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Full-length genome sequencing analysis of avian infectious bronchitis virus isolate associated with nephropathogenic infection

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ABSTRACT Infectious bronchitis virus (IBV) produces infectious bronchitis (IB) disease in poultry worldwide. In spite of proper vaccinations against the IBV, new IBV strains are continually emerging worldwide. In this study, a new highly virulent nephropathogenic IBV strain named CK/CH/XDC-2/2013 was identified from a vaccinated flock with clinical signs of IB in the Jiangsu province of China. The full-length genome sequence of the isolate was 27,714 nucleotides long, and the genome was organized similarly to classical IBV strains. Minimum divergence, phylogenetic analysis, and distance matrix of the genome showed that the CK/CH/XDC-2/2013 isolate had the highest similarity to the IBV BJ strain. The spike glycoprotein (S) gene had the greatest similarity to the nephropathogenic BJ strain and showed an 8 amino acid insertion (YSNGNSDV) at 73 to 80 sites and 3 amino acid deletion at sites 126 to 128 compared to the IBV vaccine strains. A recombination analysis of the S gene showed that the new isolate evolved from the IBV BJ strain and the KM91 vaccine strain. An animal challenge experiment showed a mortality of 60 to 80% in early-age chickens by different inoculation routes. Pathological examinations of the kidneys revealed inflammation, distention with uric acid deposits, and tubular degeneration. It indicated that the CK/CH/XDC-2/2013 isolate has robust kidney tissue tropism, and new nephropathogenic IBV strains are continuously evolving in China.

Key words: infectious bronchitis virus, genome, recombination, pathogenicity

2016 Poultry Science 95:2921–2929
http://dx.doi.org/10.3382/ps/pew259

INTRODUCTION

Infectious bronchitis virus (**IBV**) is the causative agent of infectious bronchitis (**IB**) in chickens. It is clinically characterized by respiratory distress, tracheal rales, decreased feed intake, and poor egg quality and quantity (Cook et al., 2012; Li et al., 2012). In 1931, Schalk and Hawn first identified the respiratory disease of chicken in America; and in 1936 the virus was identified as the causative agent of infectious bronchitis (Cook, Jackwood and Jones, 2012).

IBV is a member of the family Coronaviridea, order Nidoviridae, and belongs to Gamma-coronavirus group 3 (Gonzalez et al., 2003; Zaher and Girh, 2014). The virus particle is enveloped, and has positive sense with single-strand RNA of approximately 27.6 Kb. The full-length genome consists of about 10 open reading frames (**ORFs**) (Liu et al., 2009b).

The proximal two-thirds of the genome encode 2 overlapping ORFs 1a and 1b. The remaining one-third genome consists of 4 structural proteins, spike glycoprotein (**S**), envelope protein (**E**), membrane protein (**M**), and nucleocapsid protein (**N**) (Cavanagh, 2007; Li et al., 2013). The S glycoprotein is cleaved into S1 and S2 sub-units. The S1 gene is involved in attachment to the host cell receptors, transferring viral genome, neutralizing, and haemagglutination inhibition of antibodies (Liu et al., 2006b; Cavanagh, 2007; Liu et al., 2008).

New IBV isolates have been identified by diversity and evolutionary changes in the amino acids (**aa**) (Jia et al., 1995; Abro et al., 2012a; Hussein et al., 2014; Najafi et al., 2016; Seger et al., 2016). In China, the first IBV isolate was identified in 1982, and several later IB outbreaks have been reported in spite of proper vaccinations (Liu et al., 2006b; Liu et al., 2008; Sun et al., 2011; Ma et al., 2012; Afifi et al., 2015). Consequently, new IBV QX and LX4 genotypes have been identified (Liu et al., 2009b; Zeshan et al., 2010; Zhang et al., 2010b; Zou et al., 2010b; Zhou et al., 2014b). Therefore, there is a need for surveillance of recently circulating IBV strains showing genetic, antigenic, and virulence diversity.

Received April 10, 2016.
Accepted June 26, 2016.
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In this study, we reported a novel IBV strain, named CK/CH/XDC-2/2013, which was isolated from a vaccinated chicken flock. In order to test a possible relationship between genetic variation and pathogenicity in chickens, the isolate was sequenced and analyzed.

MATERIALS AND METHODS

Virus Isolation

The IBV CK/CH/XDC-2/2013 strain was isolated from a chicken flock from Jiangsu province of China in 2013 by using 10-day-old specific pathogen free (**SPF**) chicken embryonated eggs (Nanjing Tech-Bank Bio-Industry Co. Ltd., Nanjing, China). The dead embryos had shown IBV like lesions, and their allantoic fluids were collected, titration calculated, and stored at −70°C.

Genomic Sequence

The viral RNA was extracted from the IBV CK/CH/XDC-2/2013 strain using RNAiso Plus (TaKaRa Biotechnology, Dalian, China) according to the manufacturer's protocol. cDNA was synthesized using the SuperScript™ III reverse transcriptase kit (Invitrogen, Carlsbad, CA) with oligodeoxynucleotide primers (TaKaRa Biotechnology Co., Ltd., Dalian, China). A total of 21 fragments, covering the whole genome, were amplified using polymerase chain reaction (**PCR**) with PfuUltra II Fusion HS DNA Polymerase (Stratagene Corp., La Jolla, CA). All primers used for PCR amplification were designed based on the IBV A2 strain (GenBank accession number EU526388) shown in Table 1. Race PCR was performed using the 5-full race kit (TaKaRa, Shuzo, Japan), adopting primers and protocol described previously (Zhao et al., 2013). All amplified fragments were cloned into the pEASY-Blunt cloning vector (Beijing TransGen Biotech Co., Ltd., Beijing, China). The full-length genome sequence of the viral strain was assembled from the acquired fragments using the Primer Premier Version 5.0 software program (Premier Biosoft International, 3786 Corina Way, Palo Alto, CA), Nucleotide Blast (http://blast.ncbi.nlm.nih.gov/Blast.cgi), and the ORF finder program (http://www.ncbi.nlm.nih.gov/gorf/gorf.html).

