissues of experimentally infected chickens

In order to further investigate the abnormal 3a gene sequences of the IBV strains CK/CH/LSD/03I and CK/CH/LLN/98I, primers IBV-199 and IBV-200 were used to amplify gene 3 from the kidney tissues of the chickens experimentally infected with strains CK/CH/LSD/03I and CK/CH/LLN/98I, respectively. RT-PCR amplification was performed as described above. Briefly, RT was conducted using IBV primer N (−) and PCR was carried out using primers IBV-199 and IBV-200 to generate a product of approximately 1100 bp from the kidney tissues of chickens inoculated with strains CK/CH/LSD/03I and CK/CH/LLN/98I, respectively. The PCR products were purified from agarose gels by using a DNA extraction kit (Boehringer Mannheim, Germany) and sequenced directly.

2.8. Total antibody ELISA

Serum samples were assayed in single dilutions by using a commercial total antibody ELISA (IDEXX Corporation, Westbrook, Maine, USA) according to the manufacturer's instructions. Serum-to-positive ratios (S/P-ratios) were calculated as described previously (de Wit et al., 1998; Liu et al., 2006a). From these S/P-ratios, individual serum titers, expressed as $\log_2$ values, were calculated according to the manufacturer's instructions.

3. Results

3.1. Sequence determination and comparison

Table 3 presents the ORF sizes in the 3′ 6.0-kb encoding regions of the 27 IBV strains compared with IBV pf/CH/LKQ3/03 as the reference strain. The primary structures of all the 3′-encoding regions of our IBV strains containing the S protein gene, gene 3, M protein gene, gene 5, and N protein gene in this order from the 5′-end to the 3′-end were very similar to those

Table 3
Comparison of ORF sizes in the 3′-end encoding regions of IBV[a]

Strains	S2[b]	Gene 3			M	Gene 5		N
		3a	3b	3c		5a	5b	
pf/CH/LKQ3/03	1878 (625)	174 (57)	195 (64)	327 (108)	678 (225)	198 (65)	249 (82)	1230 (409)
D41	1878 (625)	174 (57)	195 (64)	327 (108)	678 (225)	198 (65)	249 (82)	1230 (409)
H94	1878 (625)	174 (57)	195 (64)	327 (108)	678 (225)	198 (65)	249 (82)	1230 (409)
HK	1878 (625)	174 (57)	195 (64)	324 (107)	672 (223)	198 (65)	249 (82)	1230 (409)
IBN	1878 (625)	174 (57)	195 (64)	327 (108)	678 (225)	198 (65)	249 (82)	1230 (409)
W93	1878 (625)	174 (57)	195 (64)	327 (108)	678 (225)	198 (65)	249 (82)	1230 (409)
J9	1878 (625)	174 (57)	195 (64)	324 (107)	672 (223)	198 (65)	249 (82)	1230 (409)
JAAS	1878 (625)	174 (57)	195 (64)	324 (107)	672 (223)	198 (65)	249 (82)	1230 (409)
Jilin	1878 (625)	174 (57)	195 (64)	324 (107)	672 (223)	198 (65)	249 (82)	1230 (409)
LX4	1878 (625)	174 (57)	192 (63)	330 (109)	678 (225)	198 (65)	249 (82)	1230 (409)
CK/CH/LDL/98I	1878 (625)	174 (57)	195 (64)	309 (102)	681 (226)	198 (65)	249 (82)	1230 (409)
CK/CH/LHN/00I	1878 (625)	174 (57)	195 (64)	324 (107)	672 (223)	198 (65)	249 (82)	1230 (409)
CK/CH/LHLJ/02I	1878 (625)	174 (57)	189 (62)	330 (109)	681 (226)	198 (65)	249 (82)	1230 (409)
CK/CH/LHLJ/95I	1878 (625)	174 (57)	189 (62)	330 (109)	681 (226)	198 (65)	249 (82)	1230 (409)
CK/CH/LHLJ/99I	1878 (625)	174 (57)	189 (62)	330 (109)	681 (226)	198 (65)	249 (82)	1230 (409)
CK/CH/LJL/04I	1878 (625)	174 (57)	189 (62)	330 (109)	681 (226)	198 (65)	249 (82)	1230 (409)
CK/CH/LLN/98I	1878 (625)	LA[c]	189 (62)	330 (109)	681 (226)	198 (65)	249 (82)	1230 (409)
CK/CH/LSC/99I	1851 (616)	174 (57)	189 (62)	327 (108)	678 (225)	198 (65)	249 (82)	1230 (409)
CK/CH/LSHH/03I	1878 (625)	174 (57)	189 (62)	330 (109)	681 (226)	198 (65)	249 (82)	1230 (409)
CK/CH/LTJ/95I	1878 (625)	174 (57)	189 (62)	330 (109)	681 (226)	198 (65)	249 (82)	1230 (409)
CK/CH/LHLJ/04XI	1878 (625)	174 (57)	189 (62)	330 (109)	681 (226)	198 (65)	249 (82)	1230 (409)
CK/CH/LHB/96I	1878 (625)	174 (57)	189 (62)	330 (109)	681 (226)	198 (65)	249 (82)	1230 (409)
CK/CH/LGD/96I	1878 (625)	174 (57)	189 (62)	330 (109)	681 (226)	198 (65)	249 (82)	1230 (409)
CK/CH/LGD/04II	1878 (625)	174 (57)	189 (62)	327 (108)	678 (225)	198 (65)	249 (82)	1230 (409)
CK/CH/LGD/04III	1878 (625)	174 (57)	219 (72)	330 (109)	681 (226)	198 (65)	249 (82)	1230 (409)
CK/CH/LDL/97I	1878 (625)	174 (57)	195 (64)	309 (102)	681 (226)	198 (65)	249 (82)	1230 (409)
CK/CH/LXJ/02I	1878 (625)	174 (57)	192 (63)	330 (109)	678 (225)	198 (65)	249 (82)	1230 (409)
CK/CH/LSD/03I	1878 (625)	144 (47)	192 (63)	327 (108)	678 (225)	198 (65)	249 (82)	1230 (409)

[a] The sequence of the IBV pf/CH/LKQ3/03 genome was used as reference, GenBank accession no. AY702085. The sizes of amino acids are indicated in parentheses.
[b] The cleavage site was not included.
[c] LA indicates that this IBV strain lacked ORF 3a.

detected in the corresponding genomic areas of the IBV pf/CH/LKQ3/03 strain. However, the sizes of corresponding ORFs of some genes varied between our strains and in comparison with the pf/CH/LKQ3/03 strain. The genes that were the most conserved in sizes were genes *5*, N, S2, and 3a in our IBV strains, as compared with the pf/CH/LKQ3/03 strain. In the IBV strains investigated, the ORFs 3b and 3c contained 189 to 219 nucleotides and 309 to 330 nucleotides, respectively. Similarly, different IBV strains had M genes of various sizes, and the ORFs comprised 672 to 681 nucleotides.

