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A new genotype of nephropathogenic infectious bronchitis virus circulating in vaccinated and non-vaccinated flocks in China

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Five strains of infectious bronchitis virus (IBV) were isolated from five layer flocks that had nephropathogenic infection in four provinces in China. Among them, three of the five flocks had been vaccinated against infectious bronchitis. Virulence studies indicated that the five Chinese IBV isolates caused 10 to 30% mortality in 15-day-old specific pathogen free chickens and gross lesions were mainly confined to the kidneys in all of the dead chickens. Two oligonucleotide pairs, S1Uni2 and S1Oligo3′ or S1Oligo5′ and S1Oligo3′, were used after propagation of the isolates in embryonated eggs to amplify the S1 protein genes of the spike protein. The cDNA derived by reverse transcriptase-polymerase chain reaction was cloned and sequenced. The nucleotide and amino acid sequence of S1 protein gene had a similar degree of identity ($\geq$ 92%) among the five Chinese IBV isolates. The nucleotide and amino acid identity of the S1 protein gene between the five Chinese IBV isolates and 16 strains of other IBVs varied from 60 to 81%. This clearly showed that the five Chinese IBV isolates comprised a separate genotype. These results demonstrated, for the first time, that there is a new genotype of nephropathogenic IBV circulating in vaccinated and non-vaccinated flocks in China.

Introduction

Infectious bronchitis (IB), caused by infectious bronchitis virus (IBV), is an acute and highly contagious disease in chickens. The disease is characterized by respiratory signs including gasping, coughing, sneezing, tracheal rales, and nasal discharge. In young chickens severe respiratory distress may occur, while in layers respiratory distress, decrease in egg production, and loss of internal and shell quality of eggs are reported. Some strains of the virus cause severe kidney damage, urolithiasis and may be associated with high mortality. IB is a major health problem affecting the chicken industry in most countries of the world. Through the use of attenuated live as well as inactivated virus vaccines, economic losses due to this disease have been significantly reduced. However, IBV variants may continue to circulate among vaccinated and non-vaccinated flocks and cause severe economic problems (reviewed by Cavanagh & Naqi, 2003).

IBV is the prototype virus of the genus *Coronavirus*, family *Coronaviridae* (Cavanagh, 1997). The genome of IBV contains an enveloped, single-stranded, positive-sense RNA of 27.6 kb. The virion has three major virus-encoded structural proteins, namely the spike (S) glycoprotein, the membrane (M) protein, and the nucleocapsid (N) protein. The spikes of IBV are formed by post-translational cleavage into two polypeptide components, designated S1 and S2. The molecular identification of IBV is based mainly on analysis of the S1 protein gene.

In China, as in other countries, IB has occurred frequently in vaccinated and non-vaccinated flocks and has caused severe economic losses in recent years. Vaccines based on Massachusetts strains such as H120 and H52 and other strains such as

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4-91 (Nobilis IB 4-91) have been used for many years on poultry farms (Farsang *et al*., 2002; Gough *et al*., 2002). However, nephropathogenic IBV strains related to the Massachusetts type have been isolated in China in recent years (Wang *et al*., 1997; Wu *et al*., 1998; Li & Yang, 2001). Furthermore, other IBV strains that had partial or no relationship to Massachusetts type in antigenic and immunogenic characterization have also been isolated in China (Wang *et al*., 1997; Wu *et al*., 1998; Yu *et al*., 2001).

In order to investigate whether there are other genotype(s) of nephropathogenic IBV besides the Massachusetts type in flocks in China, we tested five IBV isolates from layer flocks showing clinical signs of IB by sequencing and analysis of the S1 protein genes. This allowed us to evaluate the prevalence of nephropathogenic IB type(s) in vaccinated and non-vaccinated chickens in recent years in China.

Materials and Methods

Viruses

Tissue samples of kidney were collected from layers showing clinical signs suspected to be related to IB. All flocks investigated in this study contained at least 10,000 layers. Three samples were taken from chickens vaccinated with H120 Massachusetts-type vaccine (Nobilis IB H120) in Xinjiang, Shandong and Heilongjiang provinces, China (Figure 1). The other two samples were from non-vaccinated flocks in Gansu and Heilongjiang provinces, China (Table 1). Obvious nephropathogenic lesions were found in all the diseased layers.

