J. Vet. Med. B **51,** 147–152 (2004)

ISSN 0931–1793

Institute of Preventive Veterinary Medicine, Zhejiang University, Hangzhou, China

Characterization of an Avian Infectious Bronchitis Virus Isolated in China from Chickens with Nephritis

J.-Y. Zhou[1,2], D.-Y. Zhang[1], J.-X. Ye[1] and L.-Q. Cheng[1]

Addresses of authors: [1]Institute of Preventive Veterinary Medicine, Zhejiang University, 268 Kaixuan Road, Hangzhou 310029, China; [2]Corresponding author: Tel.: +86 571 8697 1698; fax.: +86 571 8697 1698; E-mail: jyzhou@zju.edu.cn

With 4 figures and 1 table

Received for publication November 10, 2003

Summary

One IBV isolate, SC021202, was isolated from the kidneys of the infected young chickens by inoculating embryonated eggs, and its morphology, physiochemical and haemagglutonating properties were detected. Virulence of the isolate SC021202 was determined with specific pathogen-free (SPF) chicken inoculation. Nucleotide acid sequence of S1 gene of the isolate SC021202 was further sequenced and analysed. The physiochemical and morphological properties of the isolate SC021202 were in accordance to that of typical infectious bronchitis virus (IBV). In a pathogenicity experiment, the clinical signs and related gross lesions resembling those of field outbreak were reproduced and the virus isolate SC021202 was re-isolated from the kidneys of the infected chicken. Sequence data demonstrated that the full length of the amplified S1 gene of the isolate SC021202 was composed of 1931 nucleotides, coding a polypeptide of 543 amino acid residues. Compared with IBV strains from GenBank, the nucleotide and deduced amino acid sequence of S1 gene of the isolate SC021202 shared 60.0–91.4% and 49.1–88.9% identities, respectively. A nucleotide fragment of 'CTTTTTAATTATACTAACGGA' was inserted at nucleotide site 208 in the S1 gene of the isolate. These results indicated that IBV isolate SC021202 was a new variant IBV isolate and responsible for field outbreak of nephritis.

Introduction

Infectious bronchitis virus (IBV) is the prototype of the family Coronaviridae, which is the causative agent of infectious bronchitis (IB) and results in a highly contagious disease of great economic importance to the poultry industry (Calnek, 1997). IBV has a single-stranded positive sense RNA genome that is 27.6 kb in size (Boursnell et al., 1987). Genome of IBV directs the synthesis of six major single-stranded polyadenylated RNA species in infected chicken embryo kidney cells. These RNAs include the intracellular form of the genome (RNA F) and five smaller RNA species (RNAs A, B, C, D and E) (Stern and Kennedy, 1980). mRNA A, C and E encode three major structural proteins: the spike protein (S), the membrane (M) and the nucleocapsid protein (N), respectively. The S protein of IBV is cleaved post-translationally into N-terminal S1 and C-terminal S2 proteins (Cavanagh and Davis, 1986).

IBV exists as many serotypes that can be identified by virus neutralization (VN) test (El-Houadfi et al., 1986; Ambali and Jones, 1990; Gelb et al., 1997). It is known that the S1 subunit was involved with infectivity and haemagglutinin activity and carries serotype-specific sequences (Cavanagh and Davis, 1986; Cavanagh et al., 1986a,b) and antigenic epitopes inducing virus neutralizing antibody. The different serotypes, subtypes or variants of IBV was thought to be generated by nucleotide point mutations, insertions, deletions or RNA recombinations of S1 genes (Kusters et al., 1987; Cavanagh et al., 1988; Cavanagh and Davis, 1988; Wang et al., 1993, 1994; Jia et al., 1995), which were responsible for outbreaks of IB in the vaccinated chicken flocks. In addition to serotype changes, the genetic variation may result in changes of the tissue tropism and pathogenicity of the virus, which lead to the generation of new IBV pathotypes.

Since classical IB was reported in 1930s (Calnek, 1997), enterotropic IBV strains (El-Houadfi et al., 1986; Ambali and Jones, 1990), nephropathogenic IBV strains (Albassam et al., 1986; Fulton et al., 1993; Zhou et al., 1998; Li and Yang, 2001), proventriculus pathogenic strains (Zhu et al., 1998; Zhou, 2000), and the strain 4/91 (Parsons et al., 1992) associated with a deep muscle myopathy have been found. In November 2002, a disease outbreak with kidney swelling has been occurred in young chicken flocks in Sichuan, China. In this study, a coronaviral isolate associated with nephritis was isolated from the kidney tissue of the infected chickens. The symptom and pathological changes similar to the field outbreaks were reproduced in specific pathogen-free (SPF) chickens and the virus isolate were molecularly characterized.

Materials and Methods

Chicken embryos and chickens

SPF white leghorn embryonated chicken eggs and chickens were purchased from Beijing Merial Vital Laboratory Animal Technology Co., Ltd (Beijing, China). Embryonated eggs were used for isolation of field isolates and re-isolation attempt of IBV from renal tissues of the experimental infected chickens. SPF chickens were used for pathogenicity experiment.

Origin of virus

The isolate SC021202 was isolated from kidney of H120-vaccinated 26-day-old young chicken flock experiencing an outbreak of nephritis, which resulted in 62% mortality rate. The cardinal clinical signs of naturally infected chickens included depression, weakness, slight respiratory signs and excretion of pale water-like egesta. At necropsy, the prominent lesion was swollen kidney with severe urate deposition. To isolate the virus, tissue sample of kidney from the diseased chickens was

homogenized (25% w/v) and the suspension was subjected to freeze-thawing for three times. This was followed by centrifugation at 7800 ***g*** at 4°C for 15 min. The suspension was filtered through 0.45 μm membrane (Acrodisc® Syringe Filters, Pall Life Sciences, New York, USA) and passaged in 10-day-old SPF-embryonated eggs by intra-allantoic route for virus isolation.

Isolation and morphology of virus

Ten-day-old SPF-embryonated eggs were inoculated with a dose of 0.4 ml of the suspension via the allantoic route and were incubated for 168 h at 37°C. The harvested allantoic fluid was used as inoculum for further serial passage (0.1 ml per embryo). Egg lethal dose $(ELD)_{50}$ of the virus isolate was detected using the embryonated eggs. The infected allantoic fluid was then negatively stained with 2% sodium phosphotungstate and examined by transmission electron microscopy.

Physical and chemical properties

The allantoic fluid containing the isolate SC021202 was exposed to 0.25% trypsinase for 3 h, 0.5 and 1.0% trypsinase for 1 h at 37°C, 20% ether, repeated freeze-thawing, acidic (pH 3.0) and alkaline (pH 12.0) conditions. Ten-day-old SPF-embryonated eggs were inoculated with such treated isolate samples (0.1 ml per embryo, $5 \times 10^{6.5}$ ELD_{50}/ml) by intra-allantoic route, and the chicken embryo lesions induced by the isolate SC021202 were checked during subsequent incubation.

Haemagglutination properties

Haemagglutinating property of the virus isolate was detected as described previously by Alexander and Chettle (1977). Briefly, the virus was first concentrated 100-fold by centrifugation at 30 000 ***g*** for 60 min. the concentrated virus was treated with the phospholipase-C type I (Sigma, St Louis, MO, USA) for 2 h at 37°C with a final enzyme concentration of 1 unit/ml. One per cent chicken erythrocytes were used for haemagglutination test (HA). Normal saline and allantoic fluid without virions were used as negative control.

In vivo pathogenicity

Fourteen-day-old SPF chickens were randomly divided into three groups with 10 birds in each group. Each bird in groups 1 and 2 was inoculated with 0.2 ml of the allantoic fluid containing the isolate SC021202 ($5 \times 10^{6.5}$ ELD_{50}/ml) and the normal allantoic fluid via trachea, respectively. The third group was used as the negative control. All chickens were reared in the isolater. The infected birds were observed for clinical signs and disease course, and were later killed by intravenous inoculation of barbiturate for observation of gross lesions, sampling of kidney, trachea and lung. These tissue samples were processed as described previously for virus re-isolation and histopathological observation.

