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A single mutation in the P_{BC} loop of VP2 is involved in the *in vitro* replication of infectious bursal disease virus

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To test whether amino acid mutations in the P_{BC} and P_{HI} loops of VP2 are involved in the replication and virulence of infectious bursal disease virus (IBDV), a pair of viruses, namely the moderately virulent IBDV (rGx-F9VP2) and the attenuated strain (rGt), were used. Residue mutations A222P (P_{BC}) and S330R (P_{HI}), selected by sequence comparison, were introduced individually into rGx-F9VP2 by using a reverse genetics system. In addition, the reverse mutation of either P222A or R330S was introduced into rGt. The four modified viruses were then rescued and evaluated *in vitro* (CEF cells) and *in vivo* (SPF chickens). Results showed that A222P elevated the replication efficiency of rGx-F9VP2 while P222A reduced that of rGt in CEF cells. A mutation at residue 330 did not alter IBDV replication. In addition, animal experiments showed that a single mutation at either residue 222 or 330 did not significantly influence the virulence of IBDV. In conclusion, residue 222 in P_{BC} of VP2 is involved in the replication efficiency of IBDV *in vitro* but does not affect its virulence *in vivo*, further facilitating our understanding of the gene-function of IBDV.

infectious bursal disease virus (IBDV), VP2, P_{BC}, replication

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INTRODUCTION

Infectious bursal disease virus (IBDV) is the causative agent of a highly contagious disease in chickens known as infectious bursal disease (IBD). IBD causes significant losses of the poultry industry owing to its high mortality and immunosuppression in young chickens via the destruction of developing B lymphocytes in the bursa (Cosgrove, 1962; Muller et al., 2003). IBDV belongs to the *Birnaviridae* family and has a genome that consists of two segments of double-stranded RNA (segments A and B). Segment B encodes the VP1 protein, the viral RNA-dependent RNA polymerase (Le Nouen et al., 2006; von Einem et al., 2004), while segment A contains two partially overlapping open reading frames (ORF). The smaller ORF encodes the non-structural protein VP5 (Mundt et al., 1995), while the larger one encodes a polyprotein which is cleaved into proteins pVP2, VP3, and VP4 (Birghan et al., 2000). VP2 and four peptides are further derived from the maturation of pVP2 (Da Costa et al., 2002).

As the sole component of the icosahedral capsid, VP2 is folded into three distinct domains, designated the base (B), shell (S), and projection (P) (Coulibaly et al., 2005; Garriga et al., 2006; Lee et al., 2006). Domains B and S are relatively well conserved, whereas domain P is more variable. The tower-like P domain contains four loops, P_{BC}, P_{HI}, P_{DE},

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and P_{FG}. It has been reported that the P_{DE} and P_{FG} domains are responsible for cell-tropism and virulence (Brandt et al., 2001; Qi et al., 2009, 2013; van Loon et al., 2002). The other two loops, P_{BC} and P_{HI}, are also located at the tip of the VP2 spike; however, their roles in viral replication and virulence are not fully understood.

Previously, a moderately virulent IBDV strain (rGx-F9VP2) and an attenuated strain (rGt) were rescued, providing a good model system for studying the viral replication and virulence of IBDV (Qi et al., 2007, 2009, 2013). In the current study, specific amino acid mutations in the P_{BC} and P_{HI} domains of VP2 were introduced into the backbone of the virulent (rGx-F9VP2) and attenuated (rGt) strains in order to evaluate the roles of these amino acid changes in replication and virulence.

RESULTS

Sequence analysis of the P_{BC} and P_{HI} domains of VP2

Sequence alignment showed that, compared to virulent IBDV including rGx-F9VP2, two amino acids mutations, one at loop P_{BC} (A222P) and another at loop P_{HI} (S330R), were observed in the attenuated strain including Gt (Figure 1).

Rescue and identification of the modified IBDV

In cells co-transfected with pCGxATA-G794CHRT/pCGxBHRT or pCGxATA-T1120AHRT/pCGxBHRT, the modified IBDV rGxHT-222 or rGxHT-330 strains were rescued, which contained a single mutation A222P in P_{BC} or S330R in P_{HI} of VP2 compared to the virulent strain rGx-F9VP2, respectively. In cells co-transfected with pCmGtA-C794GHRT/pCmGtBHRT or pCmGtA-A1120THRT/pCmGtBHRT, the modified IBDV rGt-222 or rGt-330 strain was rescued, which contained the reverse mutation P222A or R330S compared to the attenuated strain rGt.

