# Competitive replication of different genotypes of infectious bursal disease virus on chicken embryo fibroblasts

**Lixue Shi · Haibin Li · Guangpeng Ma · Jiyong Zhou ·
Lianlian Hong · Xiaojuan Zheng · Yongping Wu ·
Yongzhi Wang · Yan Yan**

**Abstract** Infectious bursal disease virus (IBDV) causes immunosuppression in chickens. We investigated the molecular changes in chicken embryo fibroblasts (CEF) adapted IBDV by genomic sequencing. IBDV were serially passaged in CEF and chickens were infected with the IBDV obtained after different numbers of passages in CEF. Chicken infections showed that 16th, 20th, and 21st passage viruses were pathogenic, while 26th and 36th passage viruses were non-pathogenic. Sequencing demonstrated that the initial changes during the serial passage comprised of a single-nucleotide deletion in the 3′ non-coding region of segment B of the virus after 19th passage, followed by changes in the VP1 gene after the 20th passage of the virus and changes in VP2, VP5 after the 21st passage of the virus. These data suggested that the attenuation of very virulent IBDV was due to multigenic mutations and there are in vitro and in vivo competitive replications in IBDV quasispecies.

## Introduction

Infectious bursal disease virus (IBDV) is a non-enveloped virus, 60 nm in diameter, with an icosahedral shell. The virus belongs to the genus *Avibirnavirus* of the family *Birnaviridae* [1]. The virus infects the precursor B lymphocytes in the bursa of Fabricius and causes severe immunosuppression or mortality in young chickens [2]. The double-stranded RNA genome of IBDV consists of segments A and B [3, 4]. Segment A is approximately 3,260 nucleotides (nts) in size and contains two open reading frames (ORFs) of 3,039 and 438 nts. The smaller ORF encodes the VP5 protein, a 17-kDa non-structural protein [5] and is located in the 5′ end of the genomic segment A. The larger ORF starts at the 5′ end of the genomic segment A and partially overlaps the VP5 ORF. The larger ORF encodes an approximately 110-kDa precursor polyprotein, which is autocatalytically cleaved by the *cis*-acting viral protease VP4 to form VP2, VP3, and VP4 [6]. Segment B is approximately 2,827 nts in size and encodes VP1, the RNA-dependent RNA-polymerase that is an essential protein for the viral replication and encapsulations [7].

Identifying the molecular determinants associated with virulence in the IBDV genome will help to understand its pathogenicity. Initially, VP2 was believed to be the primary determinant of virulence, and especially its hypervariable region was implicated in the virulence, cell tropism, and pathogenic phenotype of virulent IBDV [8, 9]. Contrary to this, another report demonstrated that VP2 was not the sole factor which determines the very virulent

L. Shi · H. Li · G. Ma · J. Zhou (✉) · L. Hong · X. Zheng ·
Y. Wu · Y. Wang · Y. Yan
Key Laboratory of Animal Epidemic Etiology and
Immunological Prevention of Ministry of Agriculture, Zhejiang
University, Hangzhou 310029, People's Republic of China
e-mail: jyzhou@zju.edu.cn

L. Shi · H. Li · J. Zhou · L. Hong · X. Zheng · Y. Wu ·
Y. Wang · Y. Yan
State Key Laboratory for Diagnosis and Treatment of Infectious
Diseases, The First Affiliated Hospital, Zhejiang University,
Hangzhou 310003, People's Republic of China

G. Ma
China Rural Technology Development Center, Beijing 100045,
People's Republic of China

phenotype (vvIBDV) [10] and the VP3 was also reported to be a determinant for virulence [11]. Meanwhile, VP5 was also identified as an important factor for the virus egress and virulence, and its N-terminus motif "MLSL" was proved to be necessary to vvIBDV phenotype [12]. Furthermore, Boot et al. [13] evaluated the role of the VP1 protein with reverse genetics technology, revealing that Asp146, Asn147, Glu242, Met390, Asp393, Pro562, Pro687, and Arg695 in VP1 were unique to the vvIBDV. Up to now, the mechanism involved in the change of IBDV virulence remains unclear. In addition, researchers have put forward the concept of viral fitness relative with viral replication. Viral fitness is often measured as the relative ability of two competing viruses to produce infectious progeny during co-infection in a given environment [14]. Some viruses have been found to exist in a competitive replication among different mutants, such as human immunodeficiency virus (HIV) [15] and infectious hematopoietic necrosis virus (IHNV) [16].

In order to analyze changes of IBDV virulence in the chicken embryo fibroblasts (CEF) and in chickens, by sequencing and analyzing the full genome of the CEF-adapted and bursa-derived IBDV, we focused on the replication of the IBDV genomes during the serial passage in the CEF monolayer.

## Materials and methods

### Virus, chicken embryos, and chickens

Bursae with edema and hemorrhage were collected from a chicken farm at Ningbo city (Zhejiang, China). To isolate the field IBDV (designated as NB isolate), the bursae were homogenized (w/v = 1:3) with sterile normal saline, and were subjected to three freeze–thaw cycles. The homogenate was centrifuged for 15 min at $12,000 \times g$ at 4°C. The SPF embryonated chicken eggs and SPF chickens, used for CEF preparation and pathogenicity tests, were purchased from Beijing Merial Vital Laboratory Animal Technology Co., Ltd (Beijing, China).

### CEF adaption of virus

CEF were prepared as previously described [17] and inoculated with the sterile bursal suspensions. After incubation for 120 h at 37°C, the harvested inoculums were passaged blindly on the CEF until cytopathic effects were observed. Serial passages of CEF-adapted virus (CEF virus) on the CEF monolayer were carried out to attenuate the pathogenicity, and $TCID_{50}$ of each CEF virus was determined using the Reed-Muench method. The CEF viruses serially passaged 36 times on CEF monolayers were designated as the CEF1 to CEF36 viruses, respectively.

### Pathogenicity of CEF-adapted IBDV

To examine the pathogenicity of the CEF-adapted virus, thirty 4-week-old SPF leghorn chickens were divided into six groups (5 chickens per group), and housed in negative pressure isolators. Each generation of CEF virus was five times serially passaged in the SPF chickens (5 chickens per passage), to observe the pathogenicity of the virus. Chickens in Groups 1 to 5 were inoculated intraocularly with 0.2 ml of CEF-16, CEF-20, CEF-21, CEF-26, and CEF-36 virus ($5 \times 10^4$ $TCID_{50}$), respectively. Chickens in Group 6 were used as a negative control without infection. Daily clinical observations of all inoculated chickens were recorded. All inoculated chickens were weighed daily and harvested at 72 h post-inoculation (p.i.). The bursa-body index (BBIX) of the inoculated chickens was calculated as bursa-to-body ratio of infected chickens divided by bursa-to-body ratio of negative control chickens. Subsequently, half of the bursa was sectioned for pathological examination and another half was homogenized to further inoculate the SPF chickens.

### Genomic amplification

Genomic dsRNA were obtained from virus (BF virus) derived from bursa of Fabricius infected with IBDV and various purified CEF-adapted viruses (CEF16, CEF19, CEF20, CEF21, CEF26, and CEF36 virus) with a Trizol LS reagent kit (Invitrogen, Carlsbad, CA, USA). To produce the first strand cDNA for both segments A and B, reverse transcription was conducted using oligo (dT) as a primer by RevertAid™ M-MuLV reverse transcriptase (Fermentas, Hanover, MD, USA). The genomic segments A (3260 nts) and B (2827 nts) were amplified, respectively, with the specific primers as follows: 5′-ATGAATTCAGGATACGATCGGTCTGACCC CGG-3′ and 5′-TAGGTACCGGGACCCGCGAACGGATC-3′ for segment A, 5′-TTAGAATTCGGATACGATGGGTCT GAC-3′ and 5′-ATTTCTAGAGGGGGGCCCCCGCAGG-3′ for segment B. And the primers were designed according to the sequence of vvIBDV strain D6948 (accession number *AF240686* and *AF240687*) and attenuated IBDV P2 strain (GenBank accession number *X84034* and *X84035*) published in GenBank. PCR amplification was performed by 35 cycles after denaturing at 95°C for 5 min. The cycling program was denaturation at 95°C for 25 s, annealing at 60.5°C for segment A and 64°C for segment B for 30 s, and extension at 72°C for 3 min.