Amplification and analysis of S1 gene for the virus isolate

Both the purified SC021202 isolate were used as the materials for RT-PCR from the allantoic fluid of the inoculated embryonated eggs with the original material of field-infected animals and experimentally infected animals. Primers for amplification of S1 gene of the isolate SC021202 was designed with reference to the nucleotide sequence of IBV-Beaudette strain (M94356) with a few modifications. S1-forward (5′-CAAAGCTTGAAAACTGAACAAAAGACA-3′) and S1-reverse (5′-TTGGATCCATAACTAACATAAGGGCAA-3′)

Table 1. Viruses and sequence accession numbers used in this study

Strains/isolates	Tissue tropism	Serotype	Country	Accession number
Ark99	Trachea	Arkansas	USA	M85244
Beaudette	Trachea	Mass	USA	X02342
B1648	Kidney	Variant	Belgium	X87238
Connecticut	Trachea	Conn	USA	L18990
CV-56b	Trachea	California	USA	AF027509
DE072	Trachea/vaccine	Delaware	USA	U77298
D3896	Trachea	Group E	Holland	X52084
D41	Trachea	Unknown	Guangdong (China)	AF036937
Florida 18288	Trachea	Florida	USA	AF027512
GA0470/98	Trachea	GA98	USA	AF274437
Gray	Kidney	JMK	USA	L14069
Holte	Kidney	Holte	USA	L18988
H120	Vaccine strain	Mass	Holland	J04329
H52	Vaccine strain	Mass	Holland	AF352315
JX/99/01	Kidney	Unknown	Jiangxi (China)	AF210735
J	Kidney	Unknown	Zhejiang (China)	AF352312
JL/97/01	Trachea	Unknown	Jilin (China)	AF258780
JMK	unkown	JMK	USA	L14070
JS/95/03	Kidney	Unknown	Jiangsu (China)	AF208239
M41	Trachea	Mass	USA	M21883
SD/97/01	Trachea	Unknown	Shandong (China)	AF208240
QXIBV	Preventriculus	Unknown	Shandong (China)	AF193423
T	Kidney	Group I	Australia	U29522
N1/88	Trachea	Group II	Australia	U29450
SC021202	Kidney	Unknown	Sichuan (China)	AY237817
TJ/96/02	Kidney	Variant	Tianjin (China)	AF257075
Z	Kidney	Unknown	Shandong (China)	AF140352
ZJ971	Preventriculus	Unknown	Zhejiang (China)	AF352313
6/82	Unknown	Group B	UK	X04723

primers flanked a 1.9-kb sequence containing the whole S1 gene and part of S2 gene The genomic RNA of the isolate SC021202 was extracted as described by Zhou and He (2000). RT-PCR was carried out as described by Zhou et al. (2003). Briefly, first strand cDNA corresponding to the S1 gene was obtained by reverse transcription using reverse transcription system (Promega, Madison, WI, USA). PCR was performed by 35 cycles of denaturation at 94°C for 1 min, annealing at 53°C for 2 min, and polymerization at 72°C for 2 min. The final polymerization step was conducted at 72°C for 10 min. The PCR products were analysed on a 1% agarose gel. The amplified cDNA fragments containing the entire S1 gene of SC021202 were purified, and transformed to *Escherichia coli* Top 10 strain. Five clones were sequenced in an automated DNA sequencer (ABI prism 377, ABI Perkin-Elmer, Foster City, CA, USA). The alignment and phylogenetic analysis of the deduced amino acid sequences of S1 gene were performed with the Jotun Hein algorithm and Clustal method by DNAStar (DNASTAR, Madison, WI, USA). GenBank accession numbers of IBV strains used in this study were shown in Table 1.

Results

Virus isolation and physiochemical properties

Embryos were inoculated with the renal suspension containing the virus isolate SC021202 and the allantoic fluids of the dying embryos were harvested for further passage in embryos. In the first passage, the embryo mortality inoculated with 0.4 ml of the kidney suspension was 100% (6/6) 70–84 h after inoculation. In following passage, 100% embryo mortality was observed with a dose of 0.1 ml of the infected allantoic fluid and death time of embryos moved to 30–48 h after inoculation. The gross lesions of the infected embryos were haemorrhage on the legs, mottled necrosis of the liver, swelling of the kidneys, dwarfing and curling (Fig. 1). In electron microscopy, the size of the virus isolate SC021202 was 80–130 nm in diameter. The virions were coronal, pear-shaped and covered with an envelope connected to pedunculate projections (Fig. 2).

The virus isolate SC021202 lost its infectivity to chicken embryos after it was exposed to 45°C for 90 min or 56°C for 15 min, ether for 20 min, acidic condition (pH 3) or alkaline conditions (pH 12) for 3 h at room temperature (25°C). Meanwhile, the virus isolate SC021202 still remained to the infectivity to chicken embryo, exposed to the repeated freeze-thawing five to 10 times, 0.5 and 1% trypsin for 1 h, and 0.25% trypsinase for 3 h at 37°C.

Haemagglutination properties

One per cent chicken erythrocytes were used for HA. Normal saline and allantoic fluid without virions was used as negative control. The infected allantoic fluid could not directly agglutinate 1% chicken erythrocytes while it could do that after being treated with the phospholipase-C type I.

Pathogenicity to chicken and virus re-isolation

All the young chickens inoculated with a dose of 0.2 ml of the virus isolate ($5 \times 10^{6.5}$ ELD_{50}/ml) exhibited respiratory signs 3 days after inoculation. Respiratory signs disappeared 24 h later and birds took much more water while less feeds. Other signs such as ruffled feather and depression were also observed. Birds excreted pale water-like egesta and then died continuously 14 days after inoculation. Lastly, the infected chickens appeared 50% mortality. Necropsy revealed that kidney of dead birds was swollen and exhibited severe urate deposition. In microscopy, kidneys of birds died early showed severe granular degeneration in epithelia of distal or proximal

Fig. 1. The chicken embryos inoculated with the allantoic fluid containing the isolate SC021202. Left: 17-day-old dwarfing and curling embryo infected by the isolate SC021202. Right: normal 17-day-old embryo.

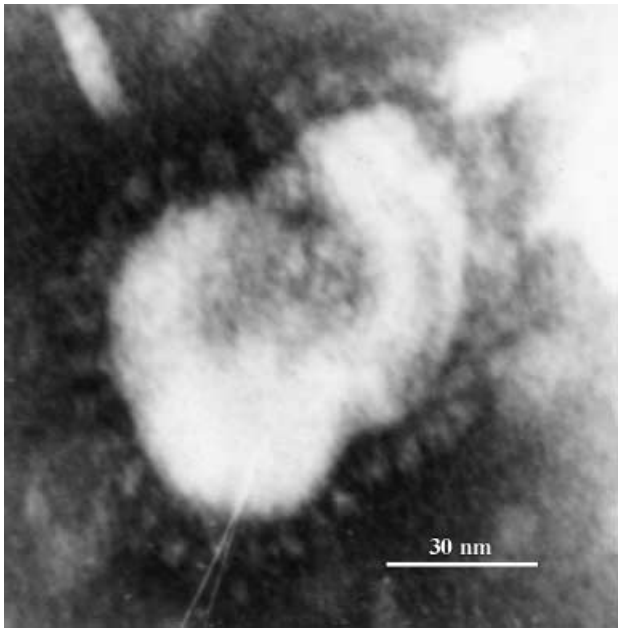

Fig. 2. Electron micrograph of virion of isolate SC021202. Virions were negatively stained with 2% sodium phosphotungstate.

convoluted; swollen or broken epithelia desquamated from basement membrane formed protein or cellular casts in renal tubule. In kidneys of birds died later, histological properties included the focal proliferation of lymphocytes around degenerated renal tubule, or epithelial atrophy of renal tubules, or dilatation of lumen of proximal or distal convoluted tubule. Local bronchitis and bronchopneumonia of dead birds were observed. After chicken embryos were inoculated with a dose of 0.4 ml of the renal suspension of the experimentally infected chickens, the infected embryo showed the similar lesions with that inoculated with the original material of the field-infected chickens.

Molecular characterization of S1 gene

The viral genomic RNA obtained from the purified SC021202 was used as template for RT-PCR. The PCR products of S1 gene were approximately 1.93 kb in size, which was consistent with expected S1 gene (Fig. 3). The full length of the amplified S1 gene of the isolate SC021202 was composed of 1931 nucleotides and coded a polypeptide of 543 amino acid residues (GenBank accession number: AY237817). Meanwhile, S1 gene of the isolate SC021202 re-isolated from the experimentally infected chickens shared 100% nucleotide and deduced amino acid identities with the original material of the field-infected chickens, indicating that there existed no nucleotide variation of S1 gene from the virus isolate SC021202.

Compared with those of other IBV strains from GenBank, the nucleotide and deduced amino acid sequences of the isolate SC021202 shared 61.9–91.4% and 49.2–88.9% identity with them, respectively. The deduced amino acid sequence of S1 gene of the isolate SC021202 was most similar to that of nephropathogenic IBV isolates J (88.9%),JX/99/01 (86.9%), TJ/96/02 (82.8%), Z (82.8%) and a preventriculus-origin IBV isolate QXIBV (82.3%) which were all isolated recently from the diseased flocks in China, and showed lower identities to other IBV strains GA0470/98 (49.2%), DE072 (49.3%), N1/88 (57.7%), Gray (75.6%), 6/82 (75.7%), Holte (76.3%), Ark99 (76.6%), D3896 (76.7%), CV-56b (77.1%), JMK (77.6%), B1648 (77.9%), Florida 1288 (78.3%), ZJ971 (78.3%), D41 (78.5%), Beaudette (78.7%), H52 (78.7%), H120 (78.9%), M41 (78.9%), SD/97/01 (78.9%), JL/97/01 (79.1%), Connecticut (79.5%) and Australian T (81.5%) (data not shown). In addition, an insertion of a 21-nt fragment (CTTTTTAATTATACTAACGGA) within the S1 gene of the virus isolates SC021202 and J at nucleotide site 208 was the most remarkable variations compared with S1 gene of other IBV strains published. As shown in Fig. 4, the predicted amino acid of S1 protein polypeptide of the isolate SC021202 showed close phylogenetic position to IBV isolate J, Z, JX/99/01, TJ/96/02 and QXIBV isolated from diseased flocks in China recently.

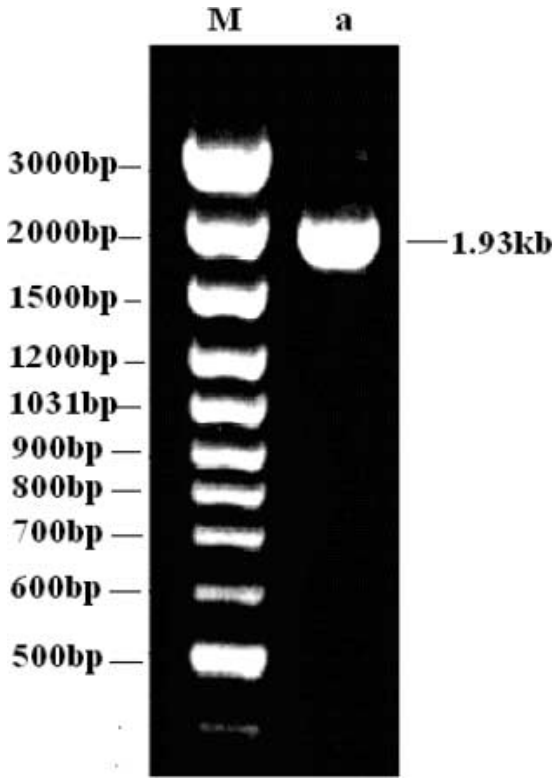

Fig. 3. RT-PCR products of S1 protein gene of the isolate SC021202. M, DNA marker; a, 1.93 kb nucleotide fragment of S1 gene.

Discussion

The isolate SC021202 recovered from kidney of the IB-vaccinated young layer flock was shown to produce dwarfing

