Identification of a Newly Isolated Avian Infectious Bronchitis Coronavirus Variant in China Exhibiting Affinity for the Respiratory Tract

Author(s): Shengwang Liu, Yu Wang, Yazhen Ma, Zongxi Han, Qingxia Zhang, Yuhao Shao, Jianfei Chen, and Xiangang Kong
Source: Avian Diseases, 52(2):306-314. 2008.
Published By: American Association of Avian Pathologists
DOI: http://dx.doi.org/10.1637/8110-091307-ResNote.1
URL: http://www.bioone.org/doi/full/10.1637/8110-091307-ResNote.1

AVIAN DISEASES 52:306–314, 2008

Research Note—

Identification of a Newly Isolated Avian Infectious Bronchitis Coronavirus Variant in China Exhibiting Affinity for the Respiratory Tract

Shengwang Liu,[A] Yu Wang, Yazhen Ma, Zongxi Han, Qingxia Zhang, Yuhao Shao, Jianfei Chen, and Xiangang Kong

Division of Avian Infectious Diseases, National Key Laboratory of Veterinary Biotechnology, Harbin Veterinary Research Institute, the Chinese Academy of Agricultural Sciences, Harbin 150001, People's Republic of China

Received 14 September 2007; Accepted and published ahead of print 5 December 2007

SUMMARY. Twelve infectious bronchitis virus (IBV) isolates obtained from commercial chickens in China between 2005 and 2006 were characterized by reverse transcriptase-polymerase chain reaction (RT-PCR) and the sequencing of the entire S1 gene. CK/CH/LSD/05I—an IBV variant, which was unlike the nephropathogenic IBV isolates found in China—exhibited an affinity for the respiratory tract. The variant was identified by phylogenic analysis and basic local alignment search tool (BLAST) searches of the entire S1 gene and by the vaccination-challenge test that was performed using heterologous strains. Further, it was demonstrated that the commercially used H120 vaccine did not provide sufficient protection against this variant; however, the attenuated heterologous IBV tl/CH/LDT3/03 P_{120}, whose parent virus was isolated in China, showed a better efficacy of protection against CK/CH/LSD/05I. This study thus may demonstrate that the use of a combination of commercially available vaccines or of attenuated heterologous strains would provide satisfactory protection against the variant CK/CH/LSD/05I. In addition, the study also revealed that IBV strains exhibiting different pathogenicities were found cocirculating in the chicken flock in China.

RESUMEN. *Nota de Investigación*—Identificación de un nuevo coronavirus variante del virus de bronquitis infecciosa en China que muestra afinidad por el tracto respiratorio.

Mediante la prueba de transcriptasa reversa-reacción en cadena por la polimerasa y la secuenciación completa del gen S1, se caracterizaron doce aislados del virus de bronquitis infecciosa obtenidos en China entre los años 2005 y 2006, a partir de aves comerciales. La cepa CK/CH/LSD/051, una variante de bronquitis que fue diferente a los aislados nefropatogénicos encontrados en China, mostró afinidad por el tracto respiratorio. La variante fue identificada mediante análisis filogenético y comparación de secuencias publicadas para todo el gen S1, y mediante el desafío de aves vacunadas usando cepas heterólogas. Además, se demostró que la vacuna H-120 usada en las aves, no proporcionó protección suficiente contra esta variante, sin embargo, la cepa atenuada heteróloga identificada como tl/CH/LDT3/03 P_{120}, aislada en China, mostró mejor eficacia en la protección contra la cepa CK/CH/LSD/051. Este estudio demuestra que el uso de una combinación de vacunas disponibles comercialmente o de cepas heterólogas atenuadas puede proporcionar protección satisfactoria contra la cepa variante. Además, este estudio también revela que cepas del virus de bronquitis que muestran patogenicidad diferente han sido encontradas circulando en lotes de aves en China.

Key words: avian infectious bronchitis, coronavirus, variant, affinity, respiratory tract

Abbreviations: BLAST = basic local alignment search tool; bp = base pair(s); EID_{50} = median embryo infectious doses; ELISA = enzyme-linked immunosorbent assay; IB = infectious bronchitis; IBV = infectious bronchitis virus; Mass = Massachusetts; OD = optical density; RT = reverse transcription; RT-PCR = reverse transcriptase-polymerase chain reaction; SG = spike glycoprotein; SPF = specific-pathogen-free; S/P-ratios = serum-to-positive ratios; UTR = untranslated region

Infectious bronchitis virus (IBV) is a common cause of respiratory disease in chickens worldwide (10). The immunization of susceptible chickens with only one antigenic type of the virus has been shown to induce partial or no protection against the other unrelated types. Thus, the isolation, typing, and antigenic study of IBV field isolates have become increasingly important for monitoring the emergence of new serotypes or variant viruses. By constantly monitoring the IBV types, we have been able to track the changes in the incidence and distribution of IBV and identify the emergence of new problematic IBV types. The identification of new field IBV strains is also essential for evaluating the existing vaccination programs and for further development of new vaccination procedures for control of infectious bronchitis (IB). Furthermore, monitoring IBV types is also very important for the study of the evolution of IBV field isolates.

An IBV particle has three major virus-encoded structural proteins, namely, the spike glycoprotein (SG), the membrane protein, and the nucleocapsid protein. The SG of IBV is posttranslationally processed into S1 and S2 subunits (9). The S1 subunit has been shown to be responsible for attachment to the host's cellular membrane, for the induction of neutralization, and for the hemagglutination of inhibiting antibodies. It is also known to induce IBV-specific cytotoxic T lymphocyte responses in chickens (6,21,34). Due to its high variability and sequence correlation with serological grouping, the S1 gene of IBV is very often used to assess the genetic diversity and direction of evolution of the IBV (13,15,19,37). Moreover, S1 gene sequence comparison has recently been shown to be a better predictor of challenge of immunity than serotyping by virus neutralization (23).

The vaccines that are administered against IBV usually contain live-attenuated or killed virus strains of Massachusetts (Mass) serotype. However, vaccination programs for IBV are costly and difficult to implement because of the high risk of vaccination failure in the case of emergence of a novel virus strain (17,24,36). IBV vaccines selected for an individual flock typically represent the virus isolates that are most likely to be found when considering the

[A]Corresponding author. E-mail: swliu@hvri.ac.cn

Table 1. Types of IBV strains isolated from chicken flocks in different provinces between 2005 and 2006 in China.

IBV isolates	Province (Capital)[A]	Year[B]	Vaccinated or non-vaccinated	Organs[C] used for virus isolation	Production type	Chicken embryo passage[D]
CK/CH/LSD/05I	Shandong (Jinan)	2005	Vaccinated with H120 vaccine	Kidney	Broiler	4
CK/CH/LJL/05I	Jilin (Changchun)	2005	Vaccinated with H120 vaccine	Kidney	Broiler	3
CK/CH/LNM/05I	Inner Mengonia (Huhehaote)	2005	Vaccinated with H120 vaccine	Kidney and trachea	Layer hen	2
CK/CH/LHLJ/05I	Heilongjiang (Harbin)	2005	Vaccinated with H120 and W93 vaccines	Kidney	Broiler	3
CK/CH/LHLJ/05VI	Heilongjiang (Harbin)	2005	Vaccinated with W93 vaccine	Kidney	Broiler	2
CK/CH/LDL/05I	Dalian	2005	Vaccinated with H120 vaccine and then boosted with killed vaccine	Kidney	Layer hen	3
CK/CH/LDL/05II	Dalian	2005	Vaccinated with H120 vaccine	Oviduct	Layer hen	3
CK/CH/LDL/05III	Dalian	2005	Vaccinated	Kidney	Layer hen	4
CK/CH/LLN/06I	Liaonin (Shenyang)	2006	Vaccinated with H120 vaccine	Trachea	Layer hen	2
CK/CH/LGS/06I	Gansu (Lanzhou)	2006	Vaccinated with H120 vaccine	Kidney and trachea	Layer hen	5
CK/CH/LHLJ/06II	Heilongjiang (Harbin)	2006	Non-vaccinated	Kidney	Layer hen	2
CK/CH/LGX/06I	Guangxi (Nannin)	2006	Vaccinated	Kidney	Layer hen	8

[A]Province (Capital) where the viruses were isolated.
[B]Year when the viruses were isolated.
[C]Kidney = swollen kidney; oviduct = atrophic oviduct.
[D]Successive passages were performed until the dwarfing and death of embryos were observed between days 2 and 8 after inoculation.

geographic region and flock characteristics (including flock genetics and purpose). When new IBV isolates are discovered, the vaccination programs selected typically attempt to include vaccines that are antigenically similar to the field virus (2,20).

The primary aim of this study was to obtain the IBV field isolates from problem farms in different geographic regions in China (done in the period between 2005 and 2006), characterize them by molecular techniques, and determine the genetic relationships that exist between recent Chinese isolates and previously known IBV strains by using sequence data analysis. The secondary aim of this study was to investigate the protection against the variant provided by both the H120 vaccine and the attenuated heterologous strains in order to yield information that would be useful in vaccine selection and in the development of future IBV vaccination strategies.

MATERIALS AND METHODS

Virus isolates, vaccines, and heterologous strains. The kidney, trachea, and oviduct of chickens with IB symptoms were collected for virus isolation in China during 2005 and 2006. The epidemiological information regarding the field IBV isolates used in this study is presented in Table 1. The viruses were isolated (16) and passaged in 9- to 11-day-old specific-pathogen-free (SPF) embryonated chicken eggs. Allantoic fluid was harvested 48–72 hr postinoculation and used for coronavirus detection using electron microscopy as previously described (29) and for viral RNA detection by reverse transcriptase-polymerase chain reaction (RT-PCR).

One commercial Mass-type vaccine, H120 (3) and three attenuated heterologous IBV strains (CK/CH/LHLJ/04V, tl/CH/LDT3/03, and CK/CH/LDL/97I), representing the different serotypes of IBV found in China (28,30,31), were used in this study for the purpose of vaccination. IBV CK/CH/LSD/05I strain, a variant according to the genotype analysis of S1 gene, was used as the challenged strain and the virus was propagated once in the 9- to 11-day-old embryonated chicken SPF eggs, as described for field isolates (29), to yield a titer of $10^{7.2}$ EID_{50} before being used in the challenge-protection test.

Extraction of RNA and RT-PCR. Viral RNA was extracted from 200 μl of infectious allantoic fluid of each isolate using TRIzol regents (Invitrogen, Grand Island, NY) as per the manufacturer's protocol. RNA from the aqueous phase was precipitated by the addition of an equal volume of isopropanol and was pelleted by centrifugation at 13,000 × *g* at 4 C for 30 min. The RNA was air-dried for 2–10 min; it was then redissolved in 25 μl of Rnase-free water and stored at –70 C until further use.

The S1 genes of all IBV isolates except CK/CH/LSD/05I were amplified by reverse transcription (RT) using two negative primers (S1Oligo3′ and IBV-212) (1,30). A positive primer, IBV-257, was used with either S1Oligo3′ or IBV-212 during PCR for S1 gene amplification. All RT procedures were performed using 20 μl of RNA in a 40 μl reaction volume as previously described (29). Two microliters of the resulting 40 μl RT mixture was used for subsequent PCR.

A modified RT-PCR procedure had to be employed to amplify the S1 gene of the CK/CH/LSD/05I isolate since its genetic variation rendered the gene difficult to amplify using the primers described above. The sequences and locations of the primers used in this study are described in Table 2. The negative-sense primer IBV-168, specific to the conserved area located in the middle portion of the S2 gene of IBV, was used for RT. The positive-sense primer IBV-167, specific to the 3′ region of the S1 gene, together with primer IBV-168 was used for PCR. The CK/CH/LSD/05I isolate generated a product of approximately 1000 base pairs (bp) that was then sequenced directly. The negative-sense primer IBV-275, specific to the 3′ region of the S2 gene, was designed according to the sequencing results; it was then used for amplifying the entire S1 gene of the CK/CH/LSD/05I isolate. PCR was performed using primers IBV-257 and IBV-275, as previously described (26).

All RT-PCR products were analyzed by electrophoresis on a 1.0% agarose gel and visualized using ethidium bromide.

Cloning, sequencing, and analysis of the S1 gene. The PCR products were first directly sequenced and then sequenced again after being cloned into pMD18-T vector (TaKaRa, Dalian, China). Each region was sequenced at least in triplicate. The sequencing study determined the nucleotides that, due to the possible heterogeneity of the virus population, were present in a majority of viral RNAs at each position.

Sequences were compiled and open reading frames were determined by comparing the sequences with those of the IBV reference strains using the Gene Runner program (version 3.00, Hastings Software, Inc., Hudson, NY) (26). Multiple alignments and phylogenetic analyses were performed using the Clustal V routine of the MegAlign program provided in the DNAStar package (Windows 4.05, DNAStar, Madison, WI). Most of the IBV isolates selected for S1 gene comparison in this study have been isolated from chickens and other birds by different

Table 2. Sequences and genome localizations of the primers used in this study.

Oligonucleotide	Sense[A]	Sequence (5′ to 3′)	Gene	Position in genome[B]
S1Oligo5′	+	TGAAAACTGAACAAAAGAC	S1	20 302 to 20 320
IBV-257	+	TATTGATTAGAGATGTGG	S1	20 356 to 20 373
S1Oligo3′	–	CATAACTAACATAAGGGCAA	S1	22 002 to 22 021
IBV-212	–	ATACAAAATCTGCCATAA	S1	22 017 to 22 034
IBV-275	–	GTATGTACTCATCTGTAAC	S1	22 147 to 22 165
IBV-167	+	GCTTCTTGAGAA(T/C)CAGTTTTA	Partial S2	21 921 to 21 941
IBV-168	–	AGACGATCAACTTGTGCATCTG	Partial S2	22 952 to 22 973
N(+)	+	GATGCCCCAGCGCCAGTCATTAAA	N	25 903 to 25 926
N(-)	–	ACGCGGAGTACGATCGAGGGTACA	N	27 484 to 27 507

[A]+ = positive-sense; – = negative-sense oligonucleotides.
[B]The nucleotide positions correspond to those in the sequence of the IBV Beaudette genome (GenBank accession number M95169).

study groups in China during the past 10 years (4,25,26,27,29, 30,35,40,41,43) and represent different IBV genotypes. The other IBV strains that were selected by basic local alignment search tool (BLAST) searches were those that were closely related to CK/CH/LSD/05I. These strains included three Korean strains, one American strain, and four IBV field strains isolated in Taiwan. In addition, Mass type IBV vaccines that were widely used in the poultry industry in China were selected, along with our 12 IBV isolates, for phylogenetic analyses in this study. The accession numbers of the S1 genes of these IBV strains used for analyses are summarized in Table 3.