### Cloning, sequencing and data analysis

The RT-PCR products of segments A and B from BF-derived and CEF-adapted viruses were ligated in a pMD18-T

**Table 1** IBDV strains used in multiple alignment analysis of segments A and B

| Strain | Country | Classification | Accession no. | |
|---|---|---|---|---|
| | | | Segment A | Segment B |
| 9109 | USA | Attenuated | AY462027 | AY459321 |
| P2 | Germany | Attenuated | X84034 | X84035 |
| B87 | China | Attenuated | DQ906921 | DQ906922 |
| CEF94 | The Netherlands | Attenuated | AF194428 | AF194429 |
| CT | France | Attenuated | AJ310185 | AJ310186 |
| Cu1 | Germany | Attenuated | X16107 | AF362775 |
| HZ2 | China | Attenuated | AF321054 | AF493979 |
| JD1 | China | Attenuated | AY321055 | AY103464 |
| OKYM | Japan | Very virulent | D49706 | D49707 |
| D6948 | The Netherlands | Very virulent | AF240686 | AF240687 |
| Harbin-1 | China | Very virulent | EF517528 | EF517529 |
| HK46 | China | Very virulent | AF092943 | AF092944 |
| TL2004 | China | Very virulent | DQ088175 | DQ118374 |
| ZJ2000 | China | Very virulent | AF321056 | DQ166818 |
| BD399 | Bangladesh | Very virulent | AF362776 | AF362770 |

**Table 2** Bursa-body index (BBIX) and microscopic lesion of the SPF chickens infected with CEF-adapted IBDV

| Gen. | BBIX | | | | | Microscopic lesion | | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| | C16 | C20 | C21 | C26 | C36 | C16 | C20 | C21 | C26 | C36 |
| 1st | 1.11 | 0.97 | 1.00 | 1.17 | 1.02 | 2/5[a] | 0/5 | 0/5 | 0/5 | 0/5 |
| 2nd | 1.03 | 0.99 | 1.02 | 1.32 | 1.33 | 5/5 | 2/5 | 0/5 | 0/5 | 0/5 |
| 3rd | 0.75 | 0.73 | 0.95 | 1.17 | 1.33 | 5/5 | 5/5 | 3/5 | 0/5 | 0/5 |
| 4th | 0.89 | 0.93 | 0.82 | 1.05 | 1.15 | 5/5 | 5/5 | 5/5 | 0/5 | 0/5 |
| 5th | 0.84 | 0.84 | 0.80 | 1.03 | 1.13 | 5/5 | 5/5 | 5/5 | 0/5 | 0/5 |

[a] Numerator: number of sick chickens/denominator: number of inoculated chickens

vector (Takara Biotechnology Co., Ltd., China) after being purified, and were transformed to *E. coli* Top10 cell. Colonies containing segment A or B were identified by restriction endonuclease digestion and confirmed by PCR, and five different clones from the same amplified reaction were sequenced. The nucleotide and deduced amino acid sequences of segments A and B were aligned with the previously published full-length IBDV sequences (Table 1) using Clustal X multiple sequence alignment program and Omiga 2.0 software (Accelrys Inc., San Diego, CA, USA). Phylogenetic analyses of IBDV strains, based on the sequences of coding- and non-coding regions, were done with MEGA vision 3.1 [18] using the minimum evolution (ME) methods and up to 1,000 bootstrapping replicates.

## Results

### Pathogenicity of CEF-adapted IBDV

When chickens were infected with different passages of CEF-adapted viruses (Table 2), only chickens inoculated with CEF16 virus exhibited pathological lesions in the lymphoid follicles in the bursa in the first inoculation. The chickens infected with CEF20 and CEF21 viruses developed bursal histopathologic lesions in the second and third passages, respectively. However, up to the fifth generation, the CEF16-, CEF20-, and CEF21-infected chickens revealed striated-like hemorrhage of leg muscle, hemorrhage in juncture of gizzard and proventriculus, hemorrhage and necrosis of bursa (Fig. 1). No pathological

changes were detected in chickens infected with CEF26 and CEF36. The data indicates that the CEF26 and CEF36 viruses were non-pathogenic to chickens, and the CEF16, CEF20, and CEF21 viruses were virulent to chickens.

### Genomic sequence of bursa-derived and CEF-adapted IBDV

We obtained the genomic sequences of the BF virus and the selected CEF-adapted viruses (CEF16, CEF19, CEF20, CEF21, CEF26, and CEF36). The genomic sequences were deposited at the GeneBank library (GenBank accession number *EU595667* to *EU595678*). Sequencing showed that the nucleotide sequences of segments A and B were composed of 3260 and 2828 nts in bursa-derived BF virus and CEF16 viruses, and were 3259 nts for segment A and 2827 nts for segment B in the CEF19 virus. In comparison with genomic sequence of the BF virus, the nucleotide mutation frequency (Table 3) was 0.3% for segment A and 0.2% for segment B in CEF16 virus, 0.3% for segment A and 0.5% for segment B in CEF19, 3.1% for segment A and 10.9% for segment B in CEF21, 4.7% for fragment A and 10.6% for segment B in CEF26 and CEF36. The nucleotide substitutions were 2.8% for segment A and 11.4% for segment B when CEF21virus was compared with CEF19 virus, but the nucleotide mutation rates of CEF26 on comparison with CEF21 were 1.8% for fragment A and 0.6% for segment B. However genomic mutation frequency of CEF36 was only 0.2% compared with CEF26, indicating that majority of nucleotide substitutions occurred between passages CEF19 and CEF21.

Notably, sequencing analysis of the CEF20 showed three genomic types, named as CEF20-1, CEF20-2, and CEF20-3. On comparison with genomic sequence of the BF virus, we observed the following nucleotide replacements in the viruses after different passage numbers: 27 nucleotide replacements and 1 nucleotide loss in segment A in the region consisting of 1000 nts in the 3′ terminal and 2 nucleotide replacements of the segment B in CEF20-1(6/15 sequenced clones), 282 nucleotide substitutions and 1

**Fig. 1** Gross and microscopic lesions of chickens infected with the 16th CEF-adapted IBDV. **a** Striated-like hemorrhage of leg muscle. **b** Purple grape-like bursa. **c** Hemorrhage in juncture of gizzard and proventriculus. **d** Lymphocyte necrosis of lymphoid follicles in bursa
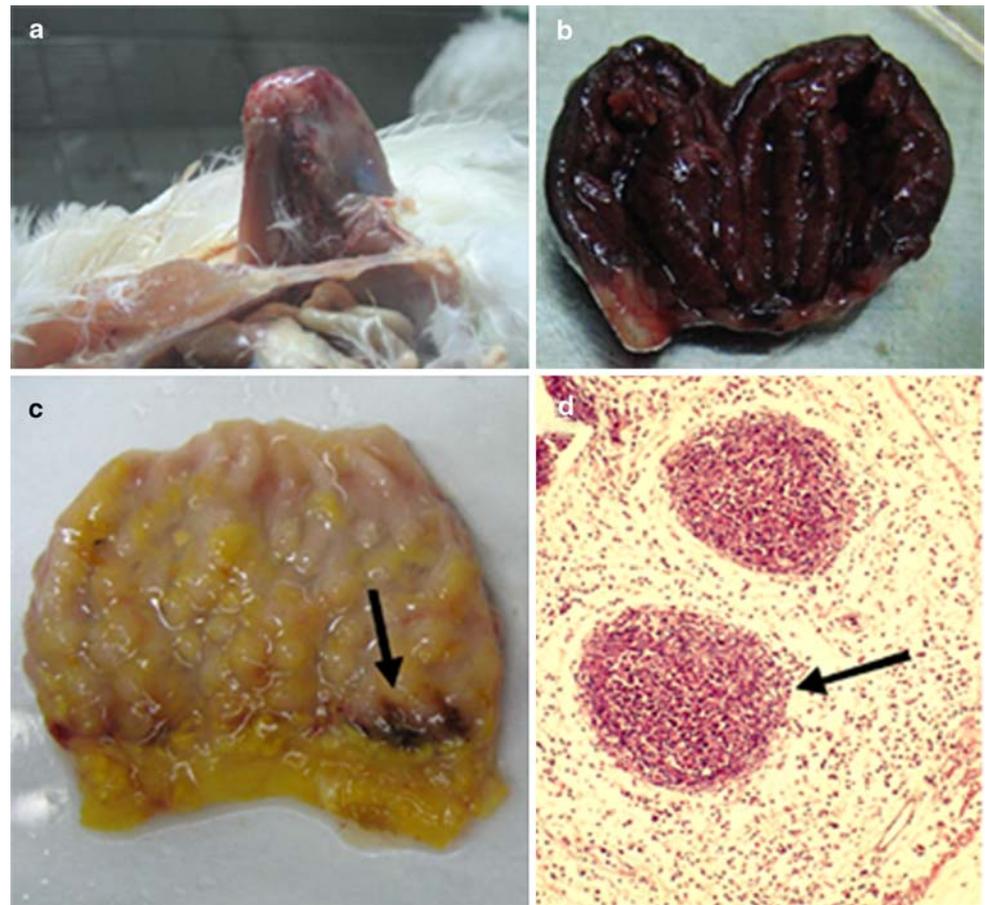


**Table 3** The nucleotide mutation frequency of CEF-adapted virus

| | Gene segment of IBDV | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| | CEF16 | | CEF19 | | CEF21 | | CEF26 | | CEF36 | |
| | A | B | A | B | A | B | A | B | A | B |
| BF virus | 0.3% | 0.2% | 0.3% | 0.5% | 3.1% | 10.9% | 4.7% | 10.6% | 4.7% | 10.6% |

nucleotide loss in segment B and 2 nucleotide substitutions in segment A in CEF20-2 (4/15 sequenced clones), 119 nucleotide substitutions in a region of 1,600 nts in the 5′ terminal of segment B and 25 nucleotide substitutions and 1 nucleotide deletion in a region of 500 nts in the 3′ end of segment B in CEF20-3 (5/15 sequenced clones) demonstrating that the CEF20 virus was a population of different genotypes of IBDV.

Phylogenetic analysis (Fig. 2) of the genomic B fragment revealed that BF virus, CEF16, CEF19, and CEF20-1 viruses were assigned to the virulent IBDV group. The CEF20-2 and CEF20-3, CEF21, CEF26, and CEF36 viruses were clustered into a clade of the non-pathogenic IBDVs. Correspondingly, the phylogenetic analysis of the genomic segment A revealed that CEF26 and CEF36 viruses belonged to the clade of non-pathogenic IBDVs,

and the bursa-derived, CEF16, CEF19, CEF20, and CEF21 viruses belonged to an evolutionary branch with the reported vvIBDV, whereas the non-coding regions of the CEF20 virus belonged to a sublineage of the non-pathogenic IBDV.

### Characterization of 5′ and 3′ non-coding regions (NCR) within IBDV

5′ NCR within the fragment A contained the promoter region of the small ORF, a conservative $^1GGAUAC GAU^C/_GGGUCUGACCC^C/_U^G/_C^G/_UGGGAGUCAC^{32}$ motif, and 18S rRNA-binding motif $^{71}CUCCUC^{76}$, and 5′ NCR within segment A of CEF26 revealed the nucleotide mutations of 44C → T, 45T → C, 47A → C, 83C → T, and 86 T→ C (Fig. 3a). In the 3′ NCR of segment A,
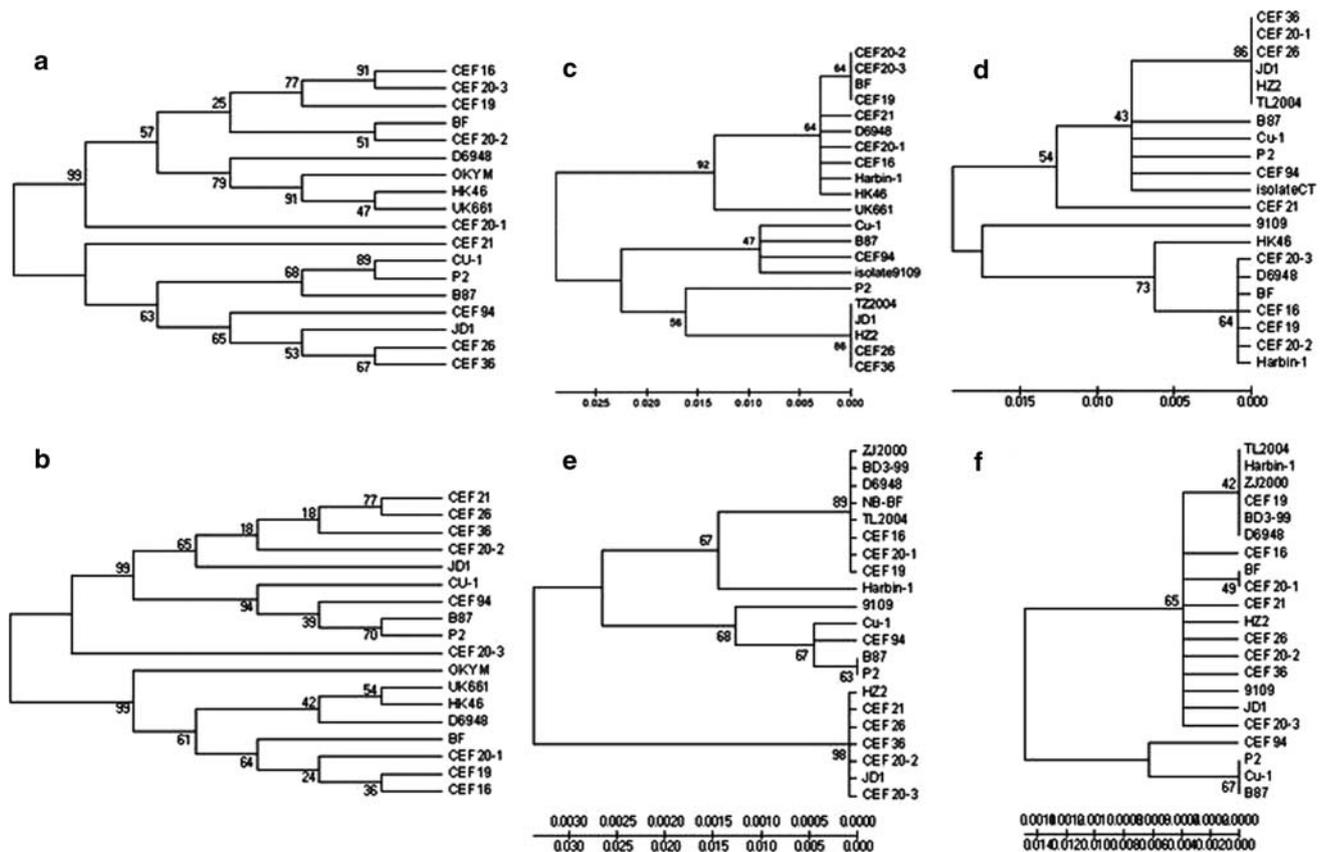
**Fig. 2** Phylogenetic tree of NCR and encoding proteins of IBDV genome. **a** VP5 + VP2/4/3. **b** VP1. **c** 5′ NCR of segment A. **d** 3′ NCR of segment A. **e** 5′ NCR of segment B. **f** 3′ NCR of segment B

CEF20-3 revealed a deletion of 3235C and mutations of 3205C → T and 3224A → T; CEF20-1 and CEF20-2 were the same as the bursa-derived virus. In segment B, the 3′ NCR displayed a deletion of 2798G and a mutation of 2807A → G in the 19th CEF virus, but the 5′ NCR exhibited 7 nucleotide mutations in CEF20-2 and CEF20-3 (Fig. 3b). These data show that nucleotide deletion only appeared in the 3′ NCR of segments A and B during CEF adaption of the field IBDV, and that nucleotide mutations of the 5′ and 3′ NCR within fragment B were earlier than those within fragment A.

Mutation of non-structural proteins of IBDV

Data in Fig. 4 shows the appearance of amino acid mutations 49R → G, 78F → I, 109S → G, 129P → S, and 137 W → R in the CEF21 virus. The amino acid residues "MLSL" believed to be present in almost all vvIBDV [19] were further lost in VP5 protein, when CEF virus was serially passaged to the 26th generation. In the predicted VP4 protein, no difference of amino acid residues was found from the BF virus to the CEF20 virus; however,

mutations were presented at 29I → V, 110R → G, 168Y → C, 173 N → K, 196A → V, 203S → P, and 239D → H in the CEF21 virus. These data showed that the amino acid mutations occurred in the VP4 and VP5 genes of IBDV during CEF adaption.

