Virus Genes (2007) 34:67–73
DOI 10.1007/s11262-006-0002-y

ORIGINAL ARTICLE

Changes in VP3 and VP5 genes during the attenuation of the very virulent infectious bursal disease virus strain Gx isolated in China

Xiaomei Wang · Houshuang Zhang · Honglei Gao · Chaoyang Fu · Yulong Gao · Yulin Ju

Received: 18 November 2005 / Accepted: 18 April 2006 / Published online: 18 August 2006

Abstract A very virulent infectious bursal disease virus (vvIBDV) Gx strain with high pathogenicity was attenuated through replication in specific-pathogen free (SPF) chicken embryos and in chicken embryo fibroblast (CEF) cell cultures. The changes in nucleotide sequences and the deduced amino acid sequences of VP3 and VP5 genes during attenuation were obtained. Sequence analysis of selected passages from numbers 0 to 20 in CEF's (designated here Gx to CEF-20) showed that there were no amino acid changes detected in the VP3 and VP5 genes before CEF-9. There were some changes in the nucleotide sequence and amino acid substitutions in the VP3 and VP5 genes at CEF-9. CEF-9 was an intermediate with some amino acid changes which were possibly related to virulence. The amino acid sequences of VP2 and VP5 genes remained unchanged from CEF-10 to CEF-20. The results of pathogenicity test showed that the mortalities of vvIBDV-Gx, CEF-5, CEF-8, and CEF-9 were 64, 60, 60, and 28%, respectively, while there were no mortalities observed for CEF-10, CEF-15 and CEF-20. There was also no bursal atrophy when chickens were inoculated with CEF-10, CEF-15, and CEF-20. Virus neutralization tests with the Gx strain and sera from inoculated chickens showed that the antigenicity was similar from Gx to CEF-20. The implications of these findings for the study of IBDV virulence and a more effective control of vvIBDV are discussed.

Keywords Infectious Bursal Disease Virus · VP3 gene · VP5 gene · Attenuation

X. Wang (✉) · H. Zhang · H. Gao · C. Fu · Y. Gao
National Key Laboratory of Veterinary Biotechnology, Harbin Veterinary Research Institute of the Chinese Academy of Agricultural Sciences, 427 Maduan Street, Harbin 150001, P. R. China
e-mail: xmw@hvri.ac.cn

Y. Ju · H. Zhang
Department of Preclinical Veterinary Medicine, Yanbian University, 977 Gongyuan Road, Yanji 133400, P. R. China

Introduction

Infectious bursal disease virus (IBDV) causes a highly contagious immunosuppressive disease in chickens by the destruction of B cells present in the bursa of Fabricius. The induced immunosuppression increases the susceptibility to other pathogens [1]. The emergence of very virulent IBDV (vvIBDV) in the last decade represents a new challenge for effective prevention and control of IBD. vvIBDV is characterized by a marked increase in virulence and high mortality rates and is antigenically similar to classical strains even though it can infect chickens with high levels of maternal antibodies. These antibodies normally provide adequate protection from classical IBDV strains [2]. Outbreaks of vvIBDV threaten the poultry industry worldwide. As a consequence, more research on the virulence of vvIBDV is needed for developing more effective vaccines. Furthermore, the identification of IBDV virulence markers would allow us to elucidate the mechanism of IBDV pathogenicity, and then design more effective vaccines against vvIBDV.

IBDV belongs to the Birnaviridae family, which is composed of nonenveloped viruses containing double stranded RNAs formed by two segments, segment A (3.2 kb), and segment B (2.9 kb) [3]. The B segment