Replication characteristics of the modified IBDV

To investigate the replication character *in vitro* of the modified IBDV strains in detail, a replication kinetics curve in CEF cells was depicted (Figure 2). Compared to the parental strain of rGx-F9VP2, rGxHT-222 replicated more efficiently, where the titers were significantly higher than rGx-F9VP2 after 36 h post-infection (p.i.) (P<0.05). rGxHT-330 showed a similar curve to rGx-F9VP2 (Figure 2A). Compared to the rGt parental strain, rGt-222 replicated less efficiently in CEF cells after 48 h p.i.. The titer of rGt-222 was $10^{6.7}$ $TCID_{50}$ mL^{-1} at 60 h p.i., which was ten times lower than that of rGt (P<0.05) (Figure 2B). rGt-330 showed a similar curve to rGt (Figure 2B).

The pathogenicity of the modified IBDV to chicken

The pathogenicity of the modified IBDV rGxHT-222 and rGxHT-330 strains was evaluated using three-week-old SPF chickens. Neither death nor clinical symptoms of IBD were observed in any of the groups during the experimental period. In order to investigate the underlying sub-clinical lesion, the BBIX at different days p.i. was calculated. The BBIX of the rGxHT-330 and rGx-F9VP2 groups were below 0.7 from 3 d p.i. to 14 d p.i., indicating signs of atrophy. In the rGxHT-222 group, a BBIX above 0.7 was observed before 3 d p.i. and then the BBIX decreased to below 0.7 (Figure 3A).

The histopathological changes of the infected bursae are presented in Figure 3B. Chickens from all groups, rGxHT-222, rGxHT-330, and rGx-F9VP2, showed persistent histopathological bursal lesions, including lymphocytic deletion and necrosis, regional atrophy, and fibrosis of the follicle (average HBLS was 4). No obvious bursal lesions were observed in the DMEM control group (HBLS was 0). RT-PCR and sequencing results confirmed that the modified viruses replicated in the bursae of chickens without causing additional mutations (data not shown).

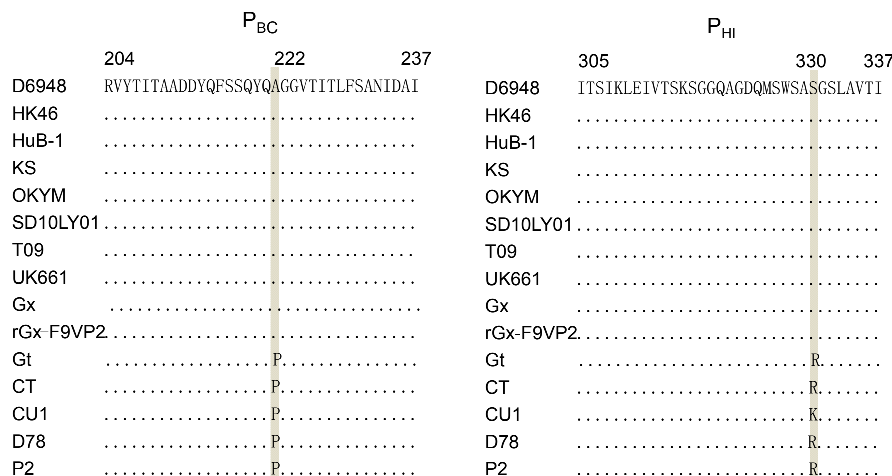

Figure 1 Comparison of the amino acid sequences of the P_{BC} (aa 204–237) and P_{HI} domain (aa 305–337) of IBDV VP2 from virulent and attenuated strains. The amino acids differences are marked.

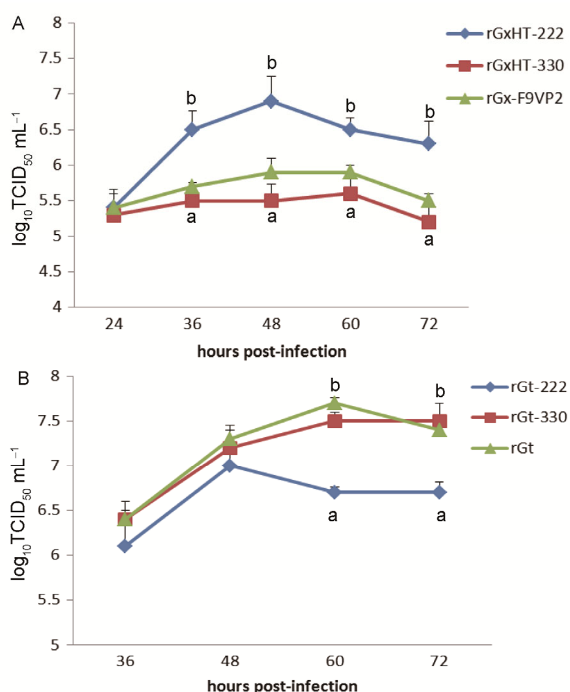

Figure 2 Replication characteristics of the modified IBDV. Replication kinetics curves *in vitro* of the modified IBDV based on backbone of rGx-F9VP2 and rGt strains are shown in (A) and (B), respectively. Average titers and standard deviations (error bars) from three independent samples are shown. Treatments sharing different lowercase letters differ significantly at a confidence level (P<0.05).

DISCUSSION

As an RNA virus, IBDV is genetically prone to mutation. In terms of disease control and early warning, it is important to fully understand the gene or residue function. Since the development of reverse genetics, the role of VP2 in replication or virulence has attracted much attention (Brandt et al., 2001; Li et al., 2015; Qi et al., 2009, 2013; van Loon et al., 2002). However, the precise molecular determinants are still somewhat unclear. The synergistic effects of multi-genes or multi-residues are indispensable (Boot et al., 2000; Le Nouen et al., 2006), although the virulence and replication of many viruses, including IBDV, mostly depend on the major corresponding gene. The four loops of VP2, P_{BC}, P_{HI}, P_{DE}, and P_{FG}, are located at the outermost surface of IBDV. Loops P_{DE} and P_{FG} influence cell-tropism and virulence and have been researched in detail (Brandt et al., 2001; Lim et al., 1999; Mundt, 1999; Qi et al., 2009, 2013; van Loon et al., 2002). While the P_{BC} and P_{HI} residues are unlikely to be involved in three-dimensional folding (Letzel et al., 2007), mutations at some residues mutations are thought to directly or indirectly alter the characteristics of VP2 via different mechanisms (Durairaj et al., 2011; Letzel et al., 2007). Therefore, we hypothesized that P_{BC} and P_{HI} might contribute to viral replication or virulence.

Sequence analysis showed that two conserved residue differences, A222P (P_{BC}) and S330R (P_{HI}), were observed in

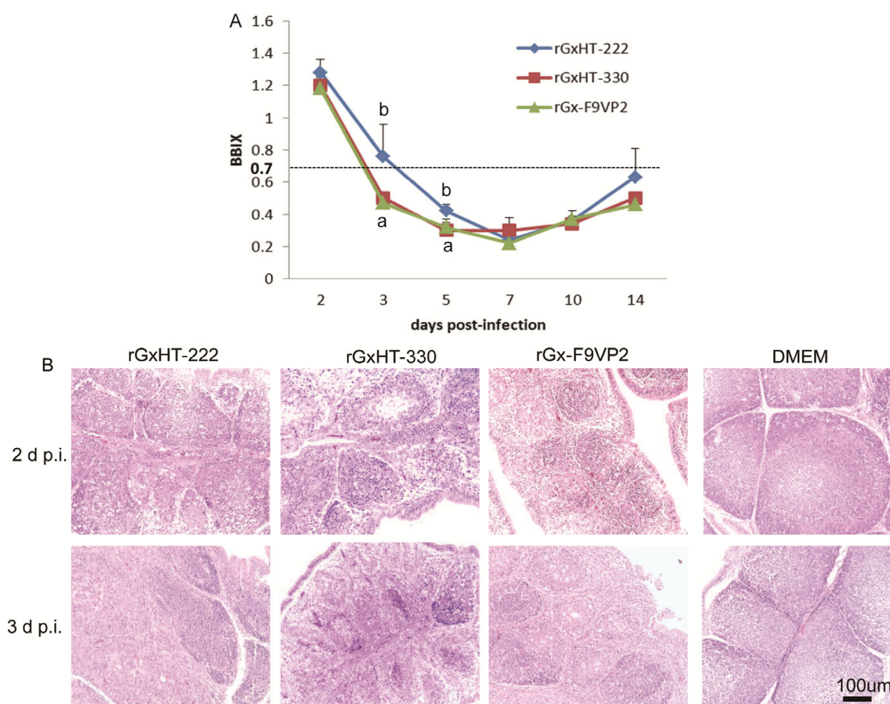

Figure 3 Evaluation of the influence of residue 222 or 330 of VP2 on the virulence of IBDV. A, Kinetics curves of bursa:body-weight index (BBIX) of SPF chickens infected with the modified virus. Average titers and standard deviations (error bars) from three independent samples are shown. Treatments sharing different lowercase letters differ significantly at a confidence level (P<0.05). B, Histopathological appearance of bursal sections (hematoxylin and eosin) derived from groups of chickens infected with the modified viruses.

the P_{BC} and P_{HI} domains between virulent and attenuated IBDV strains. The virulent IBDV strain rGx-F9VP2 and attenuated strain rGt were chosen to verify the hypothesis because they have a similar gene background but have different characters. Firstly, A222P (P_{BC}) and S330R (P_{HI}) were introduced into the backbone of rGx-F9VP2, and the resulting mutated viruses rGxHT-222 and rGxHT-330 were rescued. rGt replicated more efficiently *in vitro* compared with rGx-F9VP2. Replication of the mutated viruses was then evaluated. In CEF cells, it was clear that A222P of P_{BC} could significantly increase the replication of rGx-F9VP2 while S330R of P_{HI} could not. To further verify these results, the reverse mutation P222A or R330S was introduced into rGt and the rescued virus rGt-222 replicated less efficiently than rGt, while rGt-330 showed similar replicate curve to rGt. The data obtained from these different backbones verified that residue 222 of P_{BC} in VP2 could definitely influence the replication efficiency of IBDV *in vitro* but that a mutation in residue 330 could not.

Residue 222 of VP2 is typically A in vvIBDV and P in non-vvIBDV strains. Sometimes, T residue at position 222 of VP2 has also been observed in a few variant strains, including DEL-E, GLS, and GA988 (Jackwood and Sommer-Wagner, 2011; Letzel et al., 2007). Recently, a new occurring mutation P222S was observed in Belg strain (Letzel et al., 2007). A single substitution at position 222 (P222S or A222T) was able to alter the antigenic pattern of IBDV because an S or T residue at position 222 is the essential part of the epitope that reacts with MAb 67 (Letzel et al., 2007). Recently, a natural mutation T222A was shown to allow the Del-E strain to break through the maternal immunity induced by parenteral vaccination (Jackwood and Sommer-Wagner, 2011).

Residue 330 belongs to the heptapeptide-domain (aa 326-332) that closely follows the major hydrophilic peak B (aa 312-324 of VP2). There is usually a conserved amino acid difference at residue 330, which is R in attenuated and S in non-attenuated strains. Residue 330 had originally been thought to influence cell-tropism of IBDV; however, this has since been refuted (Brandt et al., 2001; Lim et al., 1999; Mundt, 1999). Recently, it was reported that recognition of the MAb 57 epitope involves the P_{HI} loop, and specifically a residue at position 330 (Letzel et al., 2007). The three-dimensional structure shows that residue 330 is located at the interface between subunits in the trimeric spike of VP2 and a mutation here could lead to subtle alterations in the jelly roll, ultimately leading to a different conformation of loop P_{BC} (Letzel et al., 2007). It is possible that the subtle change in the conformation of P_{BC} induced by the residue 330 mutation is not sufficient to alter the assembly and stability of the virus such that S330R cannot influence the replication of IBDV.

The virulence of rGxHT-222 and rGxHT-330 was further evaluated using SPF chickens. As was reported for rGx-F9VP2, chickens in both the rGxHT-222 and rGxHT-330 groups did not present any typical clinical symptoms. To further detect the pathological lesