Triplet amino acids located at positions 145/146/147 of the RNA polymerase of very virulent infectious bursal disease virus contribute to viral virulence

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Infectious bursal disease virus (IBDV) causes an economically significant disease of chickens worldwide. The emergence of very virulent IBDV (vvIBDV) has brought more challenges for effective prevention of this disease. The molecular basis for the virulence of vvIBDV is not fully understood. In this study, 20 IBDV strains were analysed phylogenetically and clustered in three branches based on their full-length B segments. The amino acid triplet located at positions 145/146/147 of VP1 was found highly conserved in branch I non-vvIBDVs as asparagine/glutamic acid/glycine (NEG), in branch II vvIBDVs as threonine/glutamic acid/glycine (TEG) and in branch III vvIBDVs as threonine/aspartic acid/asparagine (TDN). Further studies showed that the three amino acids play a critical role in the replication and pathogenicity of vvIBDV. Substitution of the TDN triplet with TEG or NEG reduced viral replication and pathogenicity of the vvIBDV HuB-1 strain in chickens. However, the replication of the attenuated IBDV Gt strain was reduced in chicken embryo fibroblast cells, whilst it was enhanced in the bursa by substituting NEG with TEG or TDN. The exchange of the three amino acids was also found to be capable of affecting the polymerase activity of VP1. The important role of segment B in the pathogenicity of IBDV was confirmed in this study. These results also provided new insights into the mechanism of the virulence of vvIBDVs and may offer new targets for their attenuation to develop potential vaccines using reverse genetics.

INTRODUCTION

Infectious bursal disease virus (IBDV) is one of the most economically significant viruses of poultry worldwide, causing a contagious and immunosuppressive disease of young chickens known as Gumboro disease (van den Berg, 2000). Since the first discovery of the classical strain in 1957 (Eterradossi & Saif, 2008), the isolated strains were of low virulence and caused only 1–2% specific mortality in the field (Ingrao et al., 2013). As of 1987, however, very virulent IBDV (vvIBDV) strains had emerged, causing >70 up to 100% mortality and producing lesions in other immune organs in addition to the bursa of Fabricius (Brown et al., 1994; Chettle et al., 1989; van den Berg et al., 1991). The pathogenic serotype 1 viruses can now be further categorized into classical, attenuated, very virulent and antigenic variant isolates.

IBDV belongs to the family Birnaviridae and has a bisegmented dsRNA genome (Delmas et al., 2004). The larger segment A contains two partially overlapping ORFs, encoding the non-structural protein VP5 and a polyprotein that gives rise to pVP2, VP3 and VP4 (Birghan et al., 2000; Lombardo et al., 2000). Segment B contains one large ORF encoding VP1, the RNA-dependent RNA polymerase (RdRp) responsible for the replication of the genome and synthesis of mRNAs (Pan et al., 2007; von Einem et al., 2004). The three-dimensional structure of the IBDV RdRp showed that the polypeptide chain can be divided into three domains: an N-terminal domain (residues 1–167), the central polymerase domain (residues 168–658), which contains the ‘fingers’, ‘palm’ and ‘thumb’ subdomains, and a C-terminal domain (residues 659–878) (Garriga et al., 2007; Pan et al., 2007). The N-terminal domain contains the putative self-guanylation site of IBDV RdRp and is responsible for protein priming; the central domain contains all of the structural motifs of RdRp and is suggested to be involved in nucleotide recognition and
binding; the C-terminal domain may prevent back-primed RNA synthesis during protein priming (Garriga et al., 2007; Pan et al., 2007).

Although some progress has been made in understanding the molecular biology of IBDV, the molecular basis for the pathogenicity of vvIBDV is still not fully understood. The specific amino acids that are involved in cell tropism and virulence of IBDV have been mapped in VP2 by using a reverse-genetics approach (Brandt et al., 2001; Mundt, 1999; van Loon et al., 2002). However, recent studies demonstrated that VP2 of vvIBDV was not the sole determinant for their pathogenicity and both segments contribute to the pathogenicity of vvIBDV (Boot et al., 2000; Escaffre et al., 2013; Le Nouën et al., 2012).