GenBank accession numbers of IBV sequences. The entire S1 gene nucleotide sequence, including the cleavage site, of each of the 12 IBV isolates in this study was deposited in GenBank with the following accession numbers: CK/CH/LSD/05I (EF213568), CK/CH/LGX/06I (EF213580), CK/CH/LNM/05I (EF213567), CK/CH/LDL/05I (EF213563), CK/CH/LHLJ/05VI (EF213565), CK/CH/LHLJ/05I

Table 3. IBV strains that were used for the sequence comparison of the S1 gene in this study.

Strain	Serotype	Geographic origin	Original description	Host	GenBank accession no.[A]
LX4	LX4	Xinjiang province, China	Liu and Kong, 2004 (29)	Chicken	AY189157
CK/CH/LHLJ/04V	LX4	Heilongjiang province, China	Liu *et al.*, 2006 (30)	Chicken	DQ167139
QXIBV	LX4	Shandong province, China	Pang *et al.*, unpublished	Chicken	AF193423
tl/CH/LDT3/03	tl/CH/LDT3/03	Guangdong province, China	Liu *et al.*, 2005 (26)	Teal	AY702975
CK/CH/LSC/95I	tl/CH/LDT3/03	Sichuan province, China	Liu *et al.*, 2006 (30)	Chicken	DQ167146
CK/CH/LSC/99I	CK/CH/LSC/99I	Sichuan province, China	Liu *et al.*, 2006 (30)	Chicken	DQ167147
CK/CH/LDL/97I	CK/CH/LDL/97I	Liaoning province, China	Liu *et al.*, 2006 (30)	Chicken	DQ068701
J2	CK/CH/LDL/97I	Shandong province, China	Yu *et al.*, 2001 (41)	Chicken	AF286303
D41	Mass	Vaccine strain	Liu *et al.*, 2006 (27)	Chicken	AY846836
HK	Mass	Vaccine strain	Liu *et al.*, 2006 (27)	Chicken	AY761141
W93	Mass	Vaccine strain	Liu *et al.*, 2006 (27)	Chicken	AY842862
H120	Mass	Vaccine strain, Netherlands	Bijlenga *et al.*, 2004 (3)	Chicken	M21970
TW1171/92	Taiwan group I	Taiwan	Huang and Wang, 2007 (19)	Chicken	DQ646406
TW2296/95	Taiwan group II	Taiwan	Huang and Wang, 2007 (19)	Chicken	DQ646404
TW2575/98	Taiwan group I	Taiwan	Huang and Wang, 2007 (19)	Chicken	DQ646405
3051/02	Taiwan group I	Taiwan	Huang *et al.*, 2004 (18)	Chicken	AY606318
Gray	Gray	USA	Wang and Collisson, unpublished	Chicken	L18989
SDW	Gray-like	Shandong province, China	Bing *et al.*, 2007 (4)	Chicken	DQ070840
PSH050513	Unknown	Shanghai, China	Qian *et al.*, 2006 (35)	Pigeon	DQ160004
ZJ971	Unknown	Zhejiang province, China	Zhou, 2000 (42)	Chicken	AF352313
JL/97/01	Unknown	China	Chen *et al.*, unpublished	Chicken	AF258780
SC021202	Unknown	Sichuan province, China	Zhou *et al.*, 2004 (43)	Chicken	AY237817
SC03-1	Unknown	Sichuan province, China	Xu *et al.*, 2007 (40)	Chicken	DQ459472
BJ03-1	Unknown	Beijing, China	Xu *et al.*, 2007 (40)	Chicken	DQ459475
CQ04-1	Unknown	Chongqing, China	Xu *et al.*, 2007 (40)	Chicken	DQ459476
YN05-1*	Unknown	Yunnan province, China	Xu *et al.*, 2007 (40)	Chicken	DQ459474
Partridge/GD/S14/2003	Unknown	Guangdong province, China	Fu *et al.*, unpublished	Partridge	AY646283
BJQ	Unknown	Beijing, China	Bing *et al.*, 2007 (4)	Chicken	DQ070839
NMC	Unknown	Neimenggu province, China	Bing *et al.*, 2007 (4)	Chicken	DQ973113
TJ/96/02	Unknown	Tianjing, China	Chen *et al.*, unpublished	Chicken	AF257075
TX94b	Unknown	China	Cui, unpublished	Chicken	AB274269
SH3	Unknown	Shanghai, China	Qian *et al.*, unpublished	Chicken	DQ069317
TX95	Unknown	China	Cui, unpublished	Chicken	AB274270
K514-03	KM91	Korea	Jang *et al.*, unpublished	Chicken	AY790360
K507-01	5th group of Korea	Korea	Lee *et al.*, 2004 (24)	Chicken	AY257064
K069-01	5th group of Korea	Korea	Lee *et al.*, 2004 (24)	Chicken	AY257061

[A]Based on S1 gene sequence.

Table 4. Results of serology and virus recovery postchallenge with CK/CH/LSD/05I strain (20 days postvaccination).

Group (Strains)[A]	Dose, median embryo infectious doses (log10)[B]	Morbidity (%)	Mortality (%)	Antibody (%)[C]		Virus recovery (%)[D]			
				15 days after vaccination	5 days postchallenge	Trachea	Kidney	Lung	Caecal tonsil
Group 1 (H120)	4.8	3/10 (30)	0/10 (0)	10/10 (100)	10/10 (100)	7/10 (70)	2/10 (20)	ND[E]	ND
Group 2 (CK/CH/LHLJ/04V P_{110})	5.0	0/10 (0)	0/10 (0)	7/10 (70)	10/10 (100)	10/10 (100)	0/10 (0)	ND	ND
Group 3 (tl/CH/LDT3/03 P_{120})	5.0	0/10 (0)	0/10 (0)	10/10 (100)	10/10 (100)	6/10 (60)	0/10 (0)	ND	ND
Group 4 (CK/CH/LDL/97I P_{115})	5.2	3/10 (30)	0/10 (0)	10/10 (100)	10/10 (100)	9/10 (90)	0/10 (0)	ND	ND
Group 5 (Positive control)	–	10/10 (100)	0/10 (0)	0/10 (0)	0/10 (0)	10/10 (100)	2/10 (20)	1/10 (10)	6/10 (60)
Group 6 (Negative control)	–	0/10 (0)	0/10 (0)	0/10 (0)	0/10 (0)	0/10 (0)	0/10 (0)	ND	ND

[A]Twenty chicks per group.
[B]Dose per chick, 100 µl.
[C]Number seroconverted/number inoculated.
[D]Two procedures were used for virus recovery after challenge. First, lesions in embryos that had been inoculated with individual tissue samples (trachea or kidney) were observed. Secondly, RT-PCR using oligonucleotide primers N(+) and N(−) on RNA recovered from allantoic fluid of the same eggs was conducted. The results from the two procedures were identical. Number of chicks which showed positive result after challenge/number of chicks survived after challenge.
[E]ND = not done.

(EF213560), CK/CH/LLN/06I (EF213566), CK/CH/LGS/06I (EF213564), CK/CH/LHLJ/06II (EF213561), CK/CH/LDL/05II (EF213559), CK/CH/LDL/05III (EF213558), and CK/CH/LJL/05I (EF213562).

Experimental design. Six groups of 20 White Leghorn SPF chickens (Harbin Veterinary Research Institute, China) were housed in separate isolators under negative pressure. They were provided with food and water *ad libitum*. Vaccine H120 and three attenuated heterologous IB strains were used for vaccination, and the vaccine was administered when they were 15 days old by oculonasal application. Groups 1 to 4 were inoculated with each of the four strains, and the remaining groups 5 and 6 were mock-inoculated with sterile allantoic fluid (Table 4). The actual vaccination dose per bird, as determined by the egg-titration of inocula immediately following vaccination, is summarized in Table 4. The chicks in groups 1 to 5 were challenged by the oculonasal application at 36 days of age with a virus dose per bird of $10^{5.2}$ EID_{50} of the IBV CK/CH/LSD/05I strain (group 5 was used as positive control). The chicks in group 6 were not challenged and served as a negative control.

Sampling. Ten chicks in each group were sacrificed by intravenous injection with barbiturate five days postchallenge, and tissue samples of kidney and trachea were collected (lung and cecal tonsil were also sampled in the positive-control group). Directly after sampling, each tissue was stored individually in 300 µl of virus isolation medium (50% glycerol; 50% phosphate-buffered saline) at –20 C until virus isolation. Blood samples were also collected on these days and stored at –20 C. The rest of the chicks in each group were examined daily for signs of infection for 30 days postinoculation.

Virus recovery and RT-PCR identification. Every tissue collected from each of the groups postchallenge was used for virus isolation. Individual samples containing 10,000 U penicillin and 10,000 µg streptomycin were inoculated into at least 4 SPF embryos via the allantoic cavity (0.2 ml per egg). The eggs were candled daily, and allantoic fluid from two of the inoculated embryos was collected 72 hr postinoculation for RT-PCR amplification. The remaining embryos were examined one week later for characteristic IBV lesions such as the dwarfing, stunting, or curling of embryos.

Two hundred microliters of allantoic fluid from each inoculated embryo were used for RT-PCR amplification. RNA was extracted and RT was conducted using IBV oligonucleotide N(–) (Table 2), which was specific to the 3′ untranslated region (UTR) as previously described (26). Oligonucleotides N(–) and N(+) (Table 2) were used to amplify most parts of the nucleocapsid gene and parts of the 3′-UTR from the allantoic fluids inoculated with samples collected postchallenge that were approximately 1600 bp in length (26). Both RT and PCR were carried out under identical reaction conditions as previously described (26). The PCR products were analyzed on a 1.0% agarose gel.

Total antibody enzyme-linked immunosorbent assay. Serum samples were assayed in single dilution using a commercial total antibody enzyme-linked immunosorbent assay (ELISA) (IDEXX Corporation, Westbrook, ME) as per the manufacturer's instructions. Serum-to-positive ratios (S/P-ratios) were calculated using the following formula: S/P-ratio = (OD sample – OD negative control)/(OD positive control – OD negative control), where OD stands for optical density. From these S/P-ratios, individual serum titers, expressed as $\log_2$ values, were calculated as per the manufacturer's instructions.

RESULTS

IBV CK/CH/LSD/05I strain was detected as a newly isolated variant. Virus field samples suspected of being IBV were collected from more than 37 Chinese poultry farms between 2005 and 2006; they were tested by IBV isolation and RT-PCR in this study. Twelve IBV isolates were isolated from both vaccinated and nonvaccinated birds and detected by electron microscopy examination. Almost all of the vaccinated flocks received live vaccines of the Mass serotype (Table 1). The birds were vaccinated once or twice, and at times in combination with Newcastle disease vaccines,

depending on the vaccination strategy selected. Except for the CK/CH/LLN/06I isolate, all the other isolates were obtained either from broilers with nephritis or from layers with nephritis that demonstrated poor egg quality and a decline in egg production (Table 1).

Based on our 12 isolates and 36 reference strains, a phylogenetic tree was constructed that represented most of the field and vaccine IBV strains found in China. The results of the phylogenetic analyses in this study paralleled our previous reports which categorized the Chinese IBV strains into different types (27,28,31). Based on the entire S1 gene sequences, it was demonstrated that the 12 isolates could be categorized into three clusters. Eight of them were placed into one group, together with the LX4-type strain (29). Three of the IBV isolates were clustered with the CK/CH/LSC/99I strain and belonged to the CK/CH/LSC/99I-type IBV strain. Isolate CK/CH/LSD/05I was significantly different from all the other 11 IBV strains investigated in this study, as well as from the 36 reference strains.

The S1 part of the SG protein of CK/CH/LSD/05I did not exhibit >87% identity with the reference IBV strains (Table 5). The BLAST searches that were conducted using the entire S1 gene revealed that CK/CH/LSD/05I was most closely related to the IBV isolates isolated in Taiwan and China. The isolate TW1171/92, isolated from a broiler in 1992 in Taiwan (18), shared the highest nucleotide identity (87%) with CK/CH/LSD/05I; the other strains did not share more than 86% nucleotide identity with CK/CH/LSD/05I.

The SG of IBV is first translated as a precursor protein (S0) and then cleaved into two subunits, S1 and S. The cleavage site sequences (Arg-Arg-Phe-Arg-Arg) of CK/CH/LSD/05I were the same as those of the strains CK/CH/LGX/06I and CK/CH/LNM/05I investigated in this study.

IBV CK/CH/LSD/05I exhibited an affinity for the respiratory tract. All the chicks in group 5 (positive-control group) exhibited severe respiratory clinical signs at about 4–12 days of postchallenge with the CK/CH/LSD/05I strain. These clinical signs included tracheal rales, watery eyes, nasal mucus, and sneezing, signs which were similar to those caused by other IBV strains that had an affinity for the respiratory tract (10). None of the chicks died during the experiment. Gross lesions at necropsy at five days postchallenge were mild and mainly confined to the respiratory tract. No clinical signs and gross lesions were observed in the negative-control group (group 6).

IBV CK/CH/LSD/05I were detected in the trachea by the reisolation of the virus using 9-day-old embryos and subsequent RT-PCR, and the challenge virus was detected in all of the challenge birds at five days postchallenge. However, only 1 and 2 out of 10 chicks were reported as positive for the virus, as determined by the virus recovery from their lung and kidney, respectively. Of the 10 chickens inoculated with CK/CH/LSD/05I, six were reported as positive for the virus, as revealed by the virus recovery from cecal tonsils. The virus was not detected in the trachea and kidneys of the unchallenged negative-control birds. None of 10 chicks challenged with CK/CH/LSD/05I exhibited seroconversion at five days postchallenge, and no antibodies were detected in any of the birds at five days postchallenge by ELISA.

The heterologous IBV strains provided poor respiratory protection against CK/CH/LSD/05I. As summarized in Table 4, each of the 30% of vaccinated birds in groups 1 and 4 exhibited respiratory clinical signs and mild gross lesions similar to the birds in group 5 at necropsy five days postchallenge. All the birds in groups 2 and 3 that were vaccinated with tl/CH/LDT3/03 P_{120} and CK/CH/LHLJ/05V P_{110}, respectively, did not exhibit the clinical signs. The clinical signs in the diseased birds tended to disappear gradually after 15 days of challenge. None of the chicks in the four vaccinated groups died during the experiment.

It is important to consider that the rates of challenge-virus isolation from the respiratory tract were high (50%) and were low from the kidney in birds vaccinated with the heterologous vaccine (Table 4). More than 60% of the chicks tested positive for IBV, as determined by the reisolation of the virus from the trachea at day five postchallenge in all vaccinated groups; however, the three attenuated Chinese heterologous IBV provided 100% protection to the kidney against the CK/CH/LSD/05I challenge. This result was in contrast to that obtained for the H120-vaccinated and positive-control groups in which 2-out-of-10 chicks tested positive for presence of the virus in the kidney.

The serum antibodies, as detected by ELISA in all of the vaccinated birds and challenged birds at necropsy, are summarized in Table 4. The antibodies were not detected in the nonvaccinated birds or in the nonvaccinated and unchallenged birds.

DISCUSSION

In this study, we isolated and genetically typed 12 IBV isolates from the 2005 to 2006 outbreaks in China and compared them to both previously isolated Chinese IBV isolates and to other reference strains. In general, IBV isolates circulating in the recent years mainly belonged to the LX4-type (29) and CK/CH/LSC/99I-type (30). This was consistent with the other studies conducted in China (4,35,40), suggesting that the two types of IBVs were the major problems faced by the Chinese poultry producers in recent years—even though vaccines based on the Mass-type strains such as H120 and H52 have been in use for many years in poultry farms. Considering the poor protection against the Chinese IBV isolates provided by the available commercial vaccines (27,31), the regular IBV-suspected disease occurrences, and the isolation of IBVs in vaccinated flocks (4,25,29,31,38,39,40,41,43), vaccine development and changes in the IB control programs in China require consideration.

We typed the Chinese IBV isolates into several groups according to their genotypes and protectotypes (27,29,30,31). In addition, in 2007 Bing *et al.* (4) reported a Gray-like strain that was isolated in China. In this study, we isolated an IBV strain (CK/CH/LSD/05I) and considered it to be a new IBV variant based on the following: 1) phylogenetic analyses, with respect to the entire S1 gene of 36 reference IBV strains and 11 new isolates, proved that CK/CH/LSD/05I did not belong in any of the known groups in China (Fig. 1); 2) BLAST searches using the S1 gene of CK/CH/LSD/05I revealed that the most closely related IBV strain was TW1171/92, which shared 87% nucleotide identity with CK/CH/LSD/05I; and 3) four IBV attenuated heterologous strains provided poor respiratory protection against CK/CH/LSD/05I challenge. However, IBV possesses a large RNA genome of approximately 27.6 kb. Due to the occurrence of point mutations, insertions, and deletions introduced into the viral genome by the viral RNA-dependent RNA polymerase (which lacks proofreading capabilities), as well as through genetic recombination which occurs by a genomic template-switching mechanism, IBV can undergo extensive antigenic variation (33). Thus, the definitive origin and evolution of CK/CH/LSD/05I might be clarified by further sequencing and by an analysis of the entire genome of the virus.

In addition to possessing the ability to replicate in many respiratory tissues, IBV grows on many other epithelial surfaces

Table 5. Nucleotide and amino acid similarities of the S1 protein gene[A] among our Chinese IBV isolates and other IBV strains.

	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24
Amino acid identity (%)																								
1. CK/CH/LSD/05I		86.2	84.2	85.1	80.9	81.1	80.2	81.5	81.5	80.2	84.4	80.7	80.5	80.7	80.0	80.5	81.1	79.8	80.4	80.7	81.3	80.9	74.4	75.1
2. TW1171/92	87.0		86.2	90.4	82.2	82.4	81.8	82.7	82.9	81.8	83.3	79.3	79.1	79.3	78.2	79.1	79.6	77.8	79.1	78.2	78.7	78.2	77.3	78.0
3. TW2296/95	85.7	88.3		86.5	82.2	82.4	81.6	81.6	81.3	80.5	81.1	77.1	76.9	77.1	76.2	76.7	77.5	76.0	76.9	77.3	77.6	78.0	75.6	76.4
4. TW2575/98	86.1	94.2	88.5		81.5	81.6	81.1	82.5	82.2	80.4	81.8	79.6	79.5	79.6	79.1	79.3	80.4	78.5	80.5	78.5	79.6	78.7	76.5	76.7
5. W93	81.2	81.5	82.5	81.4		99.3	97.8	77.3	77.5	77.6	79.3	76.2	76.0	76.2	75.5	76.2	76.7	74.7	75.6	75.6	76.7	76.2	73.5	74.2
6. H120	81.3	81.5	82.6	81.4	99.6		98.5	77.6	77.8	77.8	79.5	76.4	76.2	76.4	75.6	76.7	76.9	74.9	75.8	75.8	76.9	76.5	73.8	74.5
7. ZJ971	81.2	81.4	82.4	81.2	99.2	99.5		77.3	77.5	77.5	78.7	75.5	75.3	75.5	74.7	75.8	76.0	74.0	74.9	74.9	76.0	75.6	73.1	73.8
8. CK/CH/LGX/06I	81.8	82.0	81.2	81.2	79.7	79.8	79.7		97.8	87.1	84.9	77.6	77.6	77.8	77.3	77.3	78.4	75.8	78.0	77.5	77.8	77.5	74.0	74.5
9. CK/CH/LNM/05I	81.8	82.1	80.9	81.1	79.7	79.8	79.7	99.0		87.1	86.2	77.8	77.8	78.0	77.8	77.3	78.4	76.4	78.4	77.8	78.4	78.2	73.8	74.4
10. CK/CH/LDL/05I	81.5	81.7	81.4	80.5	79.7	79.8	79.8	91.4	91.1		82.7	79.5	79.5	79.6	78.9	79.1	79.8	77.8	79.6	79.3	79.6	79.3	73.8	74.5
11. tl/CH/LDT3/03	84.3	83.6	82.0	81.8	81.5	81.6	81.4	89.5	89.6	86.9		82.7	82.7	82.9	82.5	82.9	84.2	82.4	84.2	83.3	84.9	83.5	72.7	73.5
12. CK/CH/LHLJ/04V	79.4	77.7	76.3	77.2	76.8	76.9	76.7	78.9	78.7	78.7	84.4		99.6	99.5	95.6	96.5	96.9	94.7	94.4	94.5	94.9	93.6	75.5	76.0
13. CK/CH/LHLJ/05VI	79.4	77.7	76.2	77.2	76.8	76.9	76.7	78.9	78.8	78.7	84.5	99.8		99.5	95.6	96.5	96.9	94.7	94.4	94.5	94.9	93.6	75.5	76.0
14. CK/CH/LHLJ/05I	79.4	77.7	76.3	77.2	76.8	76.9	76.7	78.9	78.8	78.7	84.5	99.8	99.8		95.8	96.4	97.1	94.5	94.5	94.7	95.1	93.8	75.6	76.2
15. CK/CH/LLN/06I	80.0	77.7	76.5	77.6	77.1	77.2	77.0	78.8	78.7	78.8	84.3	97.0	96.9	97.0		94.9	95.6	92.7	93.5	93.8	94.5	93.1	74.4	74.9
16. CK/CH/LGS/06I	79.7	78.0	76.3	77.4	77.2	77.5	77.3	79.2	79.1	79.0	85.1	97.2	97.1	97.1	96.6		98.4	94.2	94.4	94.0	94.7	93.5	75.1	75.6
17. CK/CH/LHLJ/06II	79.7	78.3	76.9	77.8	77.4	77.5	77.3	79.4	79.3	79.2	85.6	97.1	97.1	97.2	96.5	98.7		94.9	95.6	95.5	96.0	94.4	75.6	76.2
18. CK/CH/LDL/05II	78.6	77.0	75.5	76.6	76.4	76.6	76.4	77.6	77.7	77.7	84.2	96.4	96.3	96.3	94.9	95.9	96.2		93.6	92.7	94.4	93.1	74.4	74.9
19. LX4	80.0	78.4	76.7	78.2	77.5	77.6	77.4	79.4	79.4	79.2	85.5	95.7	95.7	95.8	95.4	96.4	97.0	95.3		94.9	96.2	94.7	74.9	75.5
20. CK/CH/LDL/05III	79.7	78.1	76.9	77.6	77.3	77.4	77.2	79.4	79.4	79.2	85.5	95.9	95.8	95.9	95.4	96.5	97.0	95.2	97.0		96.4	94.9	74.2	74.7
21. QXIBV	80.2	78.5	77.2	78.2	77.7	77.8	77.7	79.8	79.8	79.7	86.3	96.4	96.4	96.5	95.9	96.7	97.4	96.3	97.5	97.5		95.6	75.1	75.6
22. CK/CH/LJL/05I	79.5	78.0	76.8	77.7	76.6	76.8	76.6	79.1	79.1	79.0	85.1	94.8	94.7	94.8	94.5	94.9	95.3	94.5	95.4	95.4	96.4		74.5	75.1
23. CK/CH/LDL/97I	75.6	76.1	76.3	76.4	75.5	75.6	75.4	75.3	75.4	75.2	75.8	75.4	75.4	75.5	75.3	75.7	76.0	74.8	76.0	75.9	76.4	75.2		98.9
24. J2	75.8	76.3	76.5	76.4	75.8	75.8	75.7	75.5	75.6	75.4	76.1	75.7	75.7	75.7	75.5	76.0	76.3	75.2	76.2	76.1	76.6	75.4	99.5	
Nucleotide identity (%)																								

[A]The first 1647 nucleotides, starting at the AUG translation start codon of the S1 protein genes, were compared.

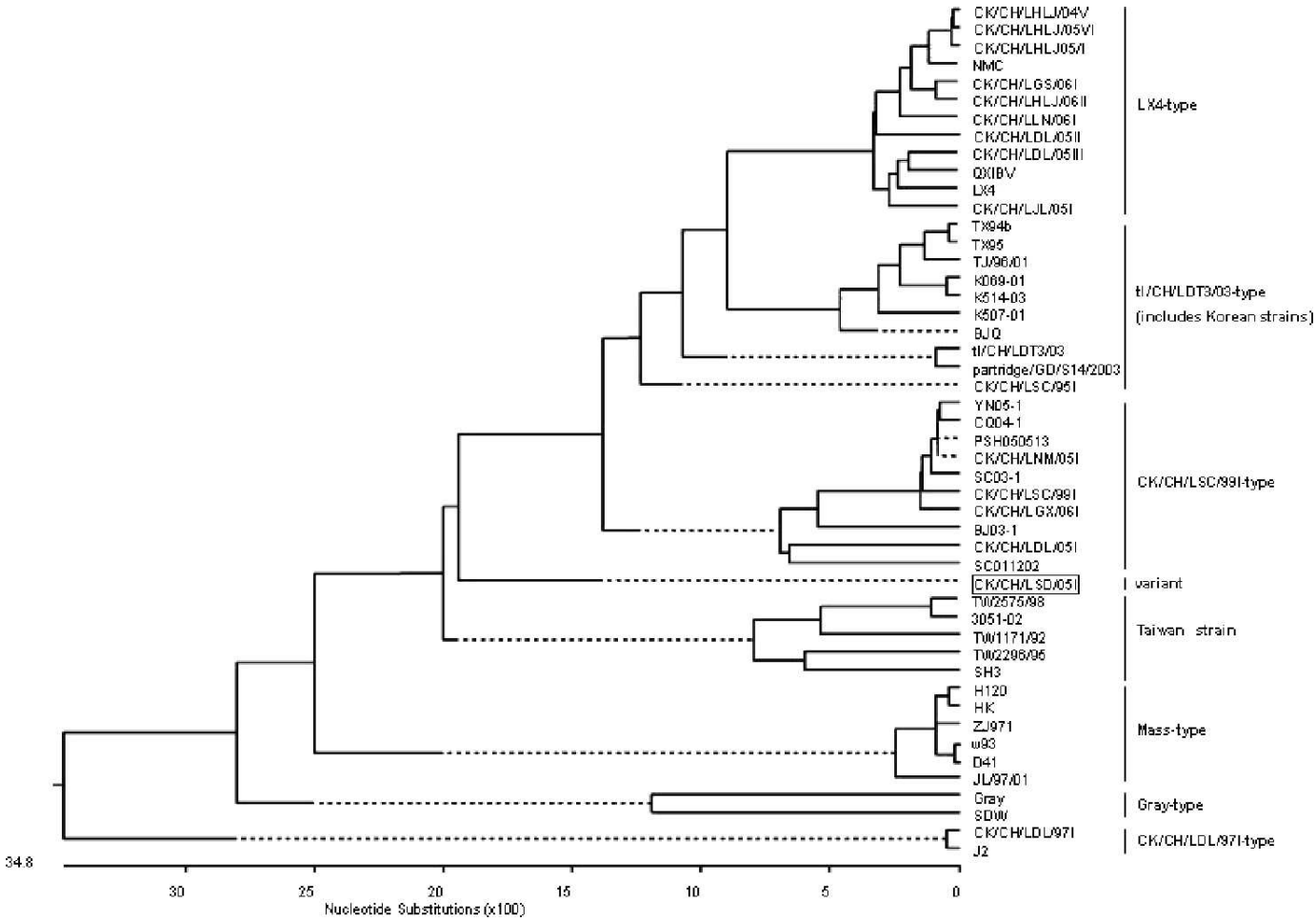

Fig. 1. Analysis of the phylogenetic relationships of newly isolated Chinese IBV strains and selected reference strains based on their entire S1 gene sequences (the first 1560 nucleotides starting at the AUG translation-initiation codon of the S protein genes) using the Clustal V routine of the MegAlign program provided in the DNAStar package (26). The IBV isolates analyzed in this study are represented in bold type.

including those in the kidneys, oviducts, testes, and in several parts of the alimentary tract. Nephropathogenic IBVs have posed a major problem to the chicken flocks in China since first reported (4,25,29,30,38,39,40,41). However, in this study no gross lesions were observed in the chicks inoculated with the variant CK/CH/LSD/05I, although this virus was isolated from the kidney of a diseased chick in the field condition. Furthermore, only 2 out of 10 chicks tested positive for virus recovery from the kidney, suggesting that this virus was not nephropathogenic. Several isolates of IBV, such as the Moroccan G strain, have been isolated from the gut and have been found to be enterotropic (11,14,32). We did not investigate the presence of the challenge virus in the oesophagus, proventriculus, duodenum, and jejunum; however, virus recovery was attempted from the cecal tonsils, and 60% of the inoculated chicks tested positive. In contrast to the kidneys, lungs, and cecal tonsils, the challenge virus was recovered from the trachea of all the chicks inoculated with CK/CH/LSD/05I, suggesting that this virus has a high affinity for the respiratory tract. For certain coronaviruses, it has been shown that the S protein is a determinant of tissue tropism (2,22); however, virtually nothing is known about the determinants of pathogenicity for IBV (8). CK/CH/LSD/05I could be a useful tool to study tissue tropism of IBV.

It was reported that the titers of live IBV are the highest in the nose and trachea within three days and remain so for an additional 2–5 days (7). Similar virus titers were detected in the lungs and air sacs. Recently, the analysis of the viral load in the trachea of birds that were administered different doses of IBV revealed that regardless of the initial dose of virus, a similar maximal amount of viral RNA was detected at five days postinoculation by real-time RT-PCR (5). For the efficacy of IBV vaccines, as outlined in the Code of Federal Regulations of America, the detection of challenge virus is required to be conducted at five days postinoculation (http://www.access.gpo.gov/nara/cfr/waisidx 99/9cfr113 99.html). Therefore, to determine the efficacy of protection in this study, virus recovery was conducted five days postchallenge. The gross lesions of the CK/CH/LSD/05I-infected chicks were mild, and serum antibody was not detected by ELISA at five days postinoculation. In the vaccination-challenge test, the respiratory clinical signs were observed at about 4–12 days postchallenge with the CK/CH/LSD/05I. Therefore, the gross lesions were delayed when compared to the time of appearance of the highest viral titer, and serum antibody was not produced at five days postinoculation. Our previous study demonstrated that different serotypes of IBV were cocirculating in the chicken populations in China (27,30,31). The present results revealed that IBV strains of different pathogenicities were also cocirculating in the chicken flocks in China.

Commercially used H120 vaccine could not provide sufficient protection against CK/CH/LSD/05I challenge in the vaccination-

challenge test in this study. This might also explain the recovery of CK/CH/LSD/05I from birds vaccinated with the IBV H120 vaccine. However, two attenuated heterologous IBV strains, tl/CH/LDT3/03 and CK/CH/LHLJ/04V, offered satisfactory clinical and kidney protection, but poor respiratory protection, against CK/CH/LSD/05I. Both IBV tl/CH/LDT3/03 and CK/CH/LHLJ/04V were nephropathogenic strains, and satisfactory kidney protection was expected. These results might suggest that the combined use of different serotypes of IB vaccines at different stages in a chicken's lifespan might help broaden the protection against the challenge of IB isolates of several different serotypes and variants, thereby eliminating the need for the development of a new IB vaccine to combat every new IB serotype that might emerge (12). The use of a combination of commercially available vaccines, or that of two attenuated heterologous strains as a means of protection against the variant CK/CH/LSD/05I, require further investigation.

REFERENCES

1. Adzhar, A., R. E. Gough, D. Haydon, K. Shaw, P. Britton, and D. Cavanagh. Molecular analysis of the 793/B serotype of infectious bronchitis virus in Great Britain. Avian Pathol. 26:625–640. 1997.

2. Alvarado, I. R., P. Villegas, J. El-Attrache, and T. P. Brown. Evaluation of the protection conferred by commercial vaccines against the California 99 isolate of infectious bronchitis virus. Avian Dis. 47:1298–1304. 2003.

3. Bijlenga, G., J. K. A. Cook, J. Gelb Jr., and J. J. de Wit. Development and use of the H strain of avian infectious bronchitis virus from the Netherlands as a vaccine: a review. Avian Pathol. 33:550–557. 2004.

4. Bing, G. X., X. Liu, J. Pu, Q. F. Liu, Q. M. Wu, and J. H. Liu. Different genotypes of nephropathogenic infectious bronchitis viruses co-circulating in chicken population in China. Virus Genes 35:333–337. 2007.

5. Callison, S. A., D. A. Hilt, T. O. Boynton, B. F. Sample, R. Robison, D. E. Swayne, and M. W. Jackwood. Development and evaluation of a real-time Taqman RT-PCR assay for the detection of infectious bronchitis virus from infected chickens. J. Virol. Meth. 138:60–65. 2006.

6. Casais, R., B. Dove, D. Cavanagh, and P. Britton. Recombinant avian infectious bronchitis virus expressing a heterologous spike gene demonstrates that the spike protein is a determinant of cell tropism. J. Virol. 77:9084–9089. 2003.

7. Cavanagh, D. Severe acute respiratory syndrome vaccine development: experiences of vaccination against avian infectious bronchitis coronavirus. Avian Pathol. 32:567–582. 2003.

8. Cavanagh, D. Coronavirus avian infectious bronchitis virus. Vet. Res. 38:281–297. 2007.

9. Cavanagh, D., P. J. Davis, D. J. Pappin, M. M. Binns, M. E. Boursnell, and T. D. Brown. Coronavirus IBV: partial amino terminal sequencing of spike polypeptide S2 identifies the sequence Arg-Arg-Phe-Arg-Arg at the cleavage site of the spike precursor propolypeptide of IBV strains Beaudette and M41. Virus Res. 4:133–143. 1986.

10. Cavanagh, D., and S. Naqi. Infectious bronchitis. In: Diseases of poultry, 11th ed. Y. M. Saif, H. J. Barnes, J. R. Glisson, A. M. Fadly, L. R. McDougald, and D. E. Swayne, eds. Iowa State University Press, Ames, IA. pp. 101–119. 2003.

11. Cook, J. K. A. The classification of new serotypes of infectious bronchitis virus isolated from poultry flocks in Britain between 1981 and 1983. Avian Pathol. 13:733–741. 1984.

12. Cook, J. K. A., S. J. Orbell, M. A. Woods, and M. B. Huggins. Breadth of protection of the respiratory tract provided by different live-attenuated infectious bronchitis vaccines against challenge with infectious bronchitis virus of heterologious serotypes. Avian Pathol. 28:477–485. 1999.

13. Dolz, R., J. Pujols, G. Ordonez, R. Porta, and N. Majo. Antigenic and molecular characterization of isolates of the Italy02 infectious bronchitis virus genotype. Avian Pathol. 35:77–85. 2006.

14. El-Houadfi, M. D., R. C. Jones, J. K. A. Cook, and A. G. Ambali. The isolation and characterisation of six avian infectious bronchitis viruses isolated in Morocco. Avian Pathol. 15:93–105. 1986.

15. Ignjatovic, J., G. Gould, and S. Sapats. Isolation of a variant infectious bronchitis virus in Australia that further illustrates diversity among emerging strains. Arch. Virol. 151:1567–1585. 2006.

16. Ignjatovic, J., and S. Sapats. Identification of previously unknown antigenic epitopes on the S and N proteins of avian infectious bronchitis virus. Arch. Virol. 150:1813–1831. 2005.

17. Hofstad, M. S. Cross-immunity in chickens using seven isolates of avian infectious bronchitis virus. Avian Dis. 25:650–654. 1981.

18. Huang, Y. P., H. C. Lee, M. C. Cheng, and C. H. Wang. S1 and N Gene analysis of avian infectious bronchitis viruses in Taiwan. Avian Dis. 48:581–589. 2004.

19. Huang, Y. P., and C. H. Wang. Sequence changes of infectious bronchitis virus isolates in the 3′7.3 kb of the genome after attenuating passage in embryonated eggs. Avian Pathol. 36:59–67. 2007.

20. Jones, R. C., K. J. Worthington, I. Capua, and C. J. Naylor. Efficacy of live infectious bronchitis vaccines against a novel European genotype, Italy02. Vet. Rec. 156:646–647. 2005.

21. Koch, G., L. Hartog, A. Kant, and D. J. van Roozelaar. Antigenic domains on the peplomer protein of avian infectious bronchitis virus: correlation with biological functions. J. Gen. Virol. 71:1929–1935. 1990.

22. Kou, L., G.-J. Godeke, M. J. B. Raamsman, P. S. Masters, and P. J. M. Rottier. Retargeting of coronavirus by substitution of the spike glycoprotein ectodomain: crossing the host cell species barrier. J. Virol. 74:1393–1406. 2000.

23. Ladman, B. S., A. B. Loupos, and J. Gelb Jr. Infectious bronchitis virus S1 gene sequence comparison is a better predictor of challenge of immunity in chickens than serotyping by virus neutralization. Avian Pathol. 35:127–133. 2006.

24. Lee, S. K., H. W. Sung, and H. M. Kwon. S1 glycoprotein gene analysis of infectious bronchitis viruses isolated in Korea. Arch. Virol. 149:481–494. 2004.

25. Li, H., and H. C. Yang. Sequence analysis of nephropathogenic infectious bronchitis virus strains of the Massachusetts genotype in Beijing. Avian Pathol. 30:535–541. 2001.

26. Liu, S., J. Chen, J. Chen, X. Kong, Y. Shao, Z. Han, L. Feng, X. Cai, S. Gu, and M. Liu. Isolation of avian infectious bronchitis coronavirus from domestic peafowl (*Pavo cristatus*) and teal (*Anas*). J. Gen. Virol. 86:719–725. 2005.

27. Liu, S., J. Chen, Z. Han, Q. Zhang, Y. Shao, X. Kong, and G. Tong. Infectious bronchitis virus: S1 gene characteristics of vaccines used in China and efficacy of vaccination against heterologous strains from China. Avian Pathol. 35:394–399. 2006.

28. Liu, S., Z. Han, J. Chen, X. Liu, Y. Shao, X. Kong, G. Tong, and J. Rong. S1 gene sequence heterogeneity of a pathogenic infectious bronchitis virus strain and its embryo-passaged, attenuated derivatives. Avian Pathol. 36:231–234. 2007.

29. Liu, S., and X. Kong. A new genotype of nephropathogenic infectious bronchitis virus circulating in vaccinated and nonvaccinated flocks in China. Avian Pathol. 33:321–327. 2004.

30. Liu, S., Q. Zhang, J. Chen, Z. Han, X. Liu, L. Feng, Y. Shao, X. Kong, J. Rong, and G. Tong. Genetic diversity of avian infectious bronchitis coronavirus strains isolated in China between 1995 and 2004. Arch. Virol. 151:1133–1148. 2006.

31. Liu, S., X. Zhang, Y. Wang, C. Li, Q. Liu, Z. Han, Q. Zhang, X. Kong, and G. Tong. Evaluation of the protection conferred by commercial vaccines and attenuated heterologous isolates in China against the CK/CH/LDL/97I strain of infectious bronchitis coronavirus. Vet. J. In press. 2007.

32. Lucia, B., and J. Fabricant. Tissue tropism of three cloacal isolates and Massachusetts strain of infectious bronchitis virus. Avian Dis. 34:865–870. 1990.

33. Masters, P. S. The molecular biology of coronaviruses. Adv. Virus Res. 66:193–292. 2006.

34. Mockett, A. P., D. Cavanagh, and T. D. Brown. Monoclonal antibodies to the S1 spike and membrane proteins of avian infectious bronchitis coronavirus strain Massachusetts M41. J. Gen. Virol. 65:2281–2286. 1984.

35. Qian, D. H., G. J. Zhu, L. Z. Wu, and G. X. Hua. Isolation and characterization of a coronavirus from pigeons with pancreatitis. Am. J. Vet. Res. 67:1575–1579. 2006.

36. Wang, C. H., M. C. Hsieh, and P. C. Chang. Isolation, pathogenicity, and H120 protection efficacy of infectious bronchitis viruses isolated in Taiwan. Avian Dis. 40:620–625. 1996.

37. Wang, C. H., and Y. C. Huang. Relationship between serotypes and genotypes based on the hypervariable region of the S1 gene of infectious bronchitis virus. Arch. Virol. 145:291–300. 2000.

38. Wang, H. N., Q. Z. Wu, Y. Huang, and P. Liu. Isolation and identification of infectious bronchitis virus from chickens in Sichuan, China. Avian Dis. 41:279–282. 1997.

39. Wu, Z. Q., Q. W. Yang, C. Fu, X. Y. Zhao, and J. Ignjatovic. Antigenic and immunogenic characterization of infectious bronchitis virus strains isolated in China between 1986 and 1995. Avian Pathol. 27:578–585. 1998.

40. Xu, C., J. Zhao, X. Hu, and G. Zhang. Isolation and identification of four infectious bronchitis virus strains in China and analyses of their S1 glycoprotein gene. Vet. Microbiol. 122:61–71. 2007.

41. Yu, L., Z. Wang, Y. Jiang, S. Low, and J. Kwang. Molecular epidemiology of infectious bronchitis virus isolates from China and Southeast Asia. Avian Dis. 45:201–209. 2001.

42. Zhou, J. Structural analysis of the S1 gene of proventriculus-pathogenic infectious bronchitis virus (ZJ971 isolate) and its structural proteins. Chin. J. Vet. Sci. 20:429–433. 2000.

43. Zhou, J.-Y., D.-Y. Zhang, J.-X. Ye, and L.-Q. Cheng. Characterization of an avian infectious bronchitis virus isolated in China from chickens with nephritis. J. Vet. Med. B 51:147–152. 2004.

ACKNOWLEGMENT

This work was supported by a grant from the National Key Technology R & D Program, Ministry of Science and Technology of the People's Republic of China (No. 2006BAD06A03).