As illustrated in Fig. 1, a 43-bp nucleotide sequence was detected at the 3′-end of the 3a gene in the virus CK/CH/LSD/03I, as compared with the normal 3a gene, resulting in a truncated 3a gene. This deletion resulted in a frameshift event and therefore, if expressed, it results in a C-terminally truncated protein with 47 deduced amino acids. However, an interesting observation is that a single mutation occurred at the corresponding position of the start codon of CK/CH/LLN/98I (ATG→ATT). This mutation resulted in the virus lacking ORF 3a and changed the primary structure of the 3′-encoding regions of CK/CH/LLN/98I, leading to a novel genomic organization of the avian coronavirus that had the S-3b-3c-M-5a-5b-N gene order from the 5′-end to the 3′-end, instead of the typical gene order in the 3′-encoding regions of group 3 coronaviruses isolated from chicken (IBV) (Boursnell et al., 1987), turkey (Breslin et al., 1999; Cavanagh et al., 2001; Lin et al., 2002), and pheasants (Cavanagh et al., 2002). The sequence profiles of RT-PCR products would represent the populations of the viral RNAs presented in a given passage. Overlapping peaks at the same position, representing different populations of viral RNAs, would be observed if several populations of viruses coexisted. However, only a single T mutation was detected in viral RNA by RT-PCR amplification prepared from CK/CH/LLN/98I-infected allantoic fluid. These abnormal sequences in ORF 3a of CK/CH/LSD/03I and CK/CH/LLN/98I were confirmed by sequencing the corresponding regions of the viruses from the kidney tissues of experimentally infected chickens.

3.2. Phylogenetic analysis

Phylogenetic analysis was performed based on alignments of nucleotide sequences with each gene in the 27 IBV strains and the Beaudette reference strain. As expected, phylogenetic analysis of S2, 3a, 3b, and 3c genes in the IBV strains in the present study revealed results parallel to its genotype (Fig. 2). However, the Chinese IBV isolates exhibited close relationships by phylogenetic analysis of the M, 5a, 5b, and N genes. Analysis of the S2, 3a, 5a, and 5b genes in the vaccine strain JAAS, which was closely related to the Australian IBV strains by S1 gene typing, revealed a greater diversity. Interestingly, in the case of CK/CH/LSD/03I and CK/CH/LLN/98I, the results were varied. Similar to the results of the S1 gene (Liu et al., 2006b), analysis of the S2, 3a, 3b, and 3c genes revealed that CK/CH/LSD/03I belonged to the LX4-type, whereas the analysis of the M, 5a, 5b, and N genes classified this strain into the Mass-type group, suggestive of a recombinant event that occurred during the origin and evolution of CK/CH/LSD/03I. The possible recombinant junction site was determined in the 3c and M genes in this study. As illustrated in Fig. 3, the nucleotide sequence alignment of the 3c and M genes of CK/CH.LSD/03I with the pf/

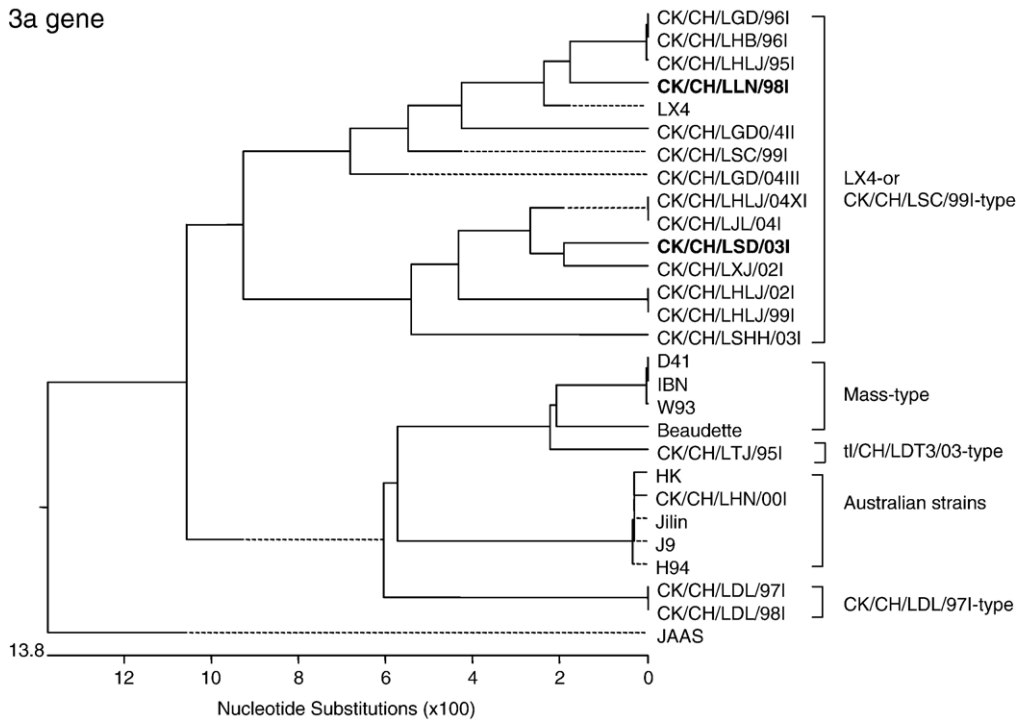


Fig. 2. Phylogenetic trees with each gene in the 27 IBV strains were generated using the MEGALIGN (DNASTAR) program. IBV pf/CH/LKQ3/03 was used as the reference strain. All the ORFs, except the S2 gene, which contained all the sequences downstream the cleavage recognition sites of the S gene, were used for phylogenetic tree construction. Sequence distance indicated by the scale was calculated using the PAM250 matrix in LASERGENE. The corresponding sequence of 3a was used for constructing the CK/CH/LLN/98I phylogenetic tree.