The phylogenetic study of different IBDV strains indicated that the genome segment B of vvIBDV was highly conserved and distinct from non-vvIBDV (Islam et al., 2001; Jackwood et al., 2012). In our previous study, we found that the vvIBDVAs had eight conserved amino acids in their VP1 proteins (Yu et al., 2010). Further, valine (position 4) was found to play a role in determining the virulence of vvIBDV (Yu et al., 2013). Interestingly, another residue that is not included in the eight conserved amino acids of VP1, threonine (position 276), was also shown to contribute to viral pathogenicity (Escaffre et al., 2013). These results provided new insights into the molecular basis of the virulence of vvIBDV. However, further studies are still needed to explore other residues of VP1 that could influence the activity of IBDV RdRp and contribute to viral virulence.

In this study, we constructed full-length cDNA clones of segments A and B of both attenuated IBDV Gt and vvIBDV HuB-1. Using the reverse-genetics system, we rescued both viruses and mutant viruses with the triplet amino acids located at positions 145/146/147 of VP1 exchanged. Viral pathogenicity, replication, and the polymerase activity of VP1 were evaluated. The data showed that the 145/146/147 amino acid triplet contributed to the virulence of vvIBDV and the substitution of this amino acid triplet affected the activity of IBDV RdRp as well as viral replication.

**RESULTS**

**Amino acid exchanges in VP1 among different IBDV strains**

The full-length genome sequences of segment B from 20 viruses were aligned and analyzed phylogenically in this study, which showed that these viruses clustered in three different branches (Fig. S1, available in the online Supplementary Material). All the non-vvIBDV strains, including the classical virulent, antigenic variant, classical attenuated and serotype 2 strains, clustered in branch I. Branch III was composed of the typical vvIBDV and part of Chinese vvIBDV strains, whilst all the viruses clustered in branch II were vvIBDV strains isolated in China. Compared with the VP1 amino acid sequences of the non-vvIBDV strains located in branch I, VP1 of the branch III vvIBDV strains has 15 conserved amino acids, whilst the vvIBDV strains in branch II only have eight conserved amino acids (Table 1). Further, the amino acids located at positions 145/146/147 of VP1 were highly conserved in branch I non-vvIBDVs as asparagine/glutamic acid/glycine (NEG), in branch II vvIBDVs as threonine/glutamic acid/glycine (TEG) and in branch III vvIBDVs as threonine/aspartic acid/asparagine (TDN), which revealed a unique motif that could distinguish the different branches of IBDV strains.

**Rescue of viruses**

Successful virus rescue was achieved when cells were co-transfected with plasmid pCAGGgtAHRT carrying segment A of the attenuated IBDV Gt strain, and with the constructs pCAGGgtBNEG, pCAGGgtBTEG or pCAGGgtBTDN containing the WT and the mutant segment B of Gt (Fig. 1), yielding the cell-adapted viruses rGtNEG, rGtTEG and rGtTDN, respectively. The results of an immunofluorescence assay (IFA) demonstrated that cells infected with these rescued viruses had detectable fluorescent signals when incubated with the VP2-specific mAb, whereas fluorescent signals were not observed in the mock-infected control (Fig. 2a–d). The cell-non-adapted viruses rHuBTDN, rHuBDNDN, rHuBTEG and rHuBNEG were also rescued successfully in specific-pathogen-free (SPF) chickens. Chickens inoculated with these viruses developed typical signs of infectious bursal disease, and an abundance of non-enveloped and icosahedral-shaped IBDV particles with a diameter of ~60 nm were observed by electron microscopy in the bursae of infected chickens (Fig. 2e–h). No virus particles were observed in the bursae of mock-infected control chickens (Fig. 2i). The rescued viruses were further confirmed by reverse transcription (RT)-PCR analysis of viral RNA template. Subsequent sequence analysis of the RT-PCR products confirmed the expected mutation in segment B of the rescued viruses.

**Substitution of the three amino acids at positions 145/146/147 of VP1 affected viral replication in vitro and in vivo**

To investigate whether substitution of the NEG triplet with TEG or TDN affected the growth of the WT virus rGtNEG, replication of the cell-adapted viruses was compared in chicken embryo fibroblast (CEF) cells and in chickens. As shown in Fig. 3(a), the kinetics and magnitude of replication for rGtTEG and rGtTDN in CEF cells were comparable to those for rGtNEG before 36 h post-infection (p.i.); however, these two mutated viruses replicated significantly slowly after 36 h p.i., yielding titres >1 log lower than that of rGtNEG at 72 h p.i. The replication of the rescued viruses in the bursae of infected chickens was determined at 7 days p.i., which showed that rGtTEG exhibited a significantly higher level of virus load than rGtNEG and the virus load of rGtTDN was even higher than that of rGtTEG (P<0.05) (Fig. 3b). The results
revealed that viral replication was reduced in vitro, whilst it was enhanced in vivo by substituting the NEG triplet with TEG or TDN. The replication of the cell-culture-non-adapted viruses carrying segment A of vvIBDV HuB-1 was evaluated in chickens, which showed that the virus load of the WT virus rHuBTDN was significantly higher than those of the mutated virus rHuBNDN, rHuBTEG and rHuBNEG at 7 days p.i. (P, 0.05) (Fig. 3c). The results indicated that the substitution of TDN with NDN, TEG or NEG reduced viral replication in chickens.

Substitution of the three amino acids at positions 145/146/147 of VP1 affected viral pathogenicity in chickens

The in vivo pathogenicity of the rescued viruses was investigated in 3-week-old SPF chickens. Throughout the experimental period, no death and no clinical symptoms of infectious bursal disease were observed for chickens receiving the cell-adapted viruses rGtNEG, rGtTEG or rGtTDN. The bursa:body weight index (BBIX) among these groups was similar and remained above the critical value of 0.7 (Fig. 4a), indicating that the bursae of chickens infected with viruses carrying segment A of attenuated Gt were not atrophic. The histopathological sections of bursae from group rGtNEG showed normal follicles and follicular connective tissues, and no microscopic lesions were observed, with the histopathologic bursal lesion scores (HBLSs) being 0–1. However, the bursae derived from one and two chickens out of five in groups rGtTEG and rGtTDN showed scattered or partial bursal damage (HBLS = 2), respectively (Fig. 4b).

The pathogenicity of the rescued viruses carrying segment A of vvIBDV HuB-1 was significantly severer than that of the cell-adapted viruses. All the bursae derived from groups rHuBTDN, rHuBNDN, rHuBTEG and rHuBNEG at 7 days p.i. were atrophic with BBIX < 0.7 (Fig. 4a), and gross bursal lesions including necrosis of lymphocytes, fibroplasias, atrophy of follicles and follicular depletion were observed (HBLS = 3–5, Fig. 4b). Nevertheless, the mortality among these groups was significantly different. The chickens infected with the parental virus rHuBTDN began to die at 3 days

### Table 1. Amino acid exchanges in VP1 among classical virulent, antigenic variant, classical attenuated, very virulent and serotype 2 IBDV strains

<table>
<thead>
<tr>
<th>Strains</th>
<th>Virulence</th>
<th>Geographical origin</th>
<th>Phylogenic branch*</th>
<th>Amino acid exchanges in VP1 at position†</th>
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<td>Variant</td>
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<td>France</td>
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*Genome sequences of segment B from 20 IBDV strains were aligned and analysed phylogenetically, forming three branches. The classical virulent, antigenic variant, classical attenuated and serotype 2 strains were clustered in branch I, whilst the very virulent IBDV strains were clustered in branches II and III.

†The VP1 amino acid sequence of the classical virulent strain F52/70 was used as a reference. Amino acid residues matching those of F52/70 are indicated as dots (.).
Amino acid 145/146/147 triplet of VP1 affects viBDV virulence

Fig. 1. Schematic diagrams of the recombinant eukaryotic expression plasmids containing segment A and mutated segment B of IBDV. (a) Based on pCAGGGtBNEG, plasmids pCAGGGtBTEG and pCAGGGtBTDN were generated by introducing one (N145T) or three (N145T, E146D and G147N) mutations at positions 145 or 145/146/147 of Gt VP1. (b) Plasmids pCAGGHuBBNDN, pCAGGHuBBTEG and pCAGGHuBBNEG were constructed by introducing one (T145N), two (D146E, N147G) or three (T145N, D146E, N147G) mutations at positions 145, 146/147 or 145/146/147 of HuB-1 VP1 using the WT pCAGGHuBBTDN as the backbone. The genome cDNA sequences are preceded by a cytomegalovirus (CMV) enhancer and a chicken β-actin promoter, and are flanked by the cDNAs of HamRz and HdvRz.

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p.i. with a high mortality of 46.7% and then the mortality soared to 86.7% at 4 days p.i. Within 5 days after inoculation, 14 out of 15 chickens (93.3%) inoculated with rHuBTDN were dead (Fig. 4c). The clinical signs of chickens inoculated with the mutated viruses rHuBNDN, rHuBTEG or rHuBNEG appeared later compared with the
chickens infected with rHuBTDN. Seven out of 15 chickens inoculated with rHuBNDN or rHuBTEG died at the end of the experiment, yielding a mortality of 46.7%. The chickens in group rHuBNEG exhibited an even lower mortality of 33.3% and only five out of 15 chickens were dead. The results indicated that the substitution of TDN with NDN, TEG or NEG reduced the pathogenicity of vvIBDV.

Substitution of the three amino acids at positions 145/146/147 of VP1 affected viral polymerase activity

To investigate whether the substitution of the amino acids at positions 145/146/147 of VP1 affected the polymerase activity of IBDV RdRp, minigenome systems derived from segment B of attenuated Gt and vvIBDV HuB-1 were developed in this study. The WT or mutated VP1 along with the GtBLP/HuBBLP and the proteins from segment A were expressed in DF1 cells, and the relative polymerase activity was represented as the expression level of the reporter gene. As shown in Fig. 5(a), the substitution of the NEG triplet with TEG or TDN reduced the polymerase activity of Gt VP1 significantly \((P<0.05)\). However, the substitution of the TDN triplet with NDN, TEG or NEG enhanced the polymerase activity of HuB-1 VP1 significantly \((P<0.05)\) (Fig. 5b). Further, the relative polymerase activity of HuBBNEG was even higher than that of HuBBNDN and HuBBTEG \((P<0.05)\). The results indicated that the substitution of the amino acid triplet at positions 145/146/147 of VP1 affected viral polymerase activity.

DISCUSSION

IBDV of chickens is one of the most economically significant viruses of poultry worldwide. The exploration of the molecular determinants of vvIBDV virulence is an area of active research (Müller et al., 2003). During the alignment of the deduced amino acid sequences of VP1, we found that the triplet amino acids at positions 145/146/147 of VP1 represented a unique characteristic among different branches. All the non-vvIBDV strains clustered in branch I and had the NEG triplet at this location. The vvIBDV strains clustered in branches II and III had TEG and TDN at this location, respectively. Based on the 5’ region of genome segment B from 117 IBDV strains, Jackwood et al. (2012) also reported that all the vvIBDV strains had TDN at this location, whilst the most common non-vvIBDV motif was NEG. In addition, the exclusive mutation from TND to NEG was found during the attenuation process of a bursal-derived vvIBDV strain by serial passage in CEF cells (Shi et al., 2009). However, it had not been identified previously if the exchange of the three amino acids could influence the pathogenicity of vvIBDV. The results of this study demonstrated that the TDN triplet is one of the molecular determinants contributing to the enhanced virulence of vvIBDV. The substitution of the amino acid triplet located at positions 145/146/147 of VP1 affected viral replication and pathogenicity.

To examine whether the three amino acids at positions 145/146/147 of VP1 could affect viral pathogenicity, we rescued the WT and a series of mutant viruses based on the parental viruses, attenuated IBDV Gt and vvIBDV HuB-1. The rescued WT viruses rGtNEG and rHuBTDN were shown to exhibit the same phenotype as their parental Gt and HuB-1 strains, as demonstrated by similar replication ability and pathogenicity in chickens (Fig. S2). In the study, the mutant viruses rGtTEG and rGtTDN did not induce mortality in chickens as did rGtNEG and their parental virus Gt, which was related to the attenuated phenotype of Gt used here as the backbone. Nonetheless, the histopathologic lesions...
caused by rGtTDN in bursae were more severe when compared with other cell-adapted viruses. With vvIBDV HuB-1 as the parental virus, however, the WT and the mutated viruses exhibited significantly different mortalities. Following the substitution of the TDN triplet with NDN, TEG or NEG, the mortality caused by rHuBTDN was reduced by 46.7, 46.7 or 60%, respectively. The results suggested that the TDN triplet contributed to the enhanced virulence of vvIBDV. Boot et al. (2000) generated a reassortant virus with segment A from attenuated CEF94 and segment B from vvIBDV D6948, and found that neither this virus nor rCEF94 caused mortality, whilst the mortality caused by rD6948 was reduced when its segment B was exchanged with the CEF94-derived cell-adapted segment B. Given the previous data and the results obtained here, we suggest that the pathogenicity of IBDV is dominated by segment A, whilst it is also influenced by segment B. Further, the results indicated that both the single mutation of T145N and the duplet substitution of DN146/147EG could reduce the pathogenicity of vvIBDV, and these two exchanges had a synergistic effect. The effects of the single mutations, D146E and N147G, on viral pathogenicity will be determined in further studies.

In this study, the viral replication was reduced in cell cultures, whilst it was enhanced in chickens by substituting the NEG triplet with TEG or TDN. However, the substitution of TDN with NDN, TEG or NEG reduced viral replication in chickens. The results revealed that the exchange of the three amino acids affected viral replication, which was consistent with the pathogenicity of the viruses. Further, the 145T and 146/147DN conserved in vvIBDV strains had a negative effect on viral replication in cell culture, whilst they had a positive effect on replication in chickens. The negative effect of the vvIBDV segment B on viral replication in cell cultures was also observed in previous studies (Boot et al., 2005; Liu & Vakharia, 2004). Boot et al. (2005) supposed that there might be factors located on segment B of cell-adapted isolates contributing to the enhanced cell culture replication. The results of this study indicated that the NEG triplet at location positions 145/146/147 of VP1 could be one such factor.

The VP1 protein is an RdRp of IBDV (von Einem et al., 2004). To determine whether the substitution of the 145/146/147 triplet could affect VP1 polymerase activity, we developed two luciferase-based minigenomes derived from segment B of Gt or HuB-1 in this study. The results showed that the substitution of NEG with the vvIBDV triplet TEG or TDN reduced the polymerase activity of Gt VP1, corresponding to the lower replication detected in cell cultures, whilst the substitution of the TDN triplet with NDN, TEG or NEG enhanced the polymerase activity of HuB-1 VP1. These data indicated that the exchange of either the single amino acid N145T or the duplet amino acids NE146/147DN affected polymerase activity. The 145N and 146/147EG conserved in non-IBDV strains had a positive effect on polymerase activity in DF1 cells relative to the vvIBDV-like 145T and 146/147DN. Yu et al. (2013)
Fig. 4. Comparison of viral pathogenicity of the WT and mutant IBDV strains in SPF chickens. Groups of 3-week-old SPF chickens were inoculated with $10^{4.4}$ TCID$_{50}$ of the cell-culture-adapted viruses rGtNEG, rGtTEG or rGtTDN, or $10^{3}$ embryo LD$_{50}$ of the bursal-derived viruses rHuBTDN, rHuBNDN, rHuBTEG or rHuBNEG by the eye and intranasal routes, respectively. Chickens inoculated with DMEM without any virus were kept as negative controls. Chickens were monitored daily for clinical signs after infection for 10 days. (a) BBIX of chickens at 7 days p.i. The BBIX was calculated as (bursa : body weight ratios)/(bursa : body weight ratios in the negative group) and presented as the mean and SD for each group. Bursae with a BBIX <0.70 were considered atrophied. (b) HBLSs of chickens at 7 days p.i. Bursae with a HBLS >1.0 were considered bursal-damaged. Mean and SD from five chickens of each group are indicated. Treatments followed by different lower-case letter are significantly different according to one-way ANOVA at the $P<0.05$ confidence level. (c) Per cent survival of chickens on different days after infection with the bursal-derived viruses. No chickens infected with the cell-culture-adapted viruses died during the experiment (data not shown).

Several mechanisms might be involved in the effects of the 145/146/147 amino acids on polymerase activity. First, the exchange of the three amino acids might influence the structure of the functional motifs of VP1, which may subsequently affect the polymerase activity. The N-terminal domain of VP1 where the 145/146/147 amino acids are located contains a putative self-guanylation site for the protein priming activity of IBDV RdRp (Pan et al., 2009). The effects of the three amino acids on primer structure activity might be due to a structural change of this site that affected the efficiency of protein priming. Positions 145/146/147 are near a $\beta$-sheet and a loop in the ‘palm’ subdomain (Garriga et al., 2007), so the mutation of these amino acids may also change the structure of the central polymerase domain. Further, these three amino acids lie on the surface of VP1 and position 145 is located in a loop, indicating that this location might be involved in the interaction between VP1 and the dsRNA genome as well as the related proteins, including VP3 and other host proteins (Stricker et al., 2002). The exchange of these three amino acids might influence VP1 interaction, which may affect viral replication and pathogenicity.

Following the alignment of the B segments, we found that the coding sequence was exclusively ACA/GAT/AAC for the TDN motif in branch III vvIBDV strains and it was AAC/GAG/GGC for the NEG motif in non-vvIBDV strains. Interestingly, the most common coding sequence for the TEG motif in branch II vvIBDV strains was ACA/GAG/GGC. Further, there examined the effects of the substitution of the eight amino acids conserved in branch III vvIBDV strains on polymerase activity and found that the polymerase activity of the HLJ-4 strain was enhanced significantly by a single V4I substitution, whilst the T145N mutation only increased slightly the polymerase activity without any statistically difference. Hence, the effects of T145N on viral pathogenicity were not further examined by Yu et al. (2013). Whilst determining the effects of different 145/146/147 triplets on pathogenicity, interestingly, we found that a single T145N substitution reduced the mortality and viral replication of rHuBTDN dramatically. In further analyses, the single mutation at position 145 of VP1 was found to be capable of changing the polymerase activity of Gt and HuB-1 significantly in DF1 cells. The difference might be due to the different background of segment B used in the experiments. In this study, we used two B segments originating from an attenuated IBDV strain in branch I and a vvIBDV strain in branch III. In the study by Yu et al. (2013), however, a segment B from a branch II vvIBDV strain was used. The original polymerase activity of the IBDV strains in different branches might not be the same, which may lead to this difference.

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Amino acid 145/146/147 triplet of VP1 affects vvIBDV virulence

Taken together, these results confirmed the important role of segment B in viral pathogenicity of vvIBDV. Further, the amino acid triplet at positions 145/146/147 of VP1 is an important determinant of viral replication and pathogenicity. The substitution of these three amino acids could also affect the polymerase activity of IBDV RdRp. This study provides more insights into the mechanism of the virulence of vvIBDVs and may offer new targets for their attenuation using reverse genetics.

**METHODS**

**Cells and plasmids.** DF-1 cells were maintained in Dulbecco’s modified Eagle’s medium (DMEM) supplemented with 10% FBS at 37 °C, 5% CO2. Primary CEF cells were prepared from 10-day-old SPF chicken embryos, and used for virus propagation and titration. The eukaryotic expression vector pCAGGS (Niwa et al., 1991) was kindly supplied by Dr. J. Miyazaki (University of Tokyo, Tokyo, Japan).

**Animals.** SPF white leghorn chickens were obtained from the Experimental Animal Center of the Harbin Veterinary Research Institute (Chinese Academy of Agricultural Science, Harbin, PR China) and housed in negative-pressure-filtered air isolators. Animal experiments were approved by the Animal Ethics Committee of the Institute, and performed in accordance with animal ethics guidelines and approved protocols.

**Alignment of the genome sequences of segment B and the deduced amino acid sequences of VP1.** The full-length genome sequences of segment B from 20 viruses, including two classical virulent, four classical attenuated, two antigenic variant, 10 very virulent and two serotype 2 IBDV strains, were aligned using the sequence analysis software MEGA version 4 (Tamura et al., 2007). After the alignment, a phylogenetic tree was reconstructed by MEGA employing the neighbour-joining method. The deduced amino acid sequences of VP1 were also aligned and the conserved amino acid exchanges in VP1 among these strains are shown in Table 1.

**Construction of plasmids for virus rescue.** Construction of plasmids pCAGGGtAHRT and pCAGGGtBNEG containing segments A and B of Gt flanked by the hammerhead ribozyme (HamRz) sequence at the 5’ ends and hepatitis delta ribozyme (HdvRz) sequence at the 3’ ends, respectively, has been described previously (Qi et al., 2007). Based on pCAGGGtBNEG, plasmids pCAGGGtBTEG and pCAGGGtTDN were generated by introducing one (N145T) or three (N145T, E146D and G147N) mutations at positions 145 or 145/146/147 of Gt VP1 using site-directed mutagenesis (Fig. 1). Plasmids pCAGGHuBAHRT and pCAGGHuBTDN containing segments A and B of vvIBDV HuB-1 flanked by the ribozyme sequences were constructed using methods described previously (Qi et al., 2007). Plasmids pCAGGHuBBNDN, pCAGGHuBTEG and pCAGGHuBBNEG were constructed by introducing one (T145N), two (D146E, N147G) or three (T145N, D146E, N147G) mutations at positions 145, 145/146 or 145/146/147 of HuB-1 VP1 using the WT pCAGGHuBTDN as the backbone. All the constructed plasmids were confirmed by sequencing, and the primers used in plasmids construction are shown in Table S1.

**Virus rescue and identification.** To rescue the cell-culture-adapted viruses rGtNEG, rGtTEG and rGtTDN, DF1 cells were co-transfected with pCAGGGtAHRT and each plasmid carrying segment B of Gt (pCAGGGtBNEG, pCAGGGtBTEG or pCAGGGtTDN) using Lipofectamine 2000 (Invitrogen). DF1 cells were also co-transfected with pCAGGHuBAHRT and each plasmid carrying segment B of

are five other amino acids conserved in branch I and II viruses in addition to 146/147EG, as shown in Table 1, and the codons for three of them (242D, 393E and 562S) were also highly conserved. The data indicated that the ‘atypical’ vvIBDV B segments of branch II viruses might result from homologous recombination between the non-vvIBDV B segments in branch I and the ‘typical’ vvIBDV B segments in branch III (He et al., 2009). The other uncommon motifs observed at positions 145/146/147 could be a consequence of missense mutation, and all the mutations that resulted in the exchanges between NEG and NED or TEG and TES were found to be caused exclusively by a transition between a guanine (G) and an adenine (A) (data not shown).
HuB-1 (pCAGGHuBBTDN, pCAGGHuBBNDN, pCAGGHuBBTEG or pCAGGHuBBNEG) to rescue the cell-non-adapted viruses rHuBDN, rHuBBNDN, rHuBBTEG and rHuBBNEG. The culture supernatant containing the virus stocks was harvested after 72 h of transfection. For rGtNEG, rGtTEG and rGtTDN, the supernatant was passaged blindly into secondary CEF cells until a visible cytopathic effect appeared. For viruses rHuBDT, rHuBBND, rHuBBTEG and rHuBBNEG, the supernatant was injected into the bursae of 3-week-old SPF chickens. Chickens were examined daily, with acute infectious bursal disease symptoms or death being a sign of vIBDV infection (Yu et al., 2012).

To characterize the rescued viruses, an IFA with anti-VP2 mAb and electron microscopy were performed for the cell-culture-adapted viruses as described previously (Qi et al., 2007). For the cell-culture-non-adapted viruses, an electron microscopy assay was performed using the bursae of infected chickens. To verify whether the rescued viruses contained the introduced mutation, the full-length genomes of the rescued viruses were amplified and sequenced (Qi et al., 2007).

**Viral replication in cell cultures.** To compare the replication kinetics of the rescued viruses *in vitro*, the one-step growth was analysed. Confluent monolayers of CEF cells in culture flasks (25 cm²) were inoculated with 10⁴ TCID₅₀ of each virus based on the titre determined in CEF cells. Infected cell cultures were harvested at 12 h intervals and the titre of infectious progeny present in the culture was determined as TCID₅₀ ml⁻¹ using the Reed–Muench formula. Mean and SD were calculated from three independent experiments.