Sequence Alignment and Pairwise Comparisons

The full-length genome nucleotide sequence of CK/CH/XDC-2/2013 was aligned and analyzed for nucleotide homology and divergence percentage (Table 2) using the MegAlign 6 and DNASTAR software programs (Madison, WI).

Table 1. Primers designed for the amplification of CK/CH/XDC-2/2013 full-length genome.

Sr:#	Length/bp	Location	Upstream primers	Downstream primers
1	1041	437-1478	ATACGACGTTTGTAGGGG	GTGTTAAGTCATTTCGCATGC
02	1426	1397-2823	CAAGGTACTAAAGGTTTTGA	TTACCGTTCTTATCAACAAGT
03	1462	2723-4185	GCTGTGATCTACGAGAAAATG	GTAAAAACCTGCCCAAATTGA
04	1383	4105-5488	TCTTACAGAGGATGGTGTTAA	CCATAAGCCCATAGTAACACC
05	1420	5405-6825	TTGCGAATTCCCACCTTCTGG	CAAAGACATTGCGCATAATA
06	1433	6757-8190	TAATACACACAGTGCGCATGC	ACTAAACAAAAGTTCTCTAAC
07	1434	8106-9540	TTCCCAATGGGTTTTGTTTAA	ACTCTCCTTGACACTAATAAT
08	1440	9469-10909	CAACCTGACAAATTAGTTACT	AATATTCACTTAAATCAATAG
09	1446	10788-12234	TCTTGTTGAGTTACACAATAA	CTTTCTCCGTAGTAGGTATTT
10	1431	12164-13595	AAGTGCAGGAAATTTAGATG	CTGTCTGGTGTGTTATACCAG
11	1453	13519-14972	TTCTAACAATTTAGTTGATCT	CAAGCGGATATGCATCTATGG
12	1417	14931-16348	GACAGAGCCTGTGGCTGTTAT	AACATATTGGTAATTTATCTT
13	1484	16273-17757	GTTGGTAGACGAGGTTAGTAT	GAGCATGGCCGTGCACATTAC
14	1451	17661-19112	GTTTATAATCCACTTTTAGTG	ACGAGGTTCAAAAGTTTCATA
15	1443	19007-20450	AGATGGAGCGAACCTGTATGT	AGCACTACATAGTGCAAACA
16	1466	20336-21802	ACTGAACAAAAAACCGACTT	AACCCTCCAGCTGCTAAATAA
17	1396	21782-23178	GCAGAACTGGCCGAGGTTTTA	CATGTCTTCCACTACCACAAA
18	1530	23100-24630	GAATTAGCCACTCAAAAAATT	ATGCGGTTATAAATAGATTAT
19	1435	24506-25941	CCGAAGAACGGTTGGAATAA	CAAGTTTTCCCTTGGAATACT
20	1446	25869-27315	ACTTTCTTAACAAAGCAGGAC	AAACTGCAACCAACAAGGGA
21	1005	26701-27706	ATTCAGCACTTGGTGAAAATGA	TTTGCTCTAACTCTATACTAGC
	5′ RACE PCR PRIMERS			
	TR1[a]		CTCCCAGATTACGGTCAAAC	
	A1[b]		GTGATTTGTGGTGGTCTTGGAC	
	A2[b]		CGGTTTCTGTAAGGGCTAGTTGA	
	R1[c]		AGTGGAGTCCCCAACAAACC	
	R2[c]		GCGACTACGAAAGCGAAAA	

Primers position is listed according to A2 strain, Accession number (EU526388).
a = 5′ Phosphate primer used to amplify 5′RACE.
b = 5′ RACE primer 1.
c = 5′ RACE primer 2.

Table 2. Representative IBV strains used in this study.

Access: Numbers	IBV strains	Pathogenesis	Country	Year of isolation
KC119407	Ck/CH/LGD/120724	N/A	China	2012
JX897900	GX-NN09032	Resp/Nephro	China	2012
JX840411	YX10	Nephropathogenic	China	2010
HQ018914	CK/CH/SC/MS10	Nephropathogenic	China	2010
KF411041	CK/CH/LGX/091109	Nephropathogenic	China	2009
HM194666	ck/CH/LHLJ/090712	Nephropathogenic	China	2009
JF732903	Sczy3	Respiratory	China	2009
HQ018896	CK/CH/GD/LZ09	Nephropathogenic	China	2009
KF853202	SDZB0808	Nephropathogenic	China	2008
EU637854	CK/CH/LSD/05I	Respiratory	China	2008
EU526388	A2	Respiratory	China	2008
HM245923	DY07	Respiratory	China	2007
FJ345395	ck/CH/LSD/07-4	Nephropathogenic	China	2007
FJ345364	CK/CH/LDL/07I	Nephropathogenic	China	2007
JQ764826	GX-YL9	Respiratory	China	2007
HQ848267	GX-YL5	Nephropathogenic	China	2005
JF893452	YN	Resp/Nephro	China	2005
DQ001338	EP3	Respiratory	China	2005
JQ764818	GX-NN6	Nephropathogenic	China	2005
DQ288927	SAIBK	Nephropathogenic	China	2005
AY842862	W93	Nephropathogenic	China	2004
AY846750	28/86	Nephropathogenic	China	2004
HM245924	CQ04-1	Nephropathogenic	China	2004
AY319651	BJ	Nephropathogenic	China	2003
EU714029	SC021202	Nephropathogenic	China	2002
AF352313	ZJ791	Proventriculitis	China	2001
DQ068701	CK/CH/LDL/97I/97	Proventriculitis	China	1997
AY561713	Ma5	Nephropathogenic	USA	2004
AY514485	California 99	Respiratory	USA	1999
AF027512	Florida-18288	Respiratory	USA	1972
GU393338	JMK	Respiratory	USA	1964
GU393334	Gray	Nephropathogenic	USA	1960
KF696629	Connecticut	Respiratory	USA	N/A
DQ834384	M41	Respiratory	USA	1956
GU393336	Holte	Nephropathogenic	USA	1954
GQ504724	Massachusetts	Respiratory	USA	1941
DQ646405	TW2575/98	Nephropathogenic	Taiwan	1998
DQ646406	TW1171/92	Nephropathogenic	Taiwan	1992
AF250006	A1211	Respiratory	Taiwan	1992
EU817497	H52	Respiratory	Netherland	1955
FJ807652	H120	Respiratory	Netherland	1955
JQ088078	CK/SWE/0658946/10	Reproductive	Sweden	2010
KF377577	4/91	Respiratory	UK	1991
JQ977698	KM91	Nephropathogenic	South Korea	1991
DQ001339	p65	Respiratory	Singapore	2005
DQ490221	Vic	Nephropathogenic	Australia	2006

N/A - date not available

Phylogenetic Analyses, Selection Pressure and Recombination Analyses

Phylogenetic analysis of CK/CH/XDC-2/2013 was performed using MEGA version 6 (Tamura et al., 2013). The sequence of 46 IBV strains was downloaded from GenBank (Table 2). The 25 IBV full-length genome, S gene, E gene, M gene, and N gene sequences, and 46 S1 partial genome sequence were used for phylogenetic and molecular evolutionary analyses using the Neighbor–Joining method and Kimura-2 parameter method with bootstraps (1,000 replicates).

To assess the selective pressure on the spike gene, a codon based Morkov model of substitution was applied by using the PAML package (ver.14) (Yang et al., 2000). The calculations were performed by using synonymous (dS) and non-synonymous (dNS) substitutional differences among the codons to estimate the substitution rate.

To analyze the recombination events in spike glycoprotein, IBV spike gene sequences were aligned pairwise using the MegAlign program, DNASTAR software (version 6, Madison, WI). The recombination events were confirmed using the Recombination Detection Program (RDP V.3.44) (Martin and Rybicki, 2000; Posada and Crandall, 2001), at the highest P-value as 0.05.

Animal Challenge Experiment

Eighty one-day-old SPF chickens of white leghorns (Nanjing Tech-Bank Bio-Industry Co. Ltd., Nanjing, China) were randomly divided into 4 groups with 20 chickens per group. Groups A, B, and C were

inoculated with 100 μl allantoic fluid containing 10^3 EID_{50} of IBV CK/CH/XDC-2/2013 per chicken by oral, ocular, and nasal routes, respectively. Group D was inoculated with PBS orally as a control. The animals were kept in cages and provided food and water ad libitum. The chicks were observed daily for 15 d for clinical signs, morbidity, and mortality rates. Dead chickens were examined for gross and histopathological lesions, and the lung and kidney tissues were preserved in 4% buffered formalin. These samples were routinely processed and stained with hematoxylin and eosin stain. All animal experiments were approved by the Animal Care and Ethics Committee of Nanjing Agricultural University (permit number IACECNAU20130905).

RESULTS

Comparison of Full-length Genomic Sequence of CK/CH/XDC-2/2013

The full-length genome sequence of IBV CK/CH/XDC-2/2013 strain was submitted to GenBank under accession number KM213963. The sequence was 27,714 nt in length, excluding the poly (A) tails, including: 529 nt for the 5′ UTR, 11,918 nt for ORF1ab, 7,958 nt for ORF1b, 3,509 nt for the S structural gene (1,651 nt for S1 and 1,658 nt for S2), 173 nt for ORF3a, 188 nt for ORF3b, 308 nt for E gene, 677 nt for M gene, 197 nt for ORF5a, 248 nt for ORF5b, 1223 nt for the N gene, and 508 nt for the 3′ UTR, while, a non-coding region of 364 nt was identified in between the M gene and ORF5a.

The genome sequence analysis of CK/CH/XDC-2/2013 showed high identities (86.6%) of the spike (S) glycoprotein (S1 = 85.7 and S2 = 91.7%) with nephropathogenic IBV BJ strains (Table 3). All genes and 5′ end and 3′ end compared nucleotide similarity indices are summarized in Table 3. The CK/CH/XDC-2/2013 strain full-length genome pair wise nucleotide similarity was closely related to BJ and A2 strains (92.2 and 91.9%, respectively). In contrast, maximum divergence (16.6 and 16.4%, respectively) was found with H52 and H120 vaccine strain (Table 4).

The deduced amino acid sequence of the S gene when compared with the H120, H52, and Ma5 vaccine strains showed that the new strain had an insertion of 8 aa (YSNGNSDV) from position 73 to 80 and a deletion of 3 aa at position 126 to 128 (Figure 1).

Phylogenetic Analyses

Phylogenetic trees were constructed based on the complete genome, and of the S, S1, E, M, and N genes of IBV CK/CH/XDC-2/2013 (Figure 2). This indicated that the CK/CH/XDC-2/2013 strain was closely related to the Chinese strains BJ and A2 based on the full-length genome, and S, M, and N genes. However, the phylogenetic tree of the partial S1 gene indicated that the IBV isolates were distributed into 5 clusters (Figure 2c). CK/CH/XDC-2/2013 belongs to the second group with BJ and A2 strains.

Table 3. Pairwise comparison of nucleotide homology of different ORFs between CK/CH/XDC-2/2013 and other IBV strains (%).