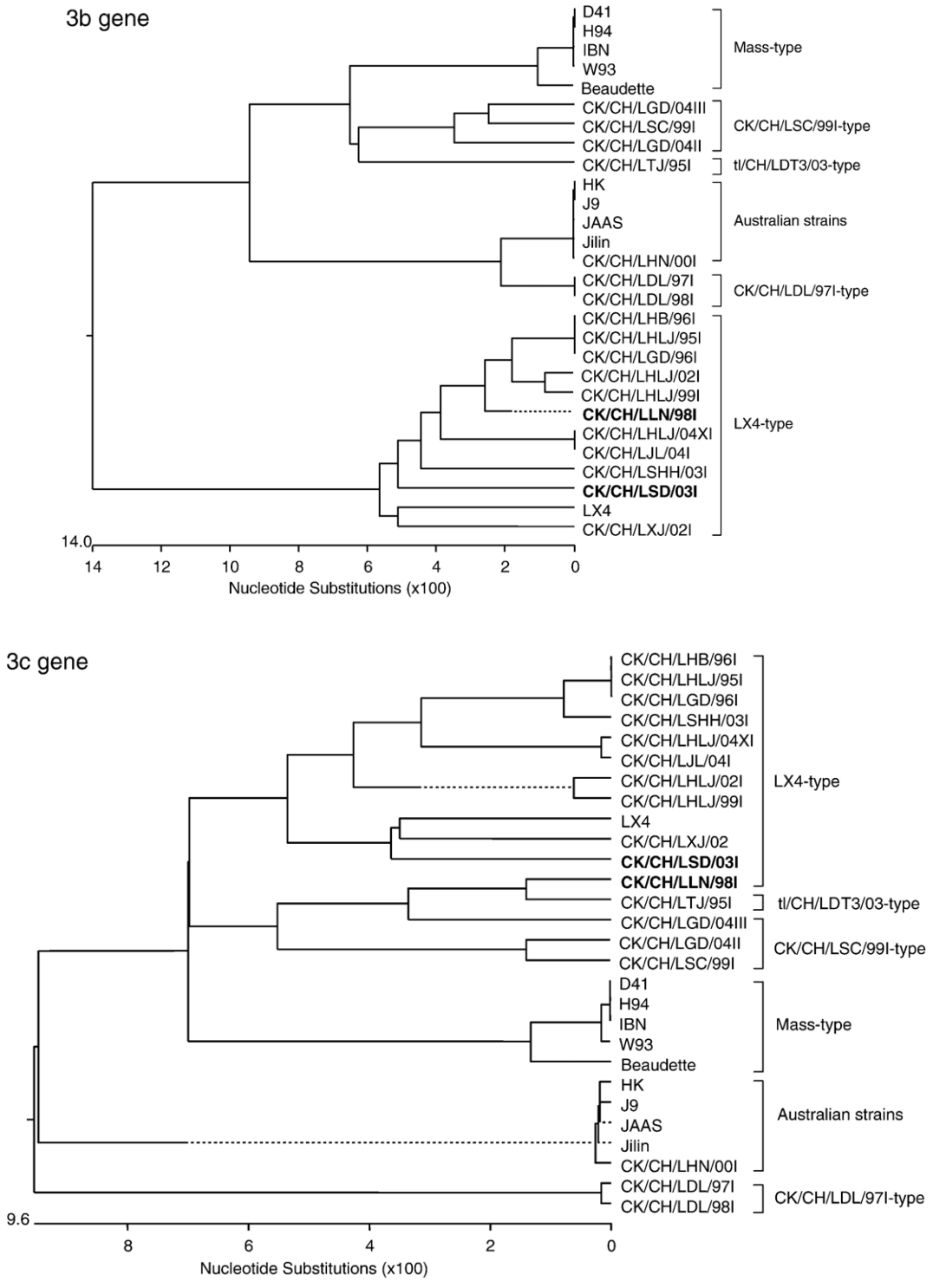


Fig. 2 (*continued*).

CH/LKQ3/03 (Mass-type) and CK/CH/SHH/03I (LX4-type) strains revealed that it shared identical sequences with CH/CK/LSHH/03I for the first 80 bp of the 3c gene; however, it bore a striking homology with the pf/CH/LKQ3/03 strain in the M gene. This shift in homology suggested that the recombinant site might be located at the 3c gene (Fig. 3). Results of the phylogenetic analysis in strain CK/CH/LLN/98I were similar to those in strain CK/CH/LSD/03I. An exchange of genetic information between CK/CH/LTJ/95-like and J9-like strains resulted in CK/CH/LLN/98I sequences in the 3′-encoding region. Sequence alignment revealed that the recombinant site might be located at the beginning of the M gene (Fig. 4).