**Animal experiments.** To assess the pathology of the rescued viruses, a total of 120 three-week-old SPF chickens were divided randomly into eight groups of 15 chickens each. Each group was maintained separately in negative-pressure isolators. Chickens in groups 1, 2 and 3 were inoculated with 10⁴.4 TCID₅₀ rGtNEG, rGtTEG or rGtTDN, respectively. Chickens in groups 4, 5, 6 and 7 were inoculated with 10⁴ embryo LDT₅₀ rHuBDT, rHuBBND, rHuBBTEG or rHuBBNEG, respectively. Group 8 received DMEM without any virus as a negative control. Each group was inoculated by the eye and intranasal routes. Chickens were monitored daily for clinical signs after infection for 10 days.

At 7 days p.i., five chickens were chosen randomly from each group and euthanized for necropsy. Each chicken was examined for signs of pathological changes. The bursa and body weights of each chicken were determined, and BBIX was calculated as: BBIX = [(bursa: body weight ratios)/(bursa: body weight ratios in the negative group)]. Bursae with a BBIX <0.70 were considered atrophied (Lucio & Hitchner, 1980). Each obtained bursa was divided into two parts. One part was used for extraction of viral RNA and the other part was fixed in 10% neutral buffered formalin for histopathological analysis from which the severity of bursal follicular necrosis was recorded using the HBLS as described previously (Schröder et al., 2000). In brief, the HBLS was scored on a scale of 0–5: 0, no lesion; 1, slight change; 2, scattered or partial bursal damage; 3, 50% or less follicle damage; 4, 51–75% follicle damage; 5, 76–100% bursal damage.

**Viral replication in bursae of infected chickens.** To evaluate the replication efficiency of the rescued viruses *in vivo*, viral RNAs obtained from the bursae of infected chickens were quantified by real-time RT-PCR as described previously (Wang et al., 2009). Mean and SD of the data obtained from three independent experiments were calculated. The viral RNAs obtained from the bursae were also amplified by RT-PCR and sequenced to confirm infection with the intended viruses.

**Luciferase-based polymerase activity assay.** Using the previously described approach (Yu et al., 2013), the minigenome plasmids GtBLP and HuBBLP were constructed based on segment B of IBDV Gt and HuB-1 strains. Briefly, the sequence coding for luciferase was amplified from vector pGL-3 (Promega) with primers GtLUCU/GtLUCCL or HuBLUCU/GuLUCCL. The 5'- and 3'-UTR sequences of segment B of Gt and HuB-1 were amplified from pCAGGGBBNEG or pCAGGHuBBTDN using primer pairs GtSU3UR/GtSU3TRL and GuSU3UR/GuSU3TR/HuBU5UTR and Gu3UTR/Gu3UTR/GtSU3TR, respectively. The luciferase ORF was flanked with the 5'- and 3'-UTR sequences by overlapping PCR using primers GtSU3TR/GtSU3TRL. The resulting fragment was fused in an antisense orientation with HdrZ and HsuZ sequences using primers P7, P8 and P9, and then inserted into the pCAGGS vector. All the constructed plasmids were confirmed by sequencing.

To determine the activity of IBDV RdRp as reflected by the expression level of the encoded reporter gene, DF1 cells were transfected with GtBLP or HuBBLP together with pTK-RL (Promega), each plasmid carrying segment A and WT or mutated segment B plasmid as summarized in Table S2. At 36 h post-transfection, the cell extracts were harvested and luciferase activity was assayed by using the Dual-Luciferase assay kit (Promega). Plasmid pTK-RL encoding Renilla luciferase was used as an internal control in this study to normalize cell viability and transfection efficiencies between wells. Transfection mixtures with the empty vector pCAGGS were used as a negative control. All experiments were performed in triplicate, and mean and SD were calculated.

**Statistical analysis.** One-way ANOVA was employed to evaluate the statistical differences among groups using SPSS 17.0 (SPSS). Statistical significance was set at *P*<0.05 for all tests.

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**REFERENCES**


Amino acid 145/146/147 triplet of VP1 affects vvIBDV virulence