IBV Strains	Full-length genome	5′UTR	1ab	1b	S	S1	S2	3a	3b	E	M	5a	5b	N	3′UTR
YX10	88.9	96.2	45.4	93.8	80.1	72.9	88.3	87.9	78.3	98.2	95	83.9	93.6	83.9	**98.6**
Connecticut	85.1	93.1	73.6	88.9	81.9	38.8	86	85.1	78.8	85.7	96.1	87.6	91.6	86.5	82.6
H52	84.5	94.3	67.4	88.7	37	74	85.3	81	79	89.5	90.9	88.2	90.4	88.8	97.9
SDZB0808	89.3	95.3	44.7	93.8	81.3	73.1	88.4	88.5	78.3	99.1	35.3	83.9	94.8	92.8	95.9
CK/CH/LSD/05I	85.7	93.6	44.1	89.6	79.5	76.7	85.8	80	68.8	85.6	**97.2**	87.1	91.6	86.5	90
BJ	**92.2**	99.4	44.7	94.4	**86.6**	**84.7**	**91.7**	89.1	78.2	92.9	92.1	**93.6**	87.6	95.6	90.6
Gray	84.5	94	45	88.7	79.3	68.8	85.5	88.5	76.9	89.5	89.9	87.6	90	90	82.2
DY07	89.5	94.7	43.5	93.9	81.5	73.2	88.5	87.9	78.3	99.1	95.1	87.1	**96.8**	93.2	97.9
GX-YL5	89	96	45.9	94	84.7	81.7	87.4	96	**89.5**	94.6	95.1	89.7	92.4	82.9	97.4
H120	84.6	94.2	67.2	89	36.6	73.9	85	81.6	78.5	88.6	95.7	88.8	91.6	88.8	76.8
Holte	83.9	93.8	74.2	89.1	78.5	69.6	85.5	82.7	75.8	90.1	90	92.9	91.2	90.3	69
EP3	84.9	92.9	44	88.8	36	74.6	86.6	78.4	78.5	89.8	**97.2**	88.4	90.8	83.8	83.9
KM91	85.4	93.8	75.1	89.7	80.3	77.8	88.6	81.9	86.6	90.9	91.9	86	90	91.1	91.7
M41	84.5	93.7	54.4	88.6	79.9	*38.4*	85.4	76.6	79	88.9	94.9	84.2	90.4	91.1	97.4
Massachusetts	84.7	93.8	54.4	88.6	80	38.9	85.4	76.6	79	89.2	94.9	84.2	90.4	90.9	96.8
SC021202	86.2	92.9	44.5	90.3	83.2	81.5	87.3	88.5	79.8	87	93.3	84.6	91.2	88.4	96.3
TW2575/98	85.5	95	**81.4**	89.3	77.7	70.6	84.1	86.2	80.2	90.7	96.6	82	90.4	**97**	90.4
YN	86.3	94.5	44.3	90.2	83	81.9	87.3	91.6	75.1	79.7	92.7	84	91.2	86.1	96.3
CK/CH/LGX/091109	89.1	95.8	47	94	85.8	82.5	88.4	87.9	77.7	**100**	93.5	84	96	93.8	97.2
Ck/CH/LGD/120724	88.9	95.9	44.6	93.8	84.5	81.5	87.4	**95.4**	87.7	91.4	90.1	88.4	95.6	89	97.1
CK/SWE/0658946/10	84	94.4	45.9	90.1	79.7	72.5	87.4	87.2	66.1	82.4	94	88.5	88	89.2	84.9
4/91	84.7	91.9	42.9	88.2	78.5	73.2	84.4	76.9	76.9	84.4	95	91.7	91.2	89.8	86.2
A2	91.9	**99.6**	47.7	**94.8**	86	73.3	88.3	88.5	66.4	92.5	94.9	85.2	**96.8**	92	95.6
SAIBK	85.4	91.4	47.2	89.3	83.3	81.3	87.8	87.4	76.1	88.7	95.2	86.5	92	84.9	97.3
GX-NN09032	86.1	96.2	46.8	**93.7**	73.8	63.4	75.4	86	83.4	87.1	93.2	82.1	90.8	81.3	92.2

Pairwise highest nucleotide homology is presented in bold numbers, and lowest nucleotide homology is underlined.

Table 4. Pairwise comparison of full-length genome sequence of CK/CH/XDC-2/2013 divergence distance with other IBV strains.

Percent Identity

	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24	25	26		
1		85.4	92.2	92.6	89.5	90.0	86.3	86.0	86.0	90.1	85.6	85.7	87.4	89.6	85.5	85.2	85.7	86.4	85.8	85.8	86.4	86.8	90.0	86.1	86.9	89.6	1	CK-CH-XDC-2-2013
2	16.3		85.5	85.7	87.1	86.0	87.6	91.9	90.3	86.3	89.6	90.0	85.1	86.0	91.8	91.0	89.7	89.3	89.5	89.5	87.6	87.5	86.0	86.8	87.4	86.2	2	4-91
3	8.2	16.2		93.6	90.0	90.8	86.7	86.6	86.1	90.6	85.9	85.8	87.3	90.1	86.0	85.8	85.8	87.2	86.0	86.0	87.5	88.0	90.5	86.4	87.9	90.0	3	A2
4	7.7	16.1	6.7		89.4	90.1	86.1	86.5	86.0	90.4	85.7	85.9	87.2	89.5	85.8	85.5	85.8	86.3	85.8	85.8	86.5	86.8	90.4	85.8	86.8	89.9	4	BJ
5	11.1	14.3	10.6	11.3		95.2	87.0	87.2	86.8	94.1	86.6	86.6	91.6	94.6	86.4	86.3	86.7	87.5	86.7	86.7	89.6	90.4	93.6	86.2	90.5	95.6	5	Ck-CH-LGD-120724
6	10.7	15.5	9.8	10.6	4.9		87.1	87.7	86.7	95.5	86.5	86.6	92.5	95.9	86.3	86.1	86.6	87.6	86.7	86.7	89.9	90.7	95.2	86.5	90.9	95.6	6	CK-CH-LGX-091109
7	15.1	13.6	14.7	15.5	14.5	14.2		88.0	90.5	87.1	88.6	89.2	85.4	87.1	88.4	88.3	88.8	89.6	88.6	88.6	88.3	88.4	87.0	90.9	88.4	87.0	7	CK-CH-LSD-05I
8	15.3	8.4	14.7	14.8	14.0	13.4	13.0		89.6	88.5	88.8	89.3	85.6	87.1	90.1	89.3	89.3	90.3	89.0	89.0	87.8	87.6	88.4	86.7	87.6	88.4	8	CK-SWE-0658946-10
9	15.6	10.3	15.5	15.5	14.7	14.7	10.2	11.1		86.6	92.8	93.7	85.5	86.8	92.3	91.9	93.4	92.1	92.8	92.8	88.2	87.9	86.6	87.8	87.7	86.7	9	Connecticut
10	10.4	15.3	10.0	10.2	6.1	4.5	14.3	12.4	14.8		86.5	86.5	92.2	94.6	86.3	86.1	86.7	87.7	86.7	86.7	89.2	89.6	97.1	86.4	89.8	96.3	10	DY07
11	16.1	11.1	15.7	15.9	14.9	15.0	12.3	12.2	7.5	15.1		91.7	85.2	86.7	92.1	92.1	91.9	90.2	93.6	93.6	87.9	87.8	86.4	87.3	87.6	86.5	11	EP3
12	15.9	10.6	15.7	15.7	14.8	14.8	11.6	11.4	6.4	15.0	8.6		85.4	86.6	91.3	91.2	93.2	91.8	92.0	92.0	88.0	87.6	86.6	87.4	87.5	86.7	12	Gray
13	13.8	16.9	14.0	14.0	8.9	7.9	16.5	16.0	16.5	8.2	16.7	16.4		92.7	85.1	84.9	85.4	85.6	85.5	85.5	87.6	88.3	92.0	85.1	88.3	92.2	13	GX-NN09032
14	11.0	15.6	10.6	11.2	5.6	4.2	14.3	14.1	14.7	5.6	14.7	14.9	7.6		86.6	86.4	86.6	88.0	86.8	86.8	90.4	90.9	94.0	86.6	91.0	94.7	14	GX-YL5
15	16.3	8.9	15.7	15.9	15.2	15.1	12.8	10.6	8.2	15.3	8.4	9.2	16.9	14.9		96.9	91.4	90.2	92.0	92.0	88.8	87.6	86.3	87.2	87.5	86.4	15	H120
16	16.5	9.9	15.9	16.2	15.3	15.3	12.9	11.5	8.6	15.5	8.3	9.3	17.1	15.2	3.2		91.0	89.8	94.8	94.8	88.7	87.6	86.0	87.0	87.4	86.2	16	H52
17	15.9	11.0	15.8	15.7	14.8	14.8	12.1	11.5	6.9	14.7	8.4	7.2	16.5	14.9	9.0	9.5		91.1	91.8	91.8	87.8	87.6	86.6	87.3	87.5	86.7	17	Holte
18	15.0	11.5	14.1	15.2	13.7	13.5	11.1	10.3	8.4	13.6	10.5	8.6	16.1	13.2	10.5	11.0	9.4		90.4	90.4	89.1	89.0	87.5	87.8	88.8	87.7	18	KM 91
19	15.8	11.2	15.6	15.8	14.7	14.7	12.3	11.9	7.5	14.8	6.5	8.3	16.5	14.7	8.4	5.2	8.5	10.2		99.9	88.0	87.9	86.7	87.3	87.8	86.8	19	M41
20	15.8	11.2	15.6	15.9	14.8	14.7	12.3	11.9	7.5	14.8	6.5	8.3	16.5	14.7	8.4	5.2	8.5	10.2	0.1		88.0	87.9	86.7	87.3	87.8	86.8	20	Massachusetts
21	15.0	13.5	13.8	15.0	11.2	10.8	12.6	13.3	12.8	11.7	13.1	12.9	13.7	10.4	12.0	12.2	13.0	11.6	13.2	13.2		94.7	88.9	88.2	94.4	89.5	21	SAIBK
22	14.6	13.7	13.2	14.6	10.4	9.9	12.6	13.4	13.3	11.2	13.4	13.5	12.8	9.8	13.6	13.7	13.6	11.9	13.3	13.3	5.5		89.4	88.1	98.2	89.9	22	SC021202
23	10.6	15.6	10.1	10.2	6.6	4.9	14.4	12.5	14.9	3.0	15.2	14.9	8.5	6.2	15.4	15.6	14.9	13.8	14.9	14.9	12.0	11.4		86.3	89.6	95.9	23	SDZB0808
24	15.5	14.7	15.1	15.9	15.3	15.0	9.7	14.7	13.5	15.2	14.1	13.9	16.8	14.8	14.3	14.5	14.1	13.4	14.1	14.1	13.0	13.1	15.3		88.1	86.5	24	TW2575-98
25	14.4	13.8	13.2	14.6	10.2	9.8	12.5	13.5	13.4	11.0	13.6	13.6	12.7	9.7	13.7	13.7	13.7	12.0	13.4	13.4	5.7	1.9	11.2	13.1		90.0	25	YN
26	11.1	15.4	10.7	10.8	4.5	4.5	14.3	12.5	14.8	3.7	15.1	14.8	8.2	5.4	15.2	15.4	14.8	13.5	14.7	14.8	11.3	10.8	4.2	15.1	10.7		26	YX10
	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24	25	26		

Divergence

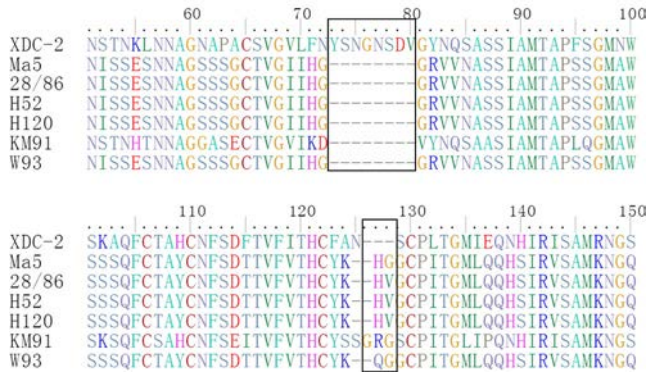

Figure 1. Alignment of spike glycoprotein of CK/CH/XDC-2/2013 strain with several representative vaccine strains. The aa insertion and deletion are indicated by boxes.

Analysis of Selection Pressure

The spike glycoprotein pairwise comparison results showed that most of the S1 sub-unit had positive codon selection of non-synonymous amino acid substitutions at specific regions encompassing positions 141 to 210, 236 to 303, 254 to 378, and 421 to 452. In contrast, most codon regions of the S2 sub-unit were highly conserved at regions 452 to 460 (Figure 3).

Recombination Analyses

The recombination hot spots of the complete S protein gene sequence of CK/CH/XDC-2/2013 were analyzed by the recombination detection program (**RDP**). The results showed true recombination between BJ (major parent) and KM91 (minor parent) with the break point at nucleotide position 2341 and the end point at nucleotide position 2566, using RDP, Geneconv, Chimaera, MaxChi, Bootscan, Siscan, and 3Seq analyses with average *P*-values of 1.040×10^{-07}, 7.686×10^{-06}, 3.637×10^{-01}, 2.358×10^{-02}, 1.070×10^{-07}, 5.068×10^{-06} and 1.070×10^{-07}, respectively (Figure 4).

Pathogenicity of the Isolate in Chickens

The infected chickens showed the earliest clinical signs 2 d after inoculation, and died between 4 and 6 days. The clinical manifestations included depression, decrease in feed intake, and ruffled feather with rapid body weight loss. The morbidity and mortality were more than 75 and 60% respectively (Table 5).

At necropsy, the chicken kidneys were prominently inflamed, and hyperemic renal tubules were distended with uric-acid crystal deposits (Figure 5). Histopathologically, there was hyperemia of the nephrons, accumulation of red blood cells, necrosis, and prominent monocytes infiltration in the epithelial cells (Figure 5). Meanwhile, no lesions were observed in the control group. In addition, IBV was detected in the kidneys and lungs from all infected chickens, using RT-PCR.

DISCUSSION

Infectious bronchitis is the most important devastating disease of the poultry industry throughout the