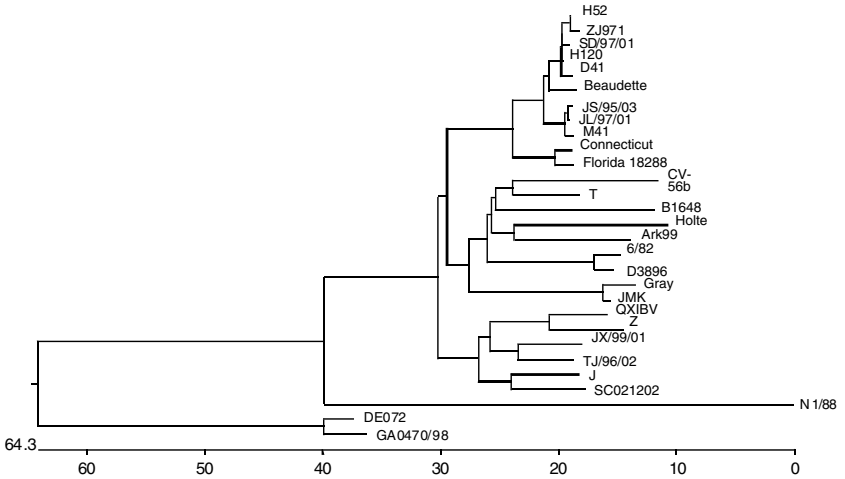

Fig. 4. Phylogenetic tree constructed on the basis of the predicted amino acid sequence of S1 protein of the isolate SC021202.

in the infected embryos and showed similar characteristics of coronavirus IBV such as size in diameter, morphology, physichemical and haemagglutinatiing properties. The clinical manifestations, gross and microscopic lesions of the infected chickens were the same as those infected with nephropathogenic IBVs (Calnek, 1997; Zhou et al., 1998), and SC021202 was re-isolated from the damaged kidneys of the infected chickens. RT-PCR products of S1 gene of the isolate SC021202 were obtained using specific primers of IBV. Sequence of S1 gene of the isolate SC021202 also showed high identity with the published certain IBV isolates. These results indicated that the characteristics of the virus isolate SC021202 was consistent with that of known IBV strains as well as the causative agent of chicken nephritis that was responsible for the field outbreaks recently.

Clinical IB case was first reported in 1970s in Guangdong province, China as a typical respiratory disease. Since 1988, many nephropathogenic IB cases have been reported in China, and nephropathogenic IB has become a familiar infectious disease in chicken farms. Unfortunately, up to now, serotypes of the different tissue tropism of field IBV isolates were not identified using virus neutralization test or monoclonal antibody. Nephropathogenic IBV usually damages kidneys and reproductive tract of chicken in addition to the slight respiratory signs, and then causes a high mortality rate in young flocks. Although the control measures of IB were widely vaccination of live attenuated IBV vaccine (Mass serotype) such as H52, H120 and Ma5 strains in China, but the chicken flocks vaccinated with live attenuated IBV vaccine usually fail to present full protection to field virulent IBV challenge, and the vaccinated chicken flocks were often subjected to the attack of nephropathogenic IBV. In our study, the virus isolate SC021202 also caused 62% natural mortality of chicken flock vaccinated with live attenuated vaccine (H120 strain) and experiencing IBV infection, indicating that Mass serotype of live attenuated IBV vaccine might be very poor in protection of chickens subjected to nephropathogenic IBV.

RT-PCR of S1 gene is a fast and precise method to identify IBV, which can not only identify IBV but also discriminate different strains or isolates. It can even help predict some of its biological characteristics. The predicted amino acid sequence of S1 gene of the IBV isolate SC021202 exhibited high identities to nephropathogenic IBV isolates J (88.9%), JX/99/01 (86.9%), TJ/96/02 (82.8%), Z(82.8%) and a preventriculus-origin IBV isolate QXIBV (82.3%) isolated recently from the diseased chicken flocks in China mainland. However, it shared lower identities with other published IBV strains, indicating that nephropathogenic IBVs in China were closely related to each other (Fig. 4). Moreover, nephropathogenic IBVs in China also showed relatively high similarity with T strain while low similarity with Gray, Holte or B1648 which were all classical nephropathogenic IBVs, indicating that the origin of nephropathogenic IBV in China might have something to do with T serotype strains. A lot of point mutation and an insertion of 'CTTTTTAATTATACTAACGGA' fragment were found in S1 gene of the isolate SC021202 and J compared with other IBV strains. Nephropathogenic IBV isolate J was isolated in 1998 and was defined to be a new variant IBV isolate by restriction fragment length polymorphism and sequence analyses of S1 gene (Zhou et al., 1998; He et al., 2000; Zhou and He, 2000). Although the S1 gene of SC021202 shared a relatively high identity with isolate J including the same insertion mutation, but a lot of point mutation had occurred in S1 gene of the isolate SC021202 compared with that of the isolate J. These characteristics of isolate SC021202 showed that it is a new variant IBV isolate and might directly evolved from other field IBV strains which was currently epidemic in China. Nevertheless, it must be pointed that genotype doesn't always agree with serotype and more work still need to be done to clarify the precise serotype of currently epidemic IBVs. As nephropathogenic IB are epidemic in many provinces in China currently and poor protection was presented by traditional vaccine, it is strongly suggested that nephropathogenic IBV strains should be screened for vaccine using.

Reported nephropathogenic IBV strains were not identical in virulence and ability to cause renal lesion (Winterfield and Albassam, 1984; Chandra, 1987). The virulence of the isolate SC021202 was relatively strong and damaged mainly kidney of chickens although slight lesions in respiratory tract were also observed. As shown in Fig. 4, the tissue tropism of IBVs didn't always agree with their similarity of S1 gene. For example, the preventriculus-origon isolate QXIBV shared 89.9% similarity in S1 protein with nephropathogenic isolate Z while it shared only 76.4% similarity in S1 protein with the preventriculus-origin isolate ZJ971(Zhou, 2000). The molecular basis for IBV tissue tropism is not known. Nevertheless, based on the S1 sequence data, amino acid residues located between 99 and 127 sites may play a role in the different pathogenesis of these viruses (Kwon and Jackwood, 1995; Sapats et al., 1996). It is known that 5′ hypervariable regions of the S1 protein (residues 56–69 and 117–131) contribute to serotyping or neutralizing activities (Koch et al., 1990) and this region shares a similarity between serotypes and genotypes of IBV (Kusters et al., 1989; Wang and Huang, 2000).

Acknowledgements

This work was financially supported by grants from the Natural Science Foundation of China (grant no. 39970030), Zhejiang provincial Natural Science Foundation (grant no. 399411) and Zhejiang Provincial Commission of Science and Technology (grant no. 991102030).

References

Albassam, M. A., R. W. Winterfield, and H. L. Thacker, 1986: Comparison of the nephropathogenicity of four strains of infectious bronchitis virus. Avian Dis. **30,** 468–476.

Alexander, D. J., and N. J. Chettle, 1977: Procedures for the hemagglutination and the hemagglutination inhibition tests for avian infectious bronchitis virus. Avian Pathol. **6,** 9–17.

Ambali A. G., and R. C. Jones, 1990: Early pathogenesis in chicks of infection with an enterotropic strain of infectious bronchitis virus. Avian Dis. **34,** 809–817.

Boursnell, M. E. G., T. D. K. Brown, and I. J. Foulds, 1987: Completion of the sequence of the genome of the coronavirus avian infectious bronchitis virus. J. Gen. Virol. **68,** 57–77.

Calnek, B. W., 1997: Diseases of Poultry, 10th edn. Iowa State University Press, Ames, IO.

Cavanagh, D., and P. J. Davis, 1986: Coronavirus IBV: removal of spike glycopolypeptide S1 by urea abolishes infectivity and haemagglutination but not attachment to cells. J. Gen. Virol. **67,** 1443–1448.

Cavanagh, D., and P. J. Davis, 1988: Evolution of avian coronavirus IBV: sequence of the matrix glycoprotein gene and intergenic region of several serotypes. J. Gen. Virol. **69,** 621–629.

Cavanagh, D., P. J. Davis, D. J. C. Pappin, M. M. Bins, M. E. G. Boursnell, and T. D. K. Brown, 1986a: Coronavirus IBV: partial amino terminal sequencing of spike polypetide S2 identifies the sequence Arg-Arg-Phe-Arg-Arg at the cleavage site of the spike precursor poplypetide of IBV strains Beaudette and M41. Virus Res. **4,** 133–143.

Cavanagh, D., P. J. Davis, J. H. Darbyshire, and R. W. Peter, 1986b: Coronavirus IBV: virus retaining spike glycopolypeptide S2 but not S1 is unable to induce virus-neutralizing or haemagglutination inhibiting antibody, or induce chicken tracheal protection. J. Gen. Virol. **67,** 1435–1442.

Cavanagh, D., P. J. Davis, and A. P. Mockett, 1988: Amino acids within hypervariable region 1 of avian coronavirus IBV (Massachusetts serotype) spike glycoprotein are associated with neutralization epitopes. Virus Res. **11,** 141–150.