encodes the VP1 protein, the putative viral RNA-dependent RNA polymerase (RdRp) [4], and segment A contains two partially overlapping open reading frames (ORFs). The larger ORF encodes a polyprotein (105 kDa) precursor in the order NH_2–pVP_2–VP_4–VP_3–COOH [5]. VP2 and VP3 are the major structural proteins [6]. VP3 coding sequence correspond to polyprotein amino acid positions 756–1012. VP3 is a 29 kDa polypeptide that forms the trimeric subunits that wrap the inner surface of the capsid [7]. VP3 does not contain virus-neutralizing epitopes. Both group and serotype-specific epitopes have been described on VP3 [8]. As with inner capsid proteins from other viruses [9], VP3 might play a role in stabilizing the genomic RNA within the particle. According to previous experimental data, VP3 might interact with the virus genome [10]. A recent study suggested that assembly required screening of the negative charges at the C terminus of VP3. The last C-terminal residue of VP3, Glutamic acid 257, controls capsid assembly of infectious bursal disease virus [11]. The smaller ORF encodes the nonstructural viral protein 5 (VP5) (145–149 amino acids, 17 kDa). This plays an important role in virus egress and virulence [12]. VP5 is dispensable for virus replication in vitro [13], but is critical for the virus' pathogenicity [14]. By using both recombinant vaccinia viruses and IBDV VP5 knockout mutants, it has been shown that VP5 is a cytolytic protein. This protein accumulates within the plasma membrane of infected cells and promotes the egress of the viral progeny [12]. Since nucleotide alignment of the highly virulent strains and attenuated strains shows that there are changes in the sequences of both segment A and segment B, it is likely that the decrease of virulence can be attributed to multiple genes.

Previously, changes of vvIBDV Gx VP2 during attenuation have been observed [15]. Due to the potential relationship between VP2, VP3 and VP5 in virulence, the changes in vvIBDV Gx VP3 and VP5 during attenuation may also be of interest. In order to confirm whether there were in fact any amino acid changes in VP3 and VP5 genes after attenuation in chicken embryo fibroblasts (CEFs) and whether these changes were correlated with virulence, we determined the amino acid sequences of VP3 and VP5 genes of vvIBDV Gx during the attenuation process. Viruses from selected passages were also tested in chickens in order to verify the relationship between the changes in genes, the virulence, pathogenicity, and antigenicity.

Materials and methods

Virus

The vvIBDV Gx strain was isolated from the Guangxi Province of China. The virus was prepared according to the International Cooperation with Developing Countries (INCO-DC) project method [11]. The results of antigen-capture enzyme-linked immuno-sorbent assay, pathogenicity testing, cloning, and analysis of genes (including VP3 and VP5) confirmed that IBDV strain Gx isolated in China was a vvIBDV [11]. Tests also revealed that the deduced amino acid sequences of strain Gx VP3 and VP5 were similar to vvIBDV UK661, which is now considered the reference strain for European vvIBDVs.

SPF chickens and SPF embryos

White Leghorn SPF chickens and SPF embryos were provided by the SPF Animal Center of Harbin Veterinary Research Institute of the Chinese Academy of Agricultural Sciences. All chickens were held in negative pressure isolators during the course of the experiment.

Virus adaptation and attenuation

The vvIBDV Gx strain was blind passaged five times in chorioallantoic membranes (CAMs) of 10-day-old SPF eggs with 0.1 ml/egg dose. The embryo-adapted vvIBDV Gx strain was purified by plaque purifying. Confluent monolayers of CEF cell were cultured in Dulbecco modified Eagle medium. A volume of 0.5 ml of 10-fold serial dilutions of embryo-adapted vvIBDV Gx strain was absorbed onto the monolayers at 37°C for 60 min. The cultures were overlaid with maintenance medium containing 1.0% Nobel agar (Difco, Detroit, Mich.). Two days post-inoculation, agarose containing 0.05% neutral red was again overlaid. After one day, a single viral clone was inoculated into CEF cells. The virus-inoculated cells were incubated at 37°C for 72–96 h. By this time, the extent of the cytopathic effect developed was approximately 70%. The cells were then harvested for the next inoculation, and further passages in CEF were carried out in a similar manner, until 20 passages (CEF-1 to CEF-20) were completed. This work was performed in a special lab that had never housed any other IBDV strain.

Purification of viral dsRNA

After being frozen and thawed three times, cell culture-adapted virus strains were harvested from the supernatants by centrifugation at 11,800 *g* for 30 min at 4°C. Virus purification was conducted by ultracentrifugation at 94,500 *g* for 3 h, and viral particles were resuspended in TNE buffer (0.01 M Tris–Cl, 0.1 M NaCl, 0.01 M EDTA, pH 7.6). Extraction of the viral RNA was conducted using TRIzol® Reagents (Invitrogen Life Technologies, Inc., UK) according to the manufacturer's instructions. All cell culture-adapted strains were treated in the same manner.

Reverse transcription polymerase chain reaction (RT-PCR)

With reference to the sequences of vvIBDV UK661 strain and attenuated IBDV P2 strain published in GenBank (accession number X92760 and X84034), two pairs of primers were designed by using the OLIGO 6.0 software (Table 1). The P1 and P2 primer sequences for amplification of VP5 gene contained an *Eco*RI site. The P3 and P4 primer sequences for amplification of VP3 gene contained an *Xba*I site. The expected sizes of the PCR products were 449 and 939 bp, respectively. RT-PCR was performed as described previously [6]. In order to avoid interactive contamination, the biological reagents were divided into disposable doses and the RT-PCRs of different passages were performed independently.

Subcloning of VP3 and VP5 genes

The VP3 and VP5 PCR products were separated on a 1% agarose gel and were purified with Wizard® PCR Preps (Promega Corporation, Madison, WI). The purified DNA was ligated into the PMD18-T vector (Takara Biotechnology Co., Ltd., Dalian, China). Recombinant plasmids containing the 939 bp VP3 fragment were confirmed by *Xba*I digestion and the 449 bp VP5 fragment by *Eco*RI digestion.

Sequencing and sequence analysis

The VP3 and VP5 genes of each passage chosen were sequenced in two ways. Purified PCR products were sequenced directly, and subcloned recombinant plasmids containing the 939 bp and 449 bp RT-PCR products, were also sequenced. The nucleotide sequences and deduced amino acid sequences of the VP3 and VP5 genes from different passages of cell culture-adapted strains were analyzed and compared with reference sequences. A phylogenetic tree was prepared with the Lasergene Software Suite (DNAstar, Madison, WI). The Accession numbers of reference sequences downloaded from Genbank are shown in Table 2.

Titration of cell culture-adapted strains and animal experimentation

CEF-5, CEF-8, CEF-9, CEF-10, CEF-15, CEF-20, and Gx were chosen for pathological examination. These viruses were separately titrated in 10-day-old SPF eggs receiving 0.2 ml/egg via CAM. The median embryo infective dose (EID_{50}) was calculated by the Reed-Muench formula. Seven groups of 25 4-week-old chickens were examined.

SPF chickens were inoculated with 2×10^3 EID_{50}/chicken from selected passages and the sixth isolator was a non-infected control group containing 10 SPF chickens. Clinical signs and the number of deaths were recorded daily. Surviving chickens were euthanatized 7 days post-inoculation. Their bursae were harvested, and the bursal weight:body weight ratios (B:B indexes) was calculated. Additionally, their sera were collected for the virus neutralization (VN) test as described previously [15], with a constant virus inoculum of Gx strain ($100TCID_{50}$/0.1 ml) against serial 10-fold dilutions of chicken serum.

Table 1 Oligonucleotide primers used in RT-PCR

Name	nucleotide Sequence (5′-3′)	Priming position
P1	CCTGAATTCATGCTATCATTGATGGTTAG	84–103
P2	CCTGAATTCTCACTCAGGCTTCCTTGGAAGGTCAC	508–533
P3	GGTCTAGAAAGTTGGCTGGTCCCGGAGCATT	2233–2255
P4	GGTCTAGAAGCCTCACTCAAGGTCCTCATCAG	3149–3172

Table 2 IBDV strains used for alignment of sequences

Strain	Virulence	Origin	Accession number
UK661	VV[a]	UK	X92760
HK46	VV	HongKong	AF092943
OKYM	VV	Japan	D49706
D6948	VV	Netherlands	AF240686
KS	VV	Germany	L42284
Cu-1	A[b]	Germany	X16107
P2	A	Germany	X84034
CEF94	A	Netherlands	AF194428
PBG	A	Israel	D00868
STC	CV[c]	UK	D00499
GLS	AV[d]	USA	M97346
OH	Serotype-2	USA	U30818
23/82	Serotype-2	UK	AF362773

[a] very virulent strains; [b] attenuated strains; [c] classical virulent strains; [d] antigentic variant strains

Results

Amplification of VP3 and VP5 genes

The expected 939 bp and 449 bp fragments were both obtained by RT-PCR for each passage strain examined.

Identification of recombinant plasmids

The plasmids obtained were digested with endonucleases. There were *Eco*RI and *Xba*I cutting sites at the 5′ terminal in Primer P1, P2 and Primer P3, P4, respectively. The expected fragments in positive recombinants were 449 and 939 bp (PCR fragment) and 2.7 kb (vector). Positive recombinants containing the VP3 and VP5 gene PCR products were obtained for each chosen passage.

Sequencing and sequence analysis

The purified PCR products were sequenced directly, as well as two or three cloned recombinants from each strain. A consensus sequence was determined after all the sequencing results were analyzed. Only the sequence from CEF-9 was not completely unanimous; the CEF-9 sequences used for our comparative analyses were generated from 2 of 3 clones. At position 163 of VP3 gene of CEF-9, the amino acid was E in some subclones whereas it was either D or E from the direct sequencing of PCR products. At CEF-10, the amino acids in the VP3 gene mutated at positions 28H>Q, 163E>D, 226P>L, 235V>A, and 250A>T, respectively. At position 78 of VP5 gene of CEF-9, the amino acid became 78I in some subclones whereas it was either F or I from the direct sequencing of PCR products. At CEF-10, the amino acids in the VP5 gene mutated at positions 18E>K, 49R>G, 78F>I, 91E>G, 104G>C, 122Y>H, 129P>S, and, 137W>R, respectively. The comparison of the deduced amino acid sequences of VP3 and VP5 is shown (Fig. 1 and Fig. 2), respectively.

The nucleotide sequences in VP3 and VP5 genes of each strain were compared with HK46 and P2 strains. A phylogenetic tree (Fig. 3) of different IBDV strains, based on the nucleotide sequences of VP3 and VP5, was made with the Lasergene Software Suite. Gx, CEF-5, CEF-8, UK661, OKYM, HK46, D6948 and KS strains belonged to the vvIBDV branch. CEF-10, CEF-15 and CEF-20 were similar to P2 and CU-1 strains in VP3 and VP5, to which CEF-9 is more closely related. The viruses in the latter group were considered typical attenuated strains.

Animal experimentation

The mortality rates produced by exposing 4-week-old SPF chickens to vvIBDV-Gx, CEF-5, CEF-8 ,CEF-9, CEF-10, CEF-15, and CEF-20 were 64, 60, 60, 28, 0, 0, and 0%, respectively (Table 3). Chickens were considered to have bursal atrophy when the B:B index was lower than 0.7, as proposed by Lucio and Hitchner. Chickens with B:B indexes of higher than 0.7 after being challenged with CEF-10, CEF-15, and CEF-20 strains were considered to have no bursal atrophy, whereas exposure to the Gx strain (0.34), CEF-5 (0.36), CEF-8 (0.37) and CEF-9 (0.53) resulted in bursal atrophy with B:B ratios of less than 0.7. The VN test revealed that the antigenicities of CEF-5, CEF-8, CEF-9, CEF-10, CEF-15, CEF-20, and Gx were similar and related to one another (Table 3).

Discussion

A non structural protein deficient IBDV mutant that fails to induce lesions was made using site-directed mutagenesis and the reverse genetics system [14]. By sequence comparisons, specific amino acids in VP2 were identified, which allow adaptation of vvIBDV to cell culture [15]. In this study, we have identified the amino acid changes in the VP3 and VP5 genes of the vvIBDV-Gx strain after attenuation in SPF embryos (for adaptation to tissue propagation) and after attenuation in 20 passages of CEF culture.

Gene variations associated with virulence are mainly in the domain of VP2 and the heptapeptide of VP2 has been regarded as a marker of virulence changes in IBDV for many years [16]. The VP2 heptapetide is conserved in virulent and low

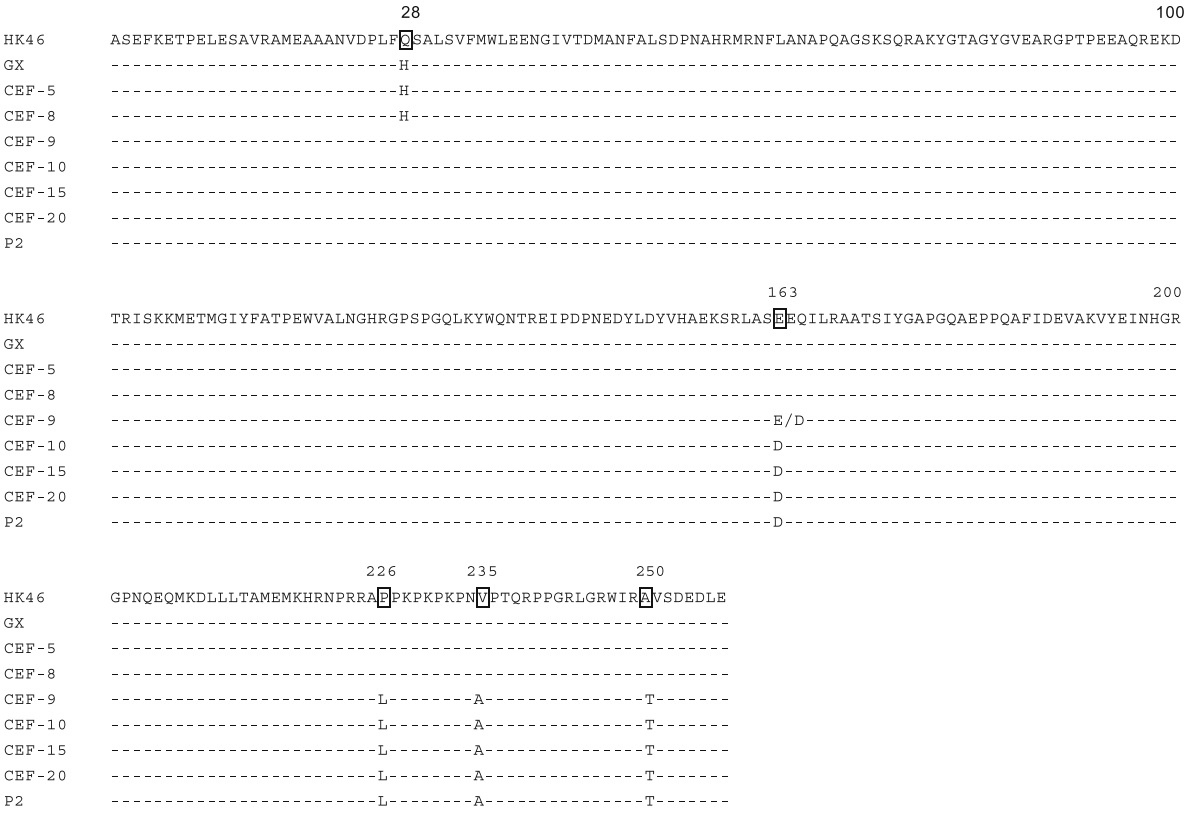

Fig. 1 Comparison of the deduced VP3 amino acid sequences of the original vvIBDV Gx strain original with its CEF-adapted strains

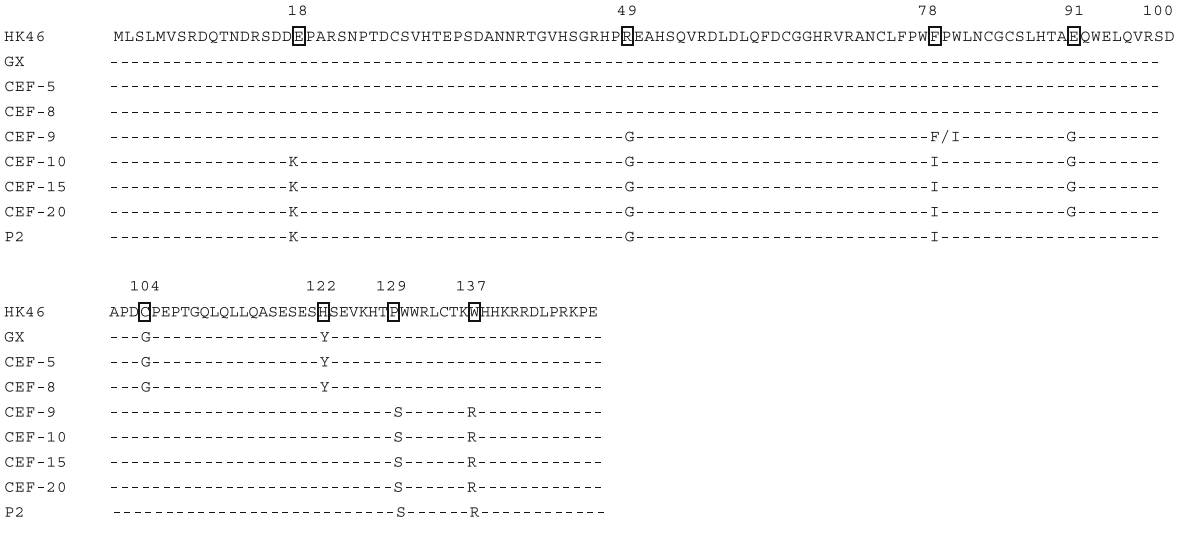

Fig. 2 Comparison of the deduced VP5 amino acid sequences of the original vvIBDV Gx strain original with its CEF-adapted strains

pathogenic strains. Our previous study [15] indicated that most amino acid changes during passage between CEF-8 and CEF-10 were identified within the VP2 variable region and there was an important change at residue 333 S > R in the heptapetide. Since the VP2 gene is not the sole determinant of the very virulent phenotype [16], amino acid changes in VP3 and VP5 genes during attenuation were examined in this study. Sequence analysis demonstrated no apparent change in the VP3 protein before CEF-8, in contrast there were many nucleotide changes and several amino acid substitutions detected in the VP3 gene of CEF-9. A few nucleotide changes identified in the VP3 gene without any associated amino acid substitutions at

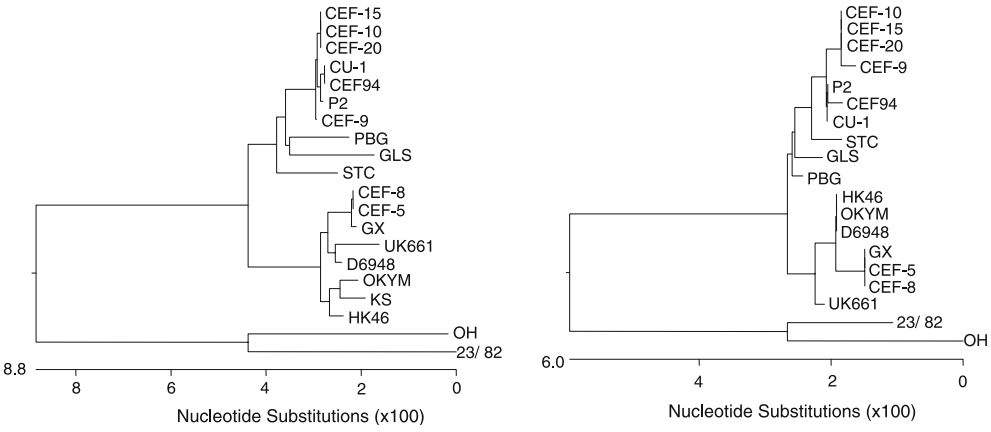

Fig. 3 The phylogenetic tree produced with the Lasergene Software Suite based on the comparison of VP3 and VP5 sequences

Table 3 Pathogenicity of each passage and virus neutralization

Group	Passage	Mortality[a]	B:B index	VN antibody titers to Gx[b]
1	Gx	64% (16/25)	0.34	4.67 ± 0.36
2	CEF-5	60% (15/25)	0.36	4.54 ± 0.30
3	CEF-8	60% (15/25)	0.37	4.34 ± 0.56
4	CEF-9	28% (7/25)	0.53	4.33 ± 0.38
5	CEF-10	0% (0/25)	0.97	4.42 ± 0.34
6	CEF-15	0% (0/25)	0.95	4.24 ± 0.26
7	CEF-20	0% (0/25)	0.97	4.15 ± 0.20
8	Control	0% (0/10)	1.05	<1

[a] Mortality of 4-week-old SPF chickens up to seven days after inoculation

[b] vvIBDV Gx strain was used in the VN assay. Data in the column are geometric mean $\log_{10}$titer ± standard deviation

CEF-8 revealed that the homology of amino acid sequence with reference vvIBDV HK46 strain was maintained at 99.6%. In the VP3 gene of CEF-10, changes were detected at amino acid 28H>Q, 163E>D, 226P>L, 235V>A, and 250A>T. Additionally, 28H>Q is a particular amino acid substitution of vvIBDV Gx. The VP3 sequence remained unchanged from CEF-10 to CEF-20 and the homology of its amino acid sequence with the reference IBDV attenuated strain P2 was up to 100%.

Changes in the VP5 gene of CEF-10 were also detected at amino acids 18E>K, 49R>G, 78F>I, 91E>G, 104G>C, 122Y>H, 129P>S, and, 137W>R, respectively. In the VP5 gene, there were no amino acid changes before CEF-8, but many nucleotide changes and some amino acid substitutions were observed at CEF-9. There were particular changes to vvIBDV Gx at amino acids 104G>C and 122Y>S. A few nucleotide changes in the VP5 gene at CEF-8, without amino acid substitutions occurring, demonstrated that the homology of amino acid sequence with HK46 strains remained at 98.7%. There were no changes in VP5 between CEF-10 and CEF-20 and the homology of the amino acid sequence with P2 was maintained at 99.3%, indicating the stability of the attenuated vvIBDV after continued passaging.

Analyses of VP3 and VP5 sequences at CEF-9 by direct sequencing of PCR products demonstrated a bimodal phenomenon in the direct nucleotide sequence of both genes. Some cloned products were observed to correspond with one peak of the bimodal position, and some cloned products correspond with the other peak. The positions of the bimodal phenomenon were confirmed to be at amino acid 163 (E/D) of VP3 gene and 78 (I/F) of VP5 gene. This bimodal phenomenon was not observed in the direct sequencing of other viral passages. CEF-9 is an intermediate point containing a mixture of virulent and attenuated virus. The pathogenicity study confirmed the transitional nature of the CEF-9 passage with a reduced mortality rate of 28%.

Sequence analyses of vvIBDV Gx showed that the protein amino acid sequences were altered between passages CEF-8 and CEF-10. Taken together with the previously published result [15], amino acid sequences of VP2, VP3 and VP5 genes all reached a point of transition in the same passage during the process of attenuation. The general structure of IBDV places the VP2 protein on the outer surface of the viral particle where changes during the process of attenuation might be expected to occur first. Interestingly changes in VP2, VP3 and VP5 all occurred within the same

passage and all were correlated with a decrease in virulence. This drop in virulence is unable to be definitively assigned to any one single change and many reside in multiple changes in multiple genes. The bimodal changes observed in the transitional passage CEF9, of which there are three, 222(A/P) of VP2 gene, 163 (E/D) of VP3 gene and 78 (I/F) of VP5 gene would appear the best candidates for further study.

Our pathogenicity study of the Gx strain showed that the mortality induced by the parent strain was 64% in 4-week-old SPF chickens. Even at CEF-5 and CEF-8, the virus remained very virulent with a mortality rate of 60%. However, pathogenicity at the CEF-9 passage was significantly reduced to a mortality rate of 28% whereas the virus was essentially non-pathogenic at CEF-10, CEF-15, and CEF-20. Lower pathogenicity of CEF-9 strongly suggested that it was a transitional point where the population balance between virulence and non-virulence was in some state of equilibrium. The CEF-20 generation was nonpathogenic and did not revert after propagation for six generations in SPF chickens (data not shown). This demonstrates that the attenuated strain has good stability. VN tests revealed no antigenic changes during the CEF cell passages. The result in this study suggests that attenuated strain may be used as a live vaccine against vvIBDV.

Acknowledgments This work was supported by grant from the International Cooperation with Developing Countries (INCO-DC) project (contract No. ERB IC18-CT98–0330) and Key Project of Chinese National Programs for Fundamental Research and Development (2005CB523200). We are very grateful to the collaborative partners in this project including Prof. Dr. Hermann Muller (University of Leipzig, Faculty of Veterinary Medicine, Institute of Virology, Germany), Dr Thierry van den Berg (Veterinary & Agrochemical Research Centre Section of Avian Virology, Belgium), Dr Nicolas Eterradossi, Mr. Didier Toquin and Mrs Gaelle Rivallan (Ploufragan Central Laboratory for Poultry and Swine Research, France). We also thank Drs Simon Fenton and Trevor Bagust (Faculty of Veterinary Science, University of Melbourne), and Dr Cui Xianlan (Landcare Research, New Zealand), for their reading of this manuscript.

References

1. H.N. Lasher, S.M. Shane, World's Poultry Sci. J. **50,** 133–166 (1994)
2. T.P. van den Berg, Avian pathol. **29,** 175–194 (2000)
3. P.J.N. Hudosh, N.M. Mchern, B.E. Power, A.A. Azad, Nucleic Acids Res. **14,** 5001–5012 (1986)
4. J.A. Bruenn, Nucliec Acids Res. **19,** 217–226 (1991)
5. A.B. Sanchez, J.F. Rodriguez, Virology **262,** 190–199 (1999)
6. H.Y. Chen, Q. Zhou, M.F. Zhang, J.J. Giambrone, Avian Dis. **42,** 762–769 (1998)
7. J.R. Caston, J.L. Martinez-Torrecuadrada, A. Maraver, E. Lombardo, J.F. Rodriguez, J.I Casal., J.L. Carrascosa, J. Virol. **75,** 10815–10828 (2001)
8. T. Yamaguchi, K. Iwata, M. Kobayashi, M. Ogawa, H. Fukushi, K. Hirai, Arch. Virol. **141,** 1493–1507 (1996)
9. B. Bottcher, N.A. Kiselev, V.Y. Stel'Mashchuk, N.A. Perevozchikova, A.V. Borisov, R.A. Crowther, J. Virol. **71,** 325–330 (1997)
10. M.G. Tacken, B.P. Peeters, A.A.M. Thomas, P.J. Rottier, H.J. Boot, J. Virol. **76,** 11301–11311 (2002)
11. X.M. Wang, C.Y. Fu, H.L. Gao, X.L. Song, X.W. Zeng, M.F. Zhang, W.B.L. Lim, Agric. Sci. China **5,** 566–572 (2003)
12. K. Yao, V. N. Vakharia, Virology **285,** 50–58 (2001)
13. E. Mundt, B. Kollner, D. Kretzschmar, J. Virol. **71,** 5647–5651 (1997)
14. K. Yao, M. A. Goodwin, V.N. Vakharia, J. Virol. **72,** 2647–2654 (1998)
15. X.M. Wang, X.W. Zeng, H.L. Gao, C.Y. Fu, P. Wei, Avian Dis. **48,** 77–83 (2004)
16. H.J. Boot, A.A.M. ter Huurne, A.J. Hoekman, B.P.H. Peeters, A.L.J. Gielkens, J. Virol. **74,** 6701–6711 (2000)