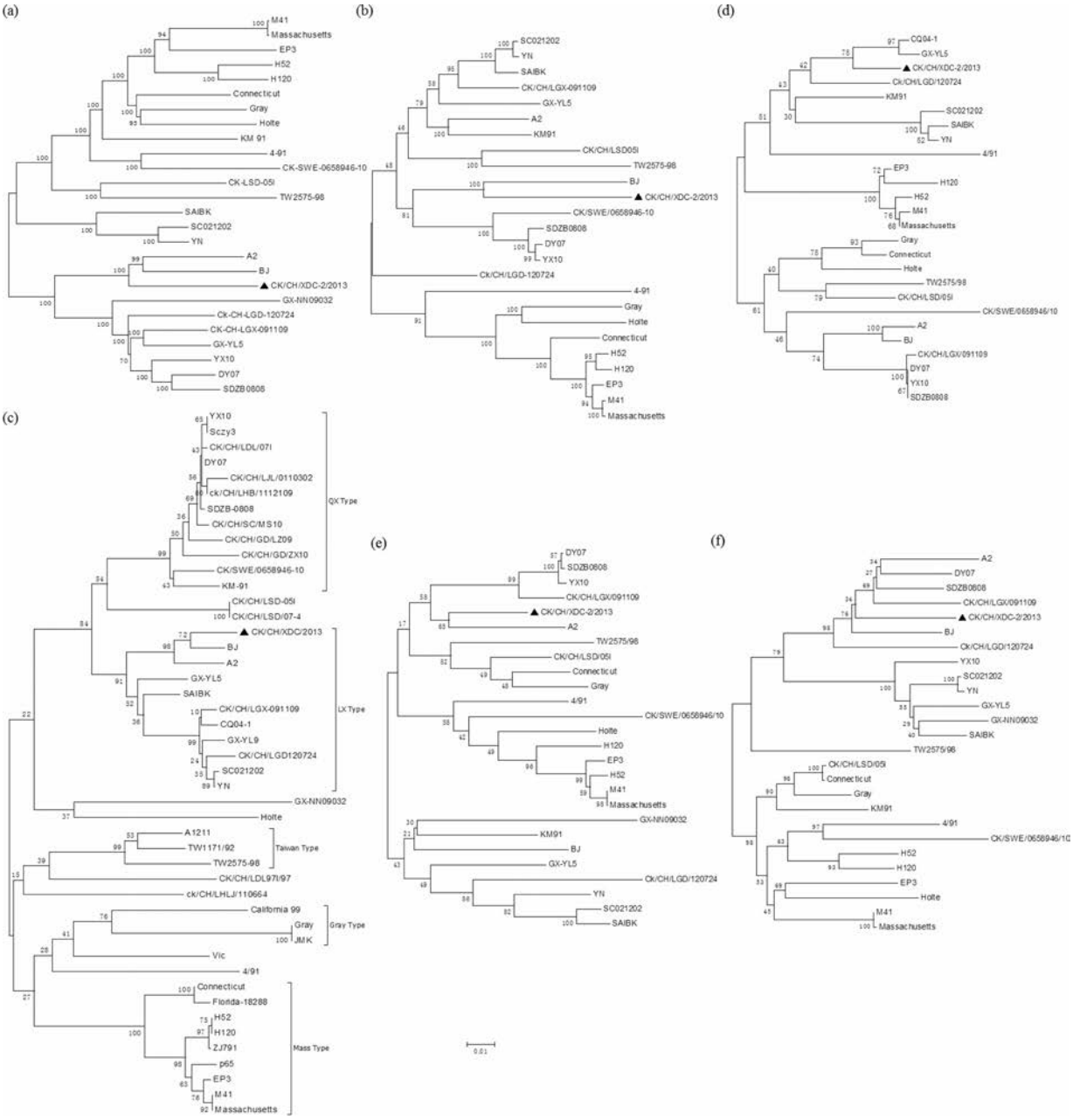

Figure 2. Phylogenetic analysis based on IBV strains nucleotide sequences of the full-length genome (a), S (b), S1 (c), E (d), M (e), and N (f) genes. The phylogenetic trees were constructed using the Neighbor-Joining method; bootstrap test (1,000 replicates) and Kimura-2 parameter method conducted in MEGA6. The bar represents the genetic distance of 0.01.

world. Many vaccines (W93, 28/86, H52, Ma5, and H120) are widely used, but they cannot provide complete protection against IBV infection (Liu and Kong, 2004).

In our study, we found a recombinant nephropathogenic IBV strain from an IBV-vaccinated chicken flock. The full-length genome sequence analyses of the IBV CK/CH/XDC-2/2013 isolate showed structural similarity (5′UTR-1ab+1b-S-3a+3b+E-M-5a+5b-N-3′UTR) to previously identified strains (Liu et al., 2009a; Zhang et al., 2010a). However, a non-coding region of an approximately 364 nt was identified as reported before (Liu et al., 2009a; Abro et al., 2012b). Phylogenetic analyses showed that the full-length genome and spike gene of CK/CH/XDC-2/2013 were closely related to the nephropathogenic IBV BJ strain, the E gene was close to the nephropathogenic GX-YL5, and the S1 gene revealed its relation to LX4 type cluster (Liu and Kong, 2004), which was circulating in more than 50% IBV strains in China (Zou et al., 2010a; Han et al., 2011). In addition, no part of the full-length genome was similar in nucleotides identity with any available vaccines (H120, H52, Connecticut, and 4/91), indicating that CK/CH/XDC-2/2013 was relatively close to previously identified clusters of Chinese strains identified between 2000 and 2012 (Liu et al., 2009b; Zou et al., 2010a; Ji et al., 2011).

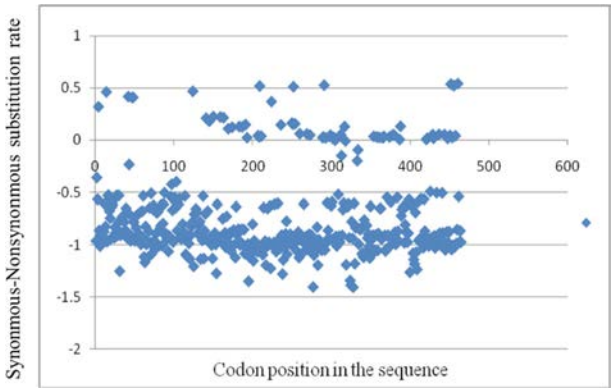

Figure 3. The selective pressure in S gene of CK/CH/XDC-2/2013 isolate illustrated that S glycoprotein gene has negative selective pressure emphasis on non-synonymous aa substitutions.

The spike glycoprotein gene always remains under pressure of mutational changes, and approximately 2 to 3% amino acid difference can decrease immune protection (Cavanagh, 2005). In this study, the S gene nucleotide identity of the CK/CH/XDC-2/2013 isolate was more dissimilar (37 and 36.6%) than those of H120 and H52 vaccine strain, respectively. Furthermore, the spike gene had an 8 amino acid insertions at the 73 to 80 site near or within HVR1 position in the S1 gene, and a 3 amino acid deletion as compared to IBV vaccines strains.

Comparison of synonymous and non-synonymous substitution rates provides vital information related to mechanisms of DNA sequence evolution. In the present study, there was evidence for positive selection in the regions of the S1 sub-unit. In contrast, no evidence for positive selection was found in the S2 sub-unit. These selective constraints in the spike gene of IBV are in accordance with a previous report (Abro et al., 2012b).

Table 5. Comparison of the morbidity and mortality of chickens challenged with different inoculation routes.

Group	Number of chickens	Morbidity rate (%)	Mortality rate (%)
A	20	85	75
B	20	80	80
C	20	75	60
D	20	00	00

Inoculation route, A = oral, B = eye, C = nasal, D = oral control.

The recombination events mostly occur naturally or mutationally in the IBV S gene (Wang et al., 1993). Here, the results showed that the S gene of CK/CH/XDC-2/2013 came from the recombination of the major parent BJ and minor parent KM91 strains, which suggested that more genotypic evolutionary and recombination events can occur under the pressure of widespread use of live attenuated IBV vaccines (Zhou et al., 2014a).

IBV isolates had been classified as nephropathogenic or respiratory, depending on clinical manifestations and lesions. Thus, gross and histopathological kidney lesions and mortality were used to assess nephropathogenicity

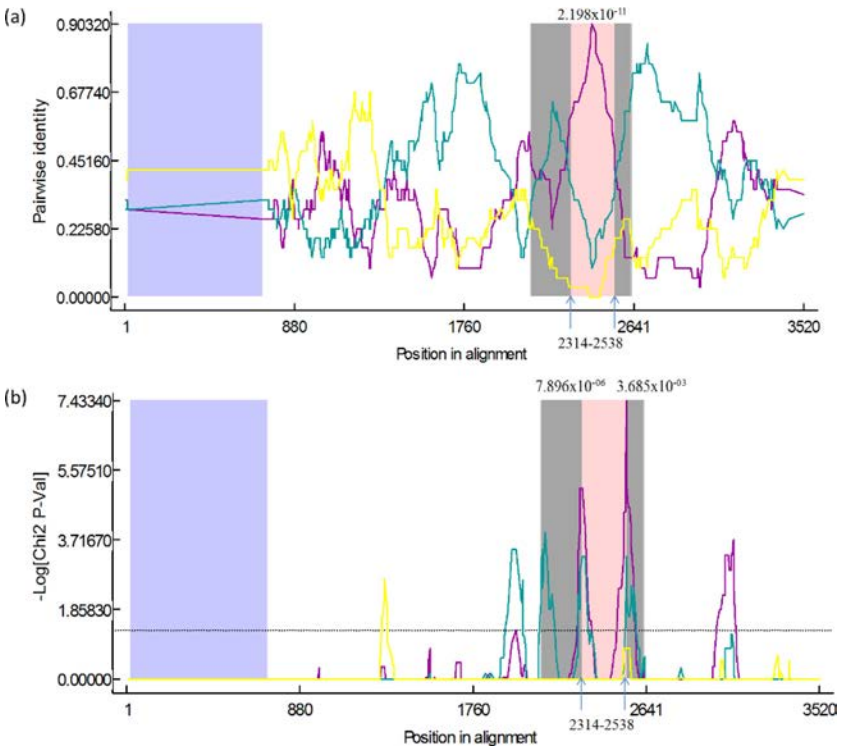

Figure 4. The recombinant event of CK/CH/XDC-2/2013 was analyzed by RDP (a) and MaxChi (b) analyses. The pink region displayed the potential recombination site; the yellow line indicates the percentage identity between the minor parent (KM91) and major parent (BJ). The green line shows the percentage identity between the major parent (BJ) and recombinant (CK/CH/XDC-2/2013). The variable size per window of RDP and MaxChi were selected at 30 and 70.

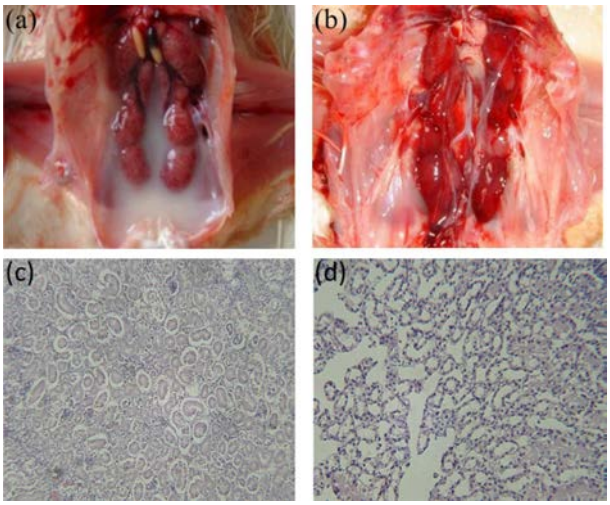

Figure 5. Gross and histopathological kidney lesions of chicken, inflammation, hyperemia, and distension with uric-acid crystal deposits in the kidneys of chickens challenged with CK/CH/XDC-2/2013 (a), non-infected control group (b), kidney nephrons and tubules showed degeneration and distension (c), hyperemic vessels with monocytes infiltration and epithelial necrosis (d) (H&E staining, 400×)

(Chong and Apostolov, 1982; Ignjatović and Sapats, 2000; Ignjatovic et al., 2002; Liu et al., 2006a; Zaher and Girh, 2014). In this study, the chicken challenge experiment showed high morbidity (85%) and mortality (80%) in one-day-old chickens, with similar clinical signs to field outbreak. All virus challenged chickens did not show prominent respiratory infection signs, such as sneezing, gasping, or coughing. At necropsy, there was no presence of pus or mucous clogging visually at the bronchi bifurcation region or in the trachea, as described (Grgiæ et al., 2008). The kidneys were highly inflamed and distended with uric acids deposits, indicating that this isolate had strong tropism to kidneys, as previously reported (Liu and Kong, 2004). Histopathological examination showed nephritis, necrosis, and monocytes infiltration in epithelial tissues in the kidneys, which were similar to previous reports (Benyeda et al., 2009). This demonstrated that this new isolated IBV strain belongs to high a nephropathogenic strain emerging from circulating field IBV strains.

CONCLUSION

The new IBV CK/CH/XDC-2/2013 isolate demonstrated characteristic features of nephropathogenic IBV in chickens. At necropsy, the chicken kidneys were prominently hyperemic, inflamed, and distended with uric-acid crystal deposits. The nucleotide sequence of the isolate showed recombination, insertions, and deletions in the spike gene, and apparent genetic variations in the ORFs regions of the genome.

ACKNOWLEDGMENTS

This work was supported mainly by the National Natural Science Foundation (31230071), grants from the Ministry of Education, China (20120097110043), and the priority academic program development of Jiangsu higher education institutions (PAPD). Authors are grateful to David Morrison, Uppsala University, Sweden, for editing the English language of this manuscript.

AUTHOR CONTRIBUTIONS

RAL and PJ designed the study and wrote the manuscript. RAL isolated the virus, completed genome sequencing and animal experiments, and analyzed the data. JB and BF carried out the PCR analyses and histopathological examinations. HW and LZ helped in collecting the samples from the poultry flocks. SHA helped in the data analysis of this manuscript.

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