Chandra, M., 1987: Comparative nephropathogenicity of different strains of infectious bronchitis virus in chickens. Poult. Sci. **66,** 954–959.

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

Fulton, R. M., W. M. Reed, and H. L. Thacker, 1993: Cellular response of the respiratory tract of chickens to infection with Massachusetts 41 and Australian T infectious bronchitis viruses. Avian Dis. **37,** 951–960.

Gelb, J. Jr, C. L. Keeler Jr , W. A. Nix, J. K. Rosenberger, and S. S. Cloud, 1997: Antigenic and S1 genomic characterization of the Delaware variant serotype of infectious bronchitis virus. Avian Dis. **41,** 661–669.

He, Y. Q., J. Y. Zhou, and L. Yu, 2000: Restriction fragment length polymorphism analysis of Zhejiang isolates of avian infectious bronchitis virus. J. Zhejiang Univ. (Agri. Life Sci.) **26,** 237–260.

Jia, W., K. Karaca, C. R. Parrish, and S. A. Naqi, 1995: A novel variant of avian infectious bronchitis virus resulting from recombination among three different strains. Arch. Virol. **140,** 259–271.

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

Kusters, J. G., H. G. M. Niesters, N. M. Bleumink-Pluym, F. G. Davelaar, M. C. Horzinek, and B. A. Zeijst, 1987: Molecular epidemiology of infectious bronchitis virus in The Netherlands. J. Gen. Virol. **68,** 343–352.

Kusters, J. G., H. G. M. Niesters, J. A. Lenstra, M. C. Horzinek, and B. A. M. van der Zeijst, 1989: Phylogeny of antigenic variants of avian coronavirus IBV. Virology **169,** 217–221.

Kwon, H. M., and M. W. Jackwood, 1995: Molecular cloning and sequence comparison of the S1 glycoprotein of the Gray and JMK strains of avian infectious bronchitis virus. Virus Genes **9,** 219–229.

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

Parsons, D., M. M. Ellis, D. Cavanagh, and J. K. A. Cook, 1992: Characterization of an infectious bronchitis virus isolated from IB-vaccinated broiler breeder flocks. Vet. Rec. **131,** 408–411.

Sapats, S. I., F. Ashton, P. J. Wright, and J. Ignjatovic, 1996: Sequence analysis of the S1 glycoprotein of infectious bronchitis viruses: identification of a novel genotypic group in Australia. J. Gen. Virol. **77,** 413–418.

Stern, D. F., and S. I. T. Kennedy, 1980: Coronavirus multiplication strategy. I. Identification and characterization of virus-specified RNA. J. Virol. **34,** 665–674.

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

Wang, L., D. Junker, and E. W. Collisson, 1993: Evidence of natural recombination within the S1 gene of infectious bronchitis virus. Virology **192,** 710–716.

Wang, L., D. Junker, L. Hock, E. Ebiary, and E. W. Collisson, 1994: Evolutionary implications of genetic variations in the S1 gene of infectious bronchitis virus. Virus Res. **34,** 327–338.

Winterfield, R. W., and M. A. Albassam, 1984: Nephropathogenicity of infectious bronchitis virus. Poult. Sci. **63,** 2358–2363.

Zhou, J. Y., 2000: 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.

Zhou, J. Y., and Y. Q. He, 2000: Cloning and sequence analysis of S1 gene of isolate J of avian nephropathogenic infectious bronchitis. J. Zhejiang Uni. (Agri. Life Sci.) **26,** 369–373.

Zhou, J. Y., L. Yu, and J. Hong, 1998: Isolation and identification of chicken's infectious bronchitis nephritis virus. J. Zhejiang Agri. Uni. **24,** 303–307.

Zhou, J. Y., J. X. Wu, L. Q. Cheng, X. J. Zheng, H. Gong, S. B. Shang, and E. M. Zhou, 2003: Expression of immunogenic S1 glycoprotein of infectious bronchitis virus in transgenic potatoes. J. Virol. **77,** 9090–9093.

Zhu, G. Q., W. W. Yan, and L. S. Sun, 1998: Coronavirus IBV strain with H95 variant isolated from chickens suffered from avian infectious proventriculus disease. Chin. J. Vet. Sci. **18,** 233–236.