Amino acid mutations of IBDV structural protein

As shown in Fig. 4, mutations of amino acid residues were not detected in the VP1 protein of the CEF16 and CEF19 viruses and in the VP2 and VP3 proteins of the CEF16 and CEF20 viruses, when compared with the BF virus. However, amino acid mutations in VP1 protein arose at positions of 390, 393, 508, 511, 562, 646, 687, and 695 in the CEF20-3 virus. Four amino acid mutations at positions of 222, 242, 256, and 279 occurred in N-terminus region of VP2 protein of CEF21 virus, and four amino acid variations at site 290, 294, 299, and 330 appeared in C terminus region of VP2 of CEF26 virus. These data indicate that the variations of the VP1 gene occurred earlier than in the VP2 and VP3 genes during CEF attenuation of the virulent IBDV.
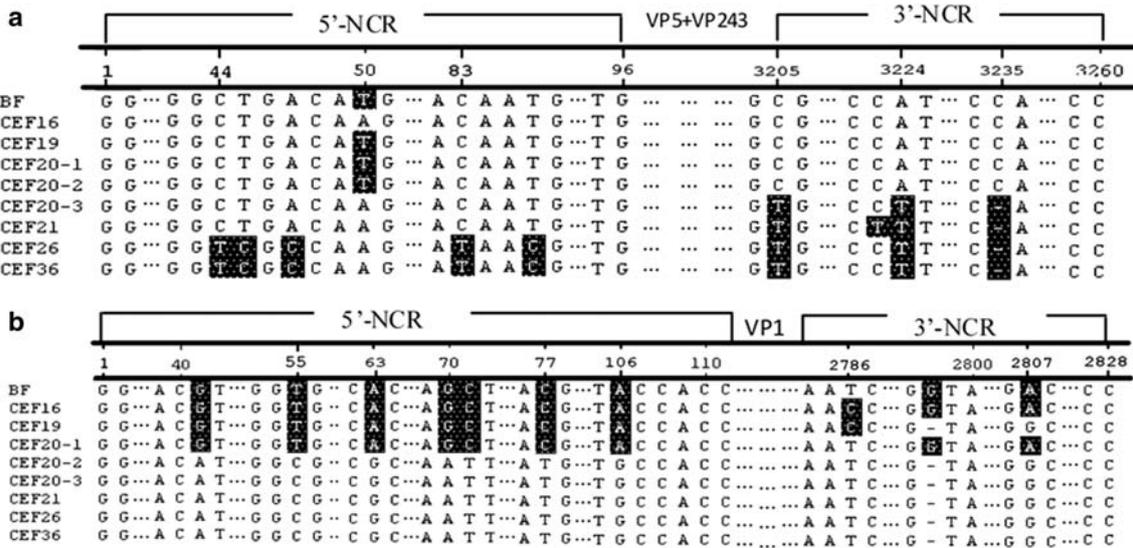
**Fig. 3** Alignment of non-coding region of segments A and B. **a** NCR of fragment A. **b** NCR of fragment B. *Shadow* indicates mutated nucleotides and "." represents the identical residues omitted
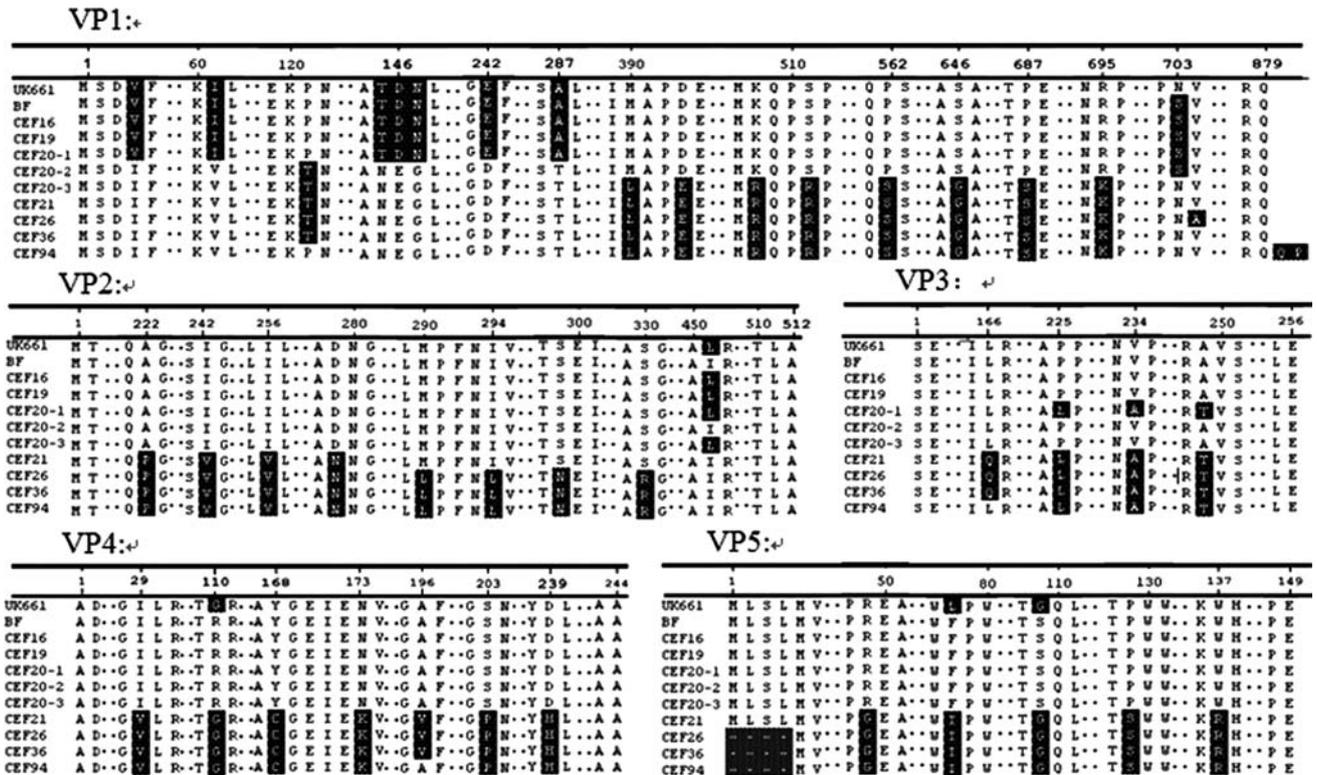