For virus isolation, samples of kidney were pooled and 10% w/v tissue suspensions were made in 0.1% phosphate-buffered saline containing 100 u penicillin and 100 μg streptomycin/ml. After 12 h at 4°C, 200 μl supernatant from the suspensions was inoculated into the allantonic cavity of 9-day-old to 11-day-old embryos of specified pathogen free chickens (Haerbin Veterinary Research Institute, China). Five eggs were used for each sample. The inoculated eggs were incubated at 37°C and candled daily. Two eggs were killed after 72 h incubation and five other eggs were inoculated with the harvested allantoic fluids. Two to 10 blind passages were performed until the dwarfing and death of embryos were observed between 2 and 7 days after inoculation. All the allantoic fluids of inoculated eggs were harvested and tested for the presence of IBV using electron microscopy. The different passages of allantoic fluids containing IBV isolates were used in subsequent experiments (Table 1).

Electron microscopy

Samples of allantoic fluids after egg passages were submitted for electron microscopy. Briefly, after low-speed centrifugation at 1500 × *g* for 30 min (Allegra™ 21R centrifuge; Beckman), the supernatant of the 1.5 ml allantoic fluids were centrifuged at 12 000 × *g* for 30 min. The resulting pellet was resuspended in a minimal volume of deionized water and examined by negative contrast electron microscope (JEM-1200, EX).

Virulence studies in chickens

Six groups each of 10 White Leghorn SPF chickens (Haerbin Veterinary Research Institute, China) were kept in isolators with negative pressure. At 15 days of age, groups 1 to 5 were inoculated intranasally with the five isolates ($\log_{10}$4.2 to $\log_{10}$5.0 median embryo infectious doses, per chick; Table 2). The remaining group 6 was mock-inoculated with sterile allantoic fluid and served as a control. The chicks were examined daily for signs of infection for 30 days after inoculation.

Extraction of RNA

Allantoic fluid (200 μl) containing virus (confirmed by electron microscopy) isolated from flocks of Xinjiang, Gansu, Heilongjiang or Shandong provinces in China, was mixed with 400 μl TRIzol Reagent (Gibco BRL) and RNA was isolated according to the description of the manufacturer. The RNA was air-dried for 2 to 10 min and re-dissolved in 25 μl Rnase-free water.

Generation of cDNA

The same general procedure was used for all the reverse transcriptase-polymerase chain reactions (RT-PCRs). S1Oligo3′ was used for the both of the RT reactions and, subsequently, the PCR with oligonucleotide S1Uni2 or S1Oligo5′ (Kwon *et al*., 1993; Adzhar *et al*., 1997). Lyophilized oligonucleotides obtained from the manufacturer were dissolved in RNase-free water at 0.1 μmol/μl to form the stock solutions.

Twenty-five microlitres of viral RNA was mixed with 50 ng S1Oligo3′ and incubated at 70°C for 10 min followed by 2 min on ice. After adding a reaction mixture consisting of 8 μl 5 × First Strand Buffer (250 mM Tris–HCl, 375 mM KCl, 15 mM $MgCl_2$), 4 μl of 2.5 mM dNTPs (Gibco BRL), 200 u RnaseH$^-$ murine Moloney leukaemia virus RT (Gibco BRL), 40 u RNAsin (Gibco BRL), the mixture was incubated at 37°C for 2 h. The reaction was terminated by heating at 98°C for 7 min and chilling on ice.

PCR, cloning and sequencing of the S1 protein genes

For the PCR reaction, the following mix was made: 15 nmol oligonucleotide S1Oligo3′ and 15 nmol oligonucleotide S1Uni2 or S1Oligo5′; 1 μl cDNA; 5 μl of 10 × PCR buffer (Mg^{2+} Plus; TaKaRa, Japan); 4 μl of 2.5 mmol dNTPs; 2 u Taq polymerase (TaKaRa, Japan); and 34 μl water. The PCR reaction was performed using the following conditions: denaturation (94°C, 1 min), annealing (50°C, 1 min), and extension (72°C, 2 min), 35 cycles followed by a final extension step (72°C, 10 min).

A product, detectable by ethidium bromide staining, of about 1700 base pairs (bp) was generated with isolates LX4 and LH2 using S1Oligo3′ and S1Oligo5′, but no product was seen with isolates LHI10, LS2 and LD3 (Table 1). Therefore, the PCR was performed for LS2, LD3 and LHI10 using S1Oligo3′ and S1Uni2, to generate a similar size product of about 1700 bp. DNA generated by PCR amplification was cloned using a T-tailed vector, pMD18-T (TaKaRa), and transformed using JM109 competent cells (TaKaRa) according to the manufacturer's instructions. Three clones of each isolate were sequenced.

Sequence analysis of the S1 protein genes

The sequences of the S1 protein gene of the five Chinese IBV isolates were assembled, aligned and compared with 16 strains of other IBV with DNAMAN version 5.2.2 (http://www.lynnon.com/). The 16 strains of IBV and their GenBank accession numbers are: Beaudette (Boursnell *et al*., 1987; accession number NC_001451), M41 (Niesters *et al*., 1987; accession number A24863), KB8523 (Sutou *et al*., 1988; accession number M21515), B1648 (Shaw *et al*., 1996; accession number X87238), Connecticut (Wang *et al*., 1994; accession number L18990), Ark99 (Jia *et al*., 1995; accession number L10384), D274 (Jordi *et al*., 1989; accession number X15832), H120 (Kusters *et al*., 1989; accession number M21970 J04329), H52 (Zhou *et al*., 2001; accession number AF352315; unpublished work), 793/B (Adzhar *et al*., 1997; accession number Z83975), Georgia GA/2787/98 (Lee *et al*., 2001; accession number AF274438), A1211 (Wang *et al*., 1996; accession number AF250006), A1171 (Wang *et al*., 1996; accession number AF250005), Q1 (Yu *et al*., 2001; accession number AF286302), T3 (Yu *et al*., 2001; accession number AF227438), and J2 (Yu *et al*., 2001; accession number AF286303).

Accession numbers

The S1 protein gene sequences of the five Chinese IBV isolates have been submitted to the GenBank database and have been assigned the following accession numbers: LX4, AY189157; LH2, AY180958; LS2, AY278246; LD3, AY277632 and LHI10, AY273193.

Figure 1. *Approximate location of provinces and capitals (★) from where the IBV strains were isolated.*

Results

Detection of IBV

IB isolates suspected to be related to IB infection were analysed in this study. The isolates were collected from flocks in four different provinces in China, showing clinical signs of IB infection and with 10 to 30% mortality. The nephritis was observed in both the vaccinated and non-vaccinated flocks and was characterized by enlarged and pale kidneys, frequently with urate deposits in the tubules, severe dehydration and weight loss. Typical signs including dwarfing and death of embryo were observed in the different passages when each of the five Chinese isolates was inoculated into 9-day-old to 11-day-old chicken embryos (Table 1). Diagnoses based on electron microscopy examination performed on allantoic fluids of different passages showed all five isolates had typical coronavirus morphology and were free of other agents such as Newcastle disease virus (results not shown).

Virulence studies

Clinical signs were observed in all of the chicks of groups 1 to 5 about 3 to 10 days after inoculation. The chicks are listless and huddled together, showed ruffled feathers and a dark, shrunken comb. Some of the chicks died during the experiment (Table 2). Gross lesions of dead chicks were mainly confined to the kidneys. The kidney par-

Table 1. *IBV strains isolated from flocks in different provinces of China*

Strain isolate	Province (capital)[a]	Year[b]	Vaccinated	Nephropathogenic lesions	Chicken embryo passage[c]
LX4	Xinjiang (Ulumuqi)	1999	Yes	Yes	4
LS2	Gansu (Lanzhou)	2002	No	Yes	2
LD3	Heilongjiang (Haerbin)	2001	No	Yes	3
LHI10	Shandong (Jinan)	2003	Yes	Yes	10
LH2	Heilongjiang (Haerbin)	2001	Yes	Yes	2

[a] Province (capital) where the viruses were isolated.
[b] Year when viruses were isolated.
[c] Different passages were performed until the dwarfing and death of embryos were observed between 2 and 7 days after inoculation.

Table 2. *Mortality in 15-day-old specific pathogen free chickens inoculated with five isolates of IBV*

Group number	Inoculated IBV isolate	Chicken embryo passage[a]	Dose, median embryo infectious doses (log_{10})[b]	Dead chicks (%)[c]
1	LX4	4	4.2	1
2	LS2	2	4.5	3
3	LD3	3	4.5	2
4	LHI10	10	5.0	2
5	LH2	2	5.0	2
6[d]	Control			

[a] Different passages used as in Table 1.
[b] Dose per chick, 200 µl.
[c] Ten chicks per group.
[d] Inoculated with 200 µl sterile allantoic fluid per chick.

enchyma of the dead birds was pale, swollen and mottled; tubules and urethras were distended with uric acid crystals. In addition, mild respiratory signs (sneezing, rales) were also observed. The clinical signs of the inoculated birds tended to disappear gradually by 20 days of inoculation.

Analysis of the S1 protein gene

The pair of oligonucleotides S1Oligo3′ and S1Oligo5′ was used to attempt the amplification of the S1 region of the spike gene from all five isolates. The RT-PCR amplified a ~1700 bp cDNA from LX4 and LH2, but not from LS2, LD3 and LHI10. Therefore, the PCR was performed for LS2, LD3 and LHI10 using S1Oligo3′ and S1Uni2.

Comparison of the S1 protein gene sequences of the five Chinese IBV isolates with those of 16 strains of other IBVs revealed that nucleotide and amino acid identities among the five Chinese IBV isolates were between 92 and 99%, but the nucleotide identity of the five Chinese IBV strains and the 16 strains of other IBV were not more than 79% and amino acid identity was not more than 78% (Table 3).

Discussion

For isolation of non-egg-adapted IBV field strains, several sequential passages can be given to increase the amounts of virus before performing subsequent experiments. The extent of changes to the infected embryo that are induced by IBV vary greatly. Especially for the field strains, the visible changes in the embryos in the first passage can be minimal. Usually, embryo mortality and dwarfing increase as the number of serial passages increases. Some field strains still caused no dwarfing on the third passage, while other methods could detect IBV using inoculated allantoic cells (reviewed by De Wit, 2000). Of the five IBV isolates in this study, they showed different adaptation to embryos. All the five IBV strains did not cause visible changes in the embryos in the first passage, and four of them caused dwarfing and death of the embryos on the second to fourth passages (Table 1). For one of the IBV isolates, LHI10, obvious changes in the inoculated embryos were observed in the 10th passage. This isolate may not grow well in chicken embryo and the virus titre may be minimal in the first several passages.

Winterfield & Hitchner (1962) first reported a nephrosis condition associated with IB in the United States. Cumming (1962) reported an IB outbreak causing severe kidney lesion in chickens in Australia in the same year. From this time, various nephropathogenic strains of IBV have been identified throughout the world (reviewed by Meulemans & van der Berg, 1998). In China, nephropathogenic IBV strains of the Massachusetts genotype have isolated in rural chicken farms of Beijing (Li & Yang, 2001). In the present study, we isolated three IBV strains from H120-vaccinated flocks and two from non-vaccinated flocks of layer chickens that experienced nephropathogenic infection in four provinces of north China. Virulence studies showed that severe kidney lesions were observed in the dead chicks that had been inoculated with each of the five Chinese IBV isolates. It was reported that nephropathogenic IB occurred in Pennsylvania from 1997 to 2000 in commercial broiler-type and layer-type chicken flocks, with mortality as high as 20% (Ziegler *et al*., 2002). Similar mortality was also found in this study by inoculating 15-day-old specific pathogen free chickens with each of the five Chinese IBV isolates (Table 2). Although the results of this experiment were not a true reflection of the situation in the flocks that the five IBV strains came from, they confirmed that the five IBV isolates are nephropathogenic.

The S1 protein genes of the five IBV isolates, LD3, LS2, LX4, LH2, and LHI10, isolated from 1999 to 2003 in China, shared 95 to 99% of nucleotide identity and 92 to 98% amino acid identity. This strikingly high identity implied a close genetic relationship and possibly indicated a common origin. Not more than 76% nucleotide and amino acid identity was shared between the five IBV isolates and three strains, Q1, T3, and J2, which were also isolated in China before 1999 (Yu *et al*., 2001). Isolates Q1, T3, and J2 were isolated from the proventricular tissues of infected chickens with a syndrome associated with lesions of alimentary and respiratory tracts. Unlike the IBV isolates in this study, nephropathogenic infections were not observed from chickens infected with those three IBV strains (Yu *et al*., 2001). In addition, nephropathogenic IBV strains that showed more than 99.1 and 97.8% nucleotide and amino acid identity with

Table 3. *Nucleotide and amino acid similarities of the S1 protein genes[a] among the five Chinese IBV isolates and other IBV strains*

Strain	LD3	LS2	LX4	LH2	LHI10	Q1	T3	J2	A1211	A1171	M41	H120	H52	UK/7/93	D274	B1648	KB8523	Beau	Conn	Ark99	GA/98
	Amino acid identity (%)																				
LD3		92	94	98	98	75	76	76	77	77	74	75	74	76	76	76	75	74	73	75	53
LS2	95		93	92	92	76	76	76	78	77	75	76	75	76	75	76	75	75	74	74	53
LX4	96	96		94	94	75	76	76	78	78	75	75	75	77	75	76	75	75	73	75	52
LH2	99	95	96		98	76	76	76	77	77	74	75	75	76	76	76	75	75	73	75	53
LHI10	99	95	96	99		76	76	76	78	77	75	75	74	76	76	76	75	75	72	75	53
Q1	76	76	76	76	76		98	99	77	77	74	74	73	75	80	76	74	73	73	76	53
T3	76	76	76	76	76	99		99	78	78	74	74	74	74	80	76	74	73	73	77	52
J2	76	76	76	76	76	99	99		77	78	82	82	82	75	80	76	75	73	73	77	52
A1211	78	78	79	78	78	77	77	77		92	82	82	82	77	77	75	82	82	79	78	53
A1171	78	78	79	78	78	77	77	77	95		82	82	82	76	77	75	81	81	79	78	53
M41	76	77	77	77	77	77	77	77	81	81		95	96	74	77	74	95	97	88	75	53
H120	76	77	77	77	77	76	76	76	81	81	97		99	74	78	74	99	96	94	76	53
H52	76	77	77	77	77	76	76	76	81	81	97	99		74	78	74	98	95	94	76	52
UK/7/93	78	78	79	78	78	78	78	78	77	77	78	78	78		78	78	75	74	73	76	48
D274	77	77	77	77	77	80	80	80	78	78	80	80	81	78		78	78	78	76	78	53
B1648	77	77	77	77	77	78	78	78	75	76	77	77	77	79	78		75	75	73	78	52
KB8523	76	77	77	77	77	76	76	76	81	81	97	99	99	78	81	77		95	87	76	53
Beau	76	77	77	76	76	76	76	76	81	81	98	97	97	78	81	78	97		86	76	53
Conn	74	74	74	74	73	74	74	74	78	78	91	91	90	75	76	74	90	90		73	51
Ark99	75	75	76	75	75	76	76	76	77	78	78	78	77	77	78	78	78	78	75		51
GA/98	60	60	60	60	60	61	61	61	61	62	62	62	62	60	61	61	62	62	61	61	
	Nucleotide identity (%)																				

Top right, amino acid identity (%); bottom left, nucleotide identity (%).

[a] The first 1605 nucleotides, starting at the AUG translation start codon, of the S1 protein genes were compared.

the S1 protein of standard IBV strain M41 were isolated in Beijing (Li & Yang, 2001). The S1 protein genes were also compared between the IBV strains isolated in this study and three Massachusetts-type IBV strains, M41, H52 and H120. The results showed that they had only 74 to 77% nucleotide and amino acid identity at the level of S1 protein gene and indicated that they belonged to different genotypes. On the basis of the genotype and pathogenicity, we can speculate that there were at least three different genotypes of IBV strains circulating in China.

The spreading of a virus from one area or country to another could be due, at least in part, to its improper introduction by the trading of birds or by the use of attenuated vaccines. With the exception of the Massachusetts strain, a very interesting aspect of IBV epidemiology, as far as it is possible to know, is the presence and the spreading of the various IBV serotypes in different continents. About 20 emergent serotypes in North America did not spread to other continents. Similarly, the European, Australian, and Asiatic serotypes apparently did not spread elsewhere. In this study, the S1 protein genes of the five Chinese isolates were compared with two IBV strains, A1211 and A1171, which were isolated in Taiwan; one Japanese isolate, KB8523; three European isolates, UK/7/93, D274, and B1648; four American isolates, GA/2787/98, Connecticut, Beaudette, and Ark99. These IBV strains shared only 48 to 79% nucleotide and amino acid identity with the S1 protein of the five Chinese isolates. Obviously, they belonged to different genotypes. However, we cannot conclude that other IBV types are not present in China because the research on the matter is rather poor and it would be worthwhile to conduct more studies.

Molecular studies have shown that a new serotype can emerge as a result of only a few changes in the amino acid composition in the S1 part of the virus spike protein, with the majority of the virus genome remaining unchanged (Cavanagh *et al*., 1992). This could be due to immunologic pressure caused by the widespread use of vaccines, to recombination as a consequence of mixed infections, or to the decrease of dominant serotypes as a result of vaccination, allowing other field strains to emerge. Further to this study, more epidemiological investigations are needed to better understand the origin, diffusion and persistence of the new genotype.

The sharing of antigens between the IBV isolates and vaccinal viruses might suggest that currently available vaccines should be able to provide protection against challenge from viruses belonging to different serotypes from the vaccine. The low nucleotide and amino acid similarities between the H120 Massachusetts vaccine and the new genotype viruses (Table 3) may account for the occurrence of the disease caused by the new genotype viruses in H120 vaccinated layer flocks.

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