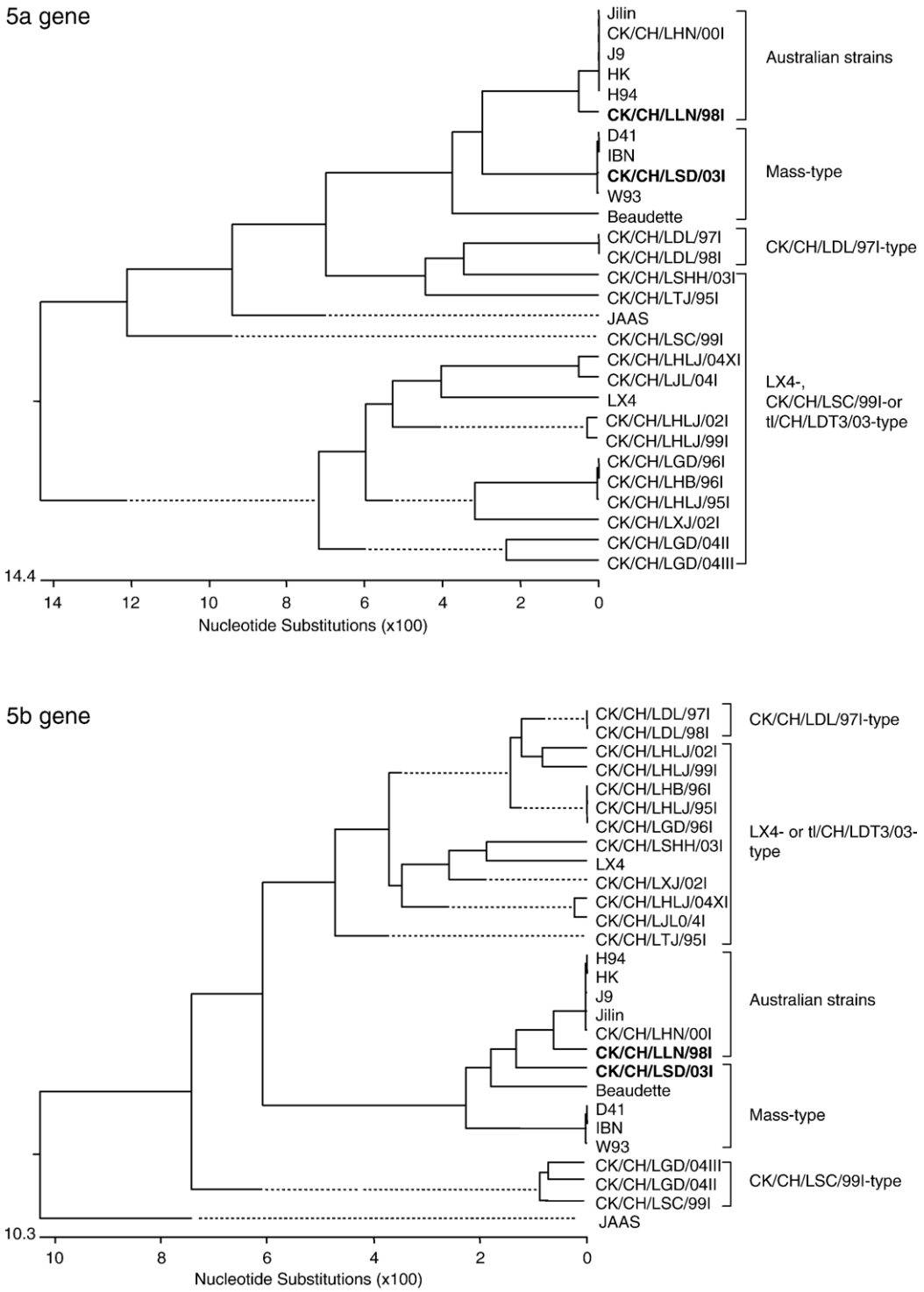
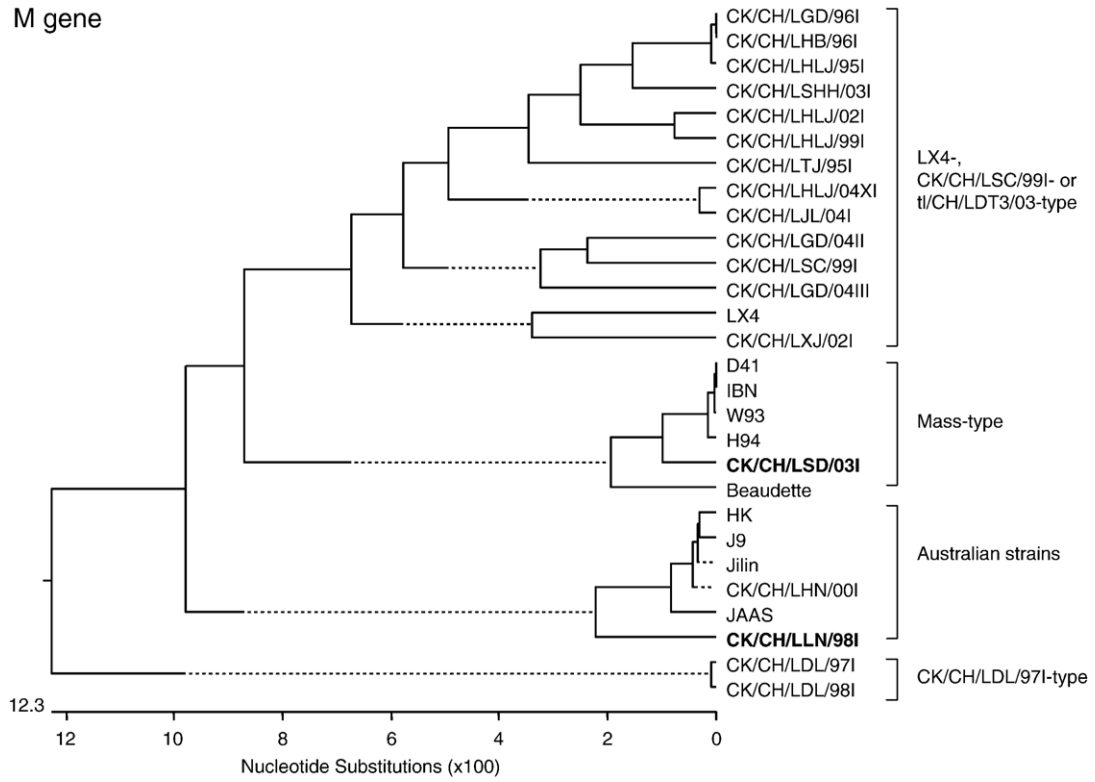


Fig. 2 (*continued*).

3.3. Propagation of the IBV strains with normal and mutant gene 3 in chicken embryos

Given that IBV ORF 3a has an important function in infection (Hodgson et al., 2006), we undertook the first experiment to investigate the potential effect of the mutations that occurred in IBV strains CK/CH/LSD/03I and CK/CH/LLN/98I, which resulted in a truncated 3a gene and absence of the 3a gene, respectively, on the growth ability of virus *in vitro*. Since no cell lines could sustain the growth of the IBV strains in this study (data not shown), the 9-day-old embryonated eggs were used to determine the growth ability of virus *in vitro*. An equal dose (10^2 EID_{50}) of each virus at passage level 3, using strains CK/CH/LJL/04I, CK/CH/LDL/97I, and CK/CH/LGD/04II carrying the normal 3a gene as controls, was used to inoculate 9-day-old embryos. The inoculated embryos were incubated at



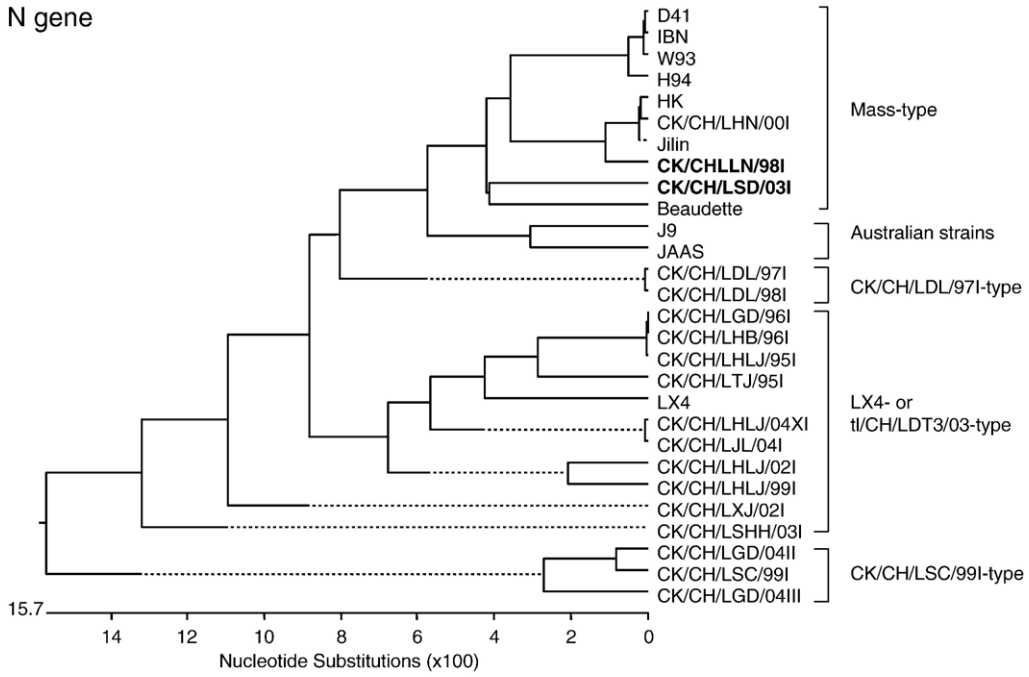


Fig. 2 (*continued*).

37 °C, and the allantoic fluid from 3 embryos of each virus was harvested at 24, 48, and 72 h, respectively, for virus titration. Based on their movement and the extent of bleeding, curling, and dwarfing, all the inoculated embryos were infected but alive within 72 h. EID_{50} was determined as the virus titer with each sample. The titers of 5 IBV strains in this study were increased gradually after inoculation with each viral strain from 24 to 72 h (Fig. 5). The virus titer of CK/CH/LLN/98I was obviously lower than those of the other 4 strains; the latter 4 viruses exhibited similar titers.

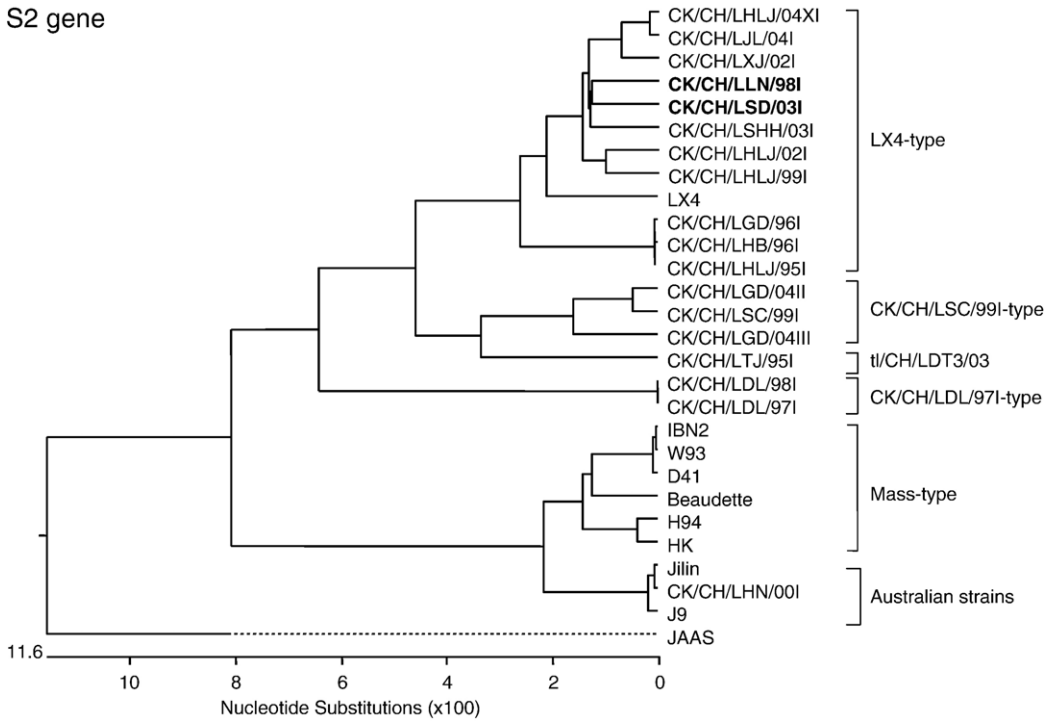


Fig. 2 (*continued*).

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CK/CH/LSSH/03I  AATAAATCGCTAGAAGAGAACGGCAGTTTTCTAACAGCACTTTATATATTTGTAGCATTTGTAGCACTTTA
CK/CH/LSD/03I   AATAAATCGCTAGAAGAGAACGGCAGTTTTCTAACAGCCCTTTATATATTTGTAGCATTTGTAGCACTTTA
pf/CH/LKQ3/03   AATAAGTCGCTAGAGGAGAATGGAAGTTTTCTAACAGCGCTTTACATATTTGTAGGATTTTTAGCACTTTA

CK/CH/LSSH/03I  TCTCCTAGGTAGAGCACTACAAGCTTTTGTACAAGCAGCTGATGCTTGTTGCTTATTCTGGTATACATGGG
CK/CH/LSD/03I   TCTTTTAGGTAGAGCGCTTCAAGCATTTGTACAAGCAGCTGATGCTTGTTGTTTATTTTGGTATACATGGG
pf/CH/LKQ3/03   TCTTCTAGGTAGAGCACTTCAAGCATTTGTACAGGCTGCTGATGCTTGTTGTTTATTTTGGTATACATGGG

CK/CH/LSSH/03I  TAATAGTTCCCGGAGCTAAGGGCACAGCCTTTGTGTACAAACATACATATGGTAAAAGACTTAACAATCCG
CK/CH/LSD/03I   TAGTAGTTCCAGGAGCTAAGGGTACAGCCTTTGTGTATAAACATACATATGGTAGAAAACTTAACAATCCG
pf/CH/LKQ3/03   TAGTAATTCCAGGAGCTAAGGGTACAGCCTTTGTATACAAGTATACATATGGTAGAAAACTTAACAATCCG

CK/CH/LSSH/03I  GAATTAGAACAGGTTATTGTTAACGAGTTCCCAAAGAACGGTTGGAATAATAAAAACCCTGCAAATTTCCA
CK/CH/LSD/03I   GAATTGGAACAGGTTATTGTTAACGAGTTCCCA**AAGAACGGTTGGAATAATAAA**AATCCAGCAAATTTTCA
pf/CH/LKQ3/03   GAATTAGAAGCAGTTATTGTCAACGAGTTTCCTAAGAACGGTTGGAATAATAAAAATCCAGCAAATTTTCA

CK/CH/LSSH/03I  AGATGTCGAATGGCACGGAAAATTGCACTCTTAGTACTCAGCAGGCAGCTGAGCTCTTCAAGGAATATAAT
CK/CH/LSD/03I   AGATGTCCAACGAGAC---AAATTGCACTCTTGACTTTGAACAGTCAGTTGAGCTTTTTAAAGAGTATAAT
pf/CH/LKQ3/03   AGATGTCCAACGAGAC---AAATTGTACTCTTGACTTTGAACAGTCAGTTGAGCTTTTTAAAGAGTATAAT

CK/CH/LSSH/03I  TTATTCATAACCGCATTCCTTTTGTTTCTAACCATACTACTTCAGTATGGATACGCGACTAGGAGTCGG
CK/CH/LSD/03I   TTATTTATAACTGCATTCTTGTTGTTCTTAACCATAATACTTCAGTATGGCTATGCAACAAGAAGTAAG
pf/CH/LKQ3/03   TTATTTATAACTGCATTCTTGTTGTTCTTAACCATAATACTTCAGTATGGCTATGCAACAAGAAGTAAG
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Fig. 3. Sequence alignment of the 3c and M genes of CK/CH/LSHH/03I, CK/CH/LSD/03I, and pf/CH/LKQ3/03. Nucleotides that are identical between 2 of the 3 IBVs but different from the rest are indicated in gray. The start codons of the M gene are enclosed in boxes and the stop codons of the 3c gene are underlined. The possible recombination junction site is in bold.

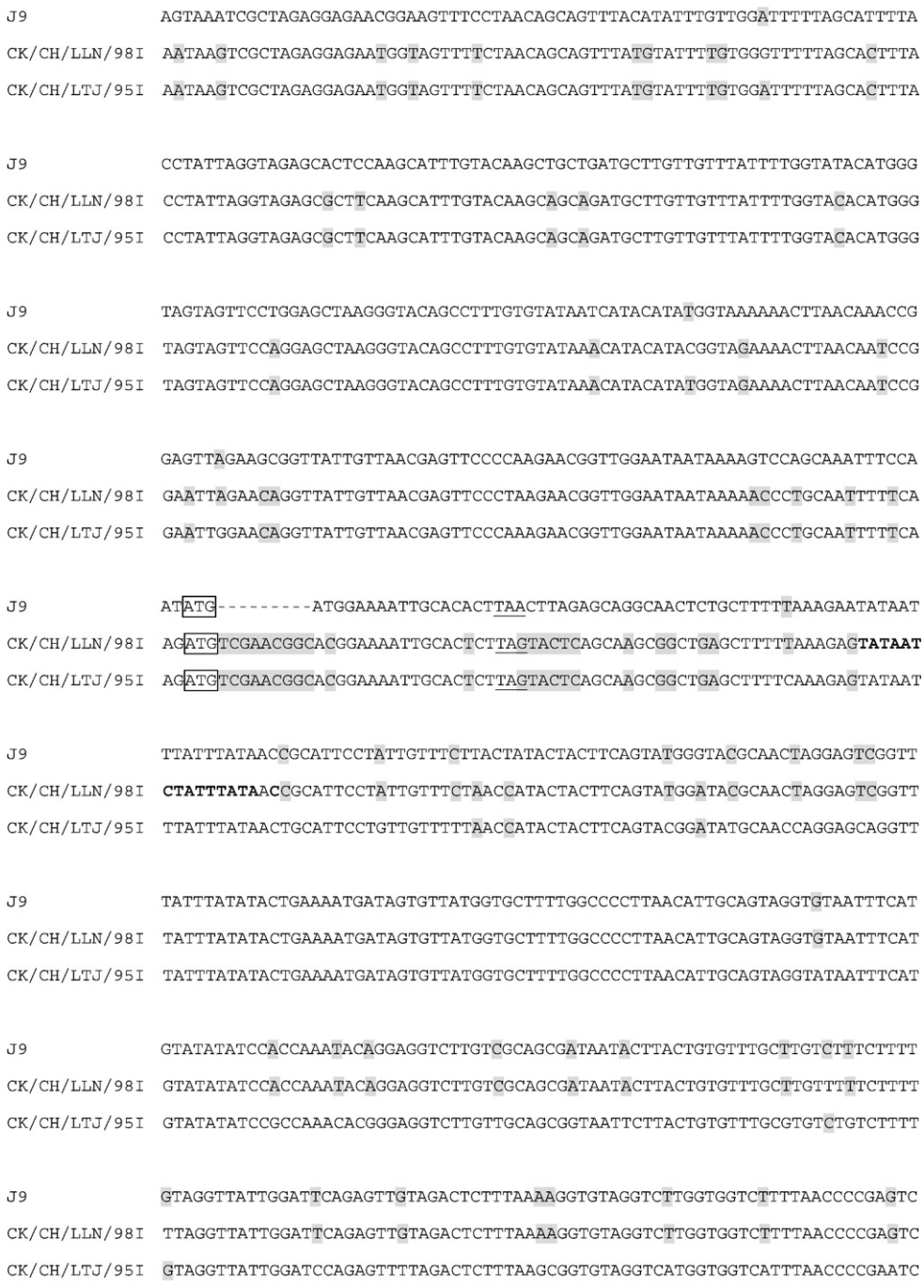

Fig. 4. Sequence alignment of the 3c and M genes of the CK/CH/LTJ/95I, CK/CH/LLN/98I, and J9 strains. Nucleotides that are identical between 2 of the 3 IBVs but different from the rest are in gray. The start codons of the M gene are enclosed in boxes and the stop codons of the 3c gene are underlined. The possible recombination junction site is in bold.

3.4. Pathogenicity of the IBV strains carrying normal and mutant gene 3 to chickens

The second experiment was extended to determine if the IBV strains with normal and mutant gene 3 have different pathogenicity to chickens. Three days after incubation with CK/CH/LSD/03I and CK/CH/LLN/98I, using CK/CH/LJL/04I, CK/CH/LDL/7I, and CK/CH/LGD/04II carrying the normal 3a gene as controls, some chicks in each virus-inoculated group exhibited respiratory clinical signs and the diseased chicks were listless and huddled together, presenting ruffled feathers and a dark, shrunken comb. As summarized in Table 4, both CK/CH/LSD/03I and CK/CH/LLN/98I could infect chickens and caused obvious clinical signs, similar to those shown by the 3 control strains. In the case of the CK/CH/LLN/98I-inoculated group, 2 chicks died between days 5 to 15 post-inoculation. Gross lesions of dead chicks were mainly confined to the kidneys. The kidney parenchyma of the dead birds was pale, swollen, and mottled; tubules and urethras were distended with uric acid crystals. These gross lesions were similar to those

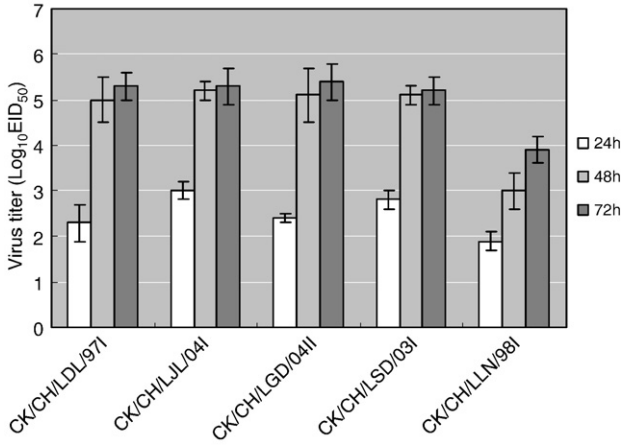

Fig. 5. Comparison of the growth of the different IBV strains in 9-day-old SPF chicken embryos evaluated by EID_{50}. The IBV strains CK/CH/LJL/04I, CK/CH/LDL/97I, and CK/CH/LGD/04II were used as positive controls.

caused by strains CK/CH/LJL/04I, CK/CH/LDL/97I, and CK/CH/LGD/04II in the present study.

Replication of the inoculated virus was assessed in the chicks of the 6 groups at days 4, 8, 12, and 16 after inoculation by virus recovery using 9-day-old embryos via the allantoic cavity and RT-PCR amplification. As shown in Table 4, virus was detected in birds of the 5 virus-inoculated groups (groups 1 to 5) and in none of the birds in the negative control group, suggestive of the similar propagation ability in chickens by all the IBV strains CK/CH/LSD/03I, CK/CH/LLN/98I, CK/CH/LJL/04I, and CK/CH/LGD/04II, although we had not quantified the viruses.

Serum antibodies were detected in the chicks of all virus-inoculated groups. The antibody was detectable from 8 days onwards in each of the virus-inoculated groups and in none of the negative control groups (Table 4), suggestive of the induction of immunity by the viruses. In addition, no obvious difference in the antibody titer induced by the 5 viruses was observed in this study (data not shown).

4. Discussion

The 27 IBV strains in this study have the same S-3-M-5-N gene order, as is the case for the other IBVs (Boursnell et al., 1987) and coronaviruses from turkeys (Breslin et al., 1999), pheasants (Cavanagh et al., 2002), mallard ducks, greylag geese, pigeons (Jonassen et al., 2005), and quails (Circella et al., 2007). IBVs have 2 genes termed genes 3 and 5 that are interspersed amongst the structural protein genes. Gene 3 has 3 ORFs, encoding proteins 3a, 3b, and 3c, of which 3c is the E protein. Gene 5 encodes 2 proteins, 5a and 5b. These genes are located as follows: -S-3a, 3b, 3c (E)-M-5a, 5b-N-. In this study, we discovered that a single mutation occurred at the corresponding position of the start codon of CK/CH/LLN/98I due to which this virus lacked ORF 3a. On the other hand, a putative transcription regulatory sequence (TRS), CTGAA-CAA, upstream the corresponding position of the 3a gene was detected. Therefore, we considered that the absence of ORF 3a in CK/CH/LLN/98I resulted due to a point mutation at the corresponding position in natural conditions, suggesting that ORF 3a was not necessary for the replication of the virus. This point mutation brought on a novel genomic organization at the 3′-encoding regions of CK/CH/LLN/98I: -S-3b, 3c-M-5a, 5b-N-. Recently, Mardani et al. submitted 3 Australian IBV strains, V6-92 and V18-91 lacking ORFs 3a, 3b, and gene 5 and Q3-88 lacking ORFs 3a, 3b, and 5a in the GenBank database (with accession numbers DQ490219, DQ490220, and DQ490212, respectively). These results and ours in the present study suggest that although the general gene order of IBV is 5′-S-3a, 3b, 3c-M-5a, 5b-N-3′ in the 3′-encoding regions, the absence of nonstructural protein genes was tolerated by the virus, as in other coronaviruses (De Haan et al., 2002). This implies that the native order is not functionally essential, as proven by engineered mutants (Casais et al., 2005; Hodgson et al., 2006).

It was believed that the S1 domain of IBV is the most divergent region of the molecule and is responsible for determining its serotype. On the contrary, apart from the S1 gene, the other parts in the 3′-encoding region were conserved

Table 4
Summary of the experimental infection of birds with 5 IBV strains

Group	Dose (log_{10} EID_{50}) [a]	Diseased chicks, *n* (%) [b]	Dead chicks, *n* (%) [b]	Antibody				Virus recovery [d]			
				4 days [c]	8 days	12 days	16 days	4 days	8 days	12 days	16 days
1 CK/CH/LDL/97I	4.1	10 (100)	1 (10)	0/10 [e]	9/10	9/10	9/10	+	+	+	−
2 CK/CH/LJL/04I	4.3	10 (100)	10 (100)	0/10	2/2	AD [f]	AD	+	+	−	−
3 CK/CH/LGD/04II	4.3	10 (100)	7 (70)	0/10	5/5	3/3	3/3	+	+	−	−
4 CK/CH/LSD/03I	4.2	10 (100)	1 (10)	0/10	10/10	10/10	10/10	+	+	−	−
5 CK/CH/LLN/98I	4.0	10 (100)	2 (20)	0/10	9/10	9/9	8/8	+	+	−	−
6 Control		0 (0)	0 (0)	0/10	0/10	0/10	0/10	−	−	−	−

[a] Dose per chick, 100 μl.
[b] Ten chicks per group.
[c] Days post-inoculation (PI).
[d] Two procedures were used for virus recovery PI. Firstly, lesions in embryos that had been inoculated with cloacal swab samples were observed. Secondly, RT-PCR using the 2 primers N (−) and N (+) on RNA recovered from allantoic fluid of the same eggs was conducted. The results from the 2 procedures were identical. + indicates positive results and − indicates negative results.
[e] Number seroconverted/number inoculated.
[f] All birds died.

among IBV serotypes. Similar to the other IBVs (Boursnell et al., 1987; Cavanagh, 2007), genes 5, N, and M of our 27 viruses were conserved both in nucleotide sequence and in length. However, the sequence length of other genes varied. The 9-bp deletion at the 3′-end of the S2 part of CK/CH/LSC/99I, the 30-bp insertion at the 5′-end of the 3b gene of CK/CH/LGD/04III, and the 21-bp deletion at the 3′-end of the 3c gene of CK/CH/LDL/97I and CK/CH/LDL/98I may indicate that the deletions or insertion of these sequences are not essential for IBV replication.

Recombination is undoubtedly a feature of the replication and evolution of IBV (Bochkov et al., 2006; Dolz et al., 2006; Kottier et al., 1995; Kusters et al., 1990; Ladman et al., 2006; Shieh et al., 2004; Wang et al., 1993) and other coronaviruses. It is believed that the conditions for recombination amongst the IBV strains in the field are as follows: an extremely large number of chickens, most maintained at high density; ease of spread of the virus; and serotype co-circulation, including proof of co-infection with more than one serotype in a given flock (Cavanagh, 2007). This is true in the case of CK/CH/LLN/98I and CK/CH/LSD/03I. In China, intensive chicken farms are concentrated in the Shandong and Liaonin provinces, where these 2 viruses have been isolated, respectively (Liu et al., 2006b). Since most of the chickens in these districts are maintained at a high density and intensively exposed to live vaccines, including Mass-type and J9, during the breeding period, the vaccines must certainly be implicated as a source for Mass- and J9-like sequences. The LX4- or CK/CH/LTJ/95I-like sources of genetic material were probably a strain that naturally infects these affected flocks (Liu and Kong, 2004; Liu et al., 2006b). The natural recombination event demonstrated here could suggest that the continuous use of live vaccines may actually contribute to natural recombination and IBV-associated disease (Wang et al., 1993). A potential recombination junction site was identified in the 5′-portion of the S1 gene upstream the HVR (Binns et al., 1986; Wang et al., 1993); however, CK/CH/LLN/98I and CK/CH/LSD/03I sites in this study were predicted to be located at the 3c and M genes, respectively. This might be due to the sequence conservation in those regions because the polymerase may attach to heterologous templates through base pairing of similar sequences on the newly synthesized strand when recombination occurred.

The predicted proteins of ORF 3a, 3b, 5a, and 5b were small and the functions of these gene products are not known. Recently, it was reported that these proteins were accessory proteins (Casais et al., 2005; Hodgson et al., 2006; Shen et al., 2003). Hodgson et al. (2006) modified gene 3 of IBV by using a reverse genetics system and discovered that the recombinant virus titers without the 3a protein produced in CK cells, embryos, and tracheal organ cultures (TOCs) were similar to those of the wild-type virus, although they declined earlier in TOCs, probably due to the absence of the 3a protein. Thus, these authors concluded that neither the tricistronic arrangement of gene 3 nor the 3a protein is essential for replication per se, suggesting that this protein may have roles *in vivo* (Hodgson et al., 2006). Our results indicate that a 43-bp nucleotide sequence was deleted at the 3′-end of the 3a gene in the IBV strain CK/CH/LSD/03I, resulting in a frameshift event and a C-terminally truncated protein, with 47 deduced amino acids. However, this strain had growth ability in embryos similar to that of other IBV strains. Moreover, the pathogenicity and replication in chicks were also identical to IBVs with the normal 3a gene in our study, suggesting that the deleted sequence was not essential for virus replication and pathogenesis either *in vitro* or *in vivo*. However, it is intriguing to note that the CK/CH/LLN/98I lacking ORF 3a had a visibly lower titer in embryos at 72 h post-inoculation compared with the other IBV strains carrying normal ORF 3a. The virus adapted ability in embryos varied among IBV strains, and we could not conclude that such a change may be due to the defect of ORF 3a. However, the other genes in the 3′-encoding region of CK/CH/LLN/98I had highly conserved sequences and structures with other strains. Although the relationship between the change in the virus titer and ORF 3a defect in this virus required confirmation by using the reverse genetics system, CK/CH/LLN/98I lacking ORF 3a as a wild-type IBV strain could be a more useful study tool as compared to the mutant engineered using the reverse genetic system. It is hypothesized that gene 3 may play important roles in the coronavirus pathogenesis to avian species. The replication and pathogenicity of CK/CH/LLN/98I was compared to those of the other IBV strains with normal ORF 3a by infecting chickens at a similar dose (EID_{50}) of virus. All 5 IBV strains could infect chickens, induce specific antibody, and secrete offspring viruses from the respiratory tract. Although we did not qualify the secreted viruses and evaluate the precise differences of replication ability from these viruses in chickens, our observation suggested that CK/CH/LLN/98I lacking ORF 3a could effectively replicate *in vivo* and infect chickens.

Acknowledgements

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