**Fig. 4** Amino acid comparison of encoding proteins of bursa-derived and CEF-adapted IBDV. *Shadow* indicates mutated amino acid residues and "." represents the identical residues omitted. UK661 and CEF94 are the European reference pathogenic and non-pathogenic IBDV strains, respectively

## Discussion

Since IBDV was identified in 1962, researchers have tried to attenuate the virulence of field IBDV in order to develop an appropriate attenuated vaccine. The conventional procedure for attenuating the virulence of field IBDV was to infect chicken embryonated eggs first, then pass on the infective virus to monolayers of chicken embryo bursal

cells, next to chicken embryo kidney cell line, and finally passaged on CEF monolayer. Although the field IBDV can adapt to primary cells or cell lines, e.g., CEF, Vero, and BGM-70 [17], the present study adapted the field IBDV NB isolate directly on to CEF monolayer. However, no gross or microscopic lesions of bursa were exhibited in the infected chickens during five serial passages of CEF26 and CEF36 viruses. Genomic nucleotide sequences also revealed that CEF26 and CEF36 had the most prominent characteristics of the reportedly attenuated IBDVs [20]. These observations confirmed that the virulence of CEF26 and CEF36 viruses were attenuated.

Identifying the virulence markers of IBDV is an interesting and much needed research focus. In the previous studies, VP2, especially the amino acid residues Q253, D279, and A284 located in hypervariable region, were identified to have a significant effect on virulence [8, 20]. Jackwood et al. [9] found that the mutation of the residue H253 to Q or N within VP2 would markedly increase the virulence of an attenuated IBDV. VP3 and VP5, as well as the segment B have also been identified to have an important relation with virulence [11, 13]. Our data also showed that the CEF26 and CEF36 viruses had lost their pathogenicity in the infected chickens, confirming that the residues Q253, D279, and A284 are involved in IBDV virulence. However we also observed that the earliest nucleotide mutations occurred at the 3′ NCR of segment B in the CEF19 virus, followed by nucleotide substitutions at the 3′ NCR of fragment A, the 5′ NCR of fragment B, and the VP1 gene in the CEF20 virus. Finally, the coding region of VP2 and VP5 displayed nucleotide mutations in the CEF21 virus. Based on these data, we determined that the loss of pathogenicity of IBDV is a process originating from mutation in the 3′ NCR of segment B and followed by multigenic mutations, rather than single gene mutation during CEF adaption.

In the present study, genomic sequences showed that the CEF20 virus was a mixed population of three genotypes of IBDV, homologous to the pathogenic BF virus and also the non-pathogenic CEF26 virus (Fig. 4). Interestingly, this phenomenon was not detected except in CEF-20 virus. Further experiments showed that the CEF20 virus population gradually changed to a single pathogenic IBDV after repeated chicken passages, and likewise the mixed population purified into a single non-pathogenic IBDV (CEF26 virus) after serial passages in CEF. Therefore, it was reasonable to believe that the different genotypes of IBDV, as observed in CEF20 population, underwent competitive replication during in vitro and in vivo serial passages. This competitive replication phenomenon has been observed in poliovirus and some DNA viruses [21, 22]. The selective pressures and mechanism involved in the competitive replication of different genotypes of IBDV requires further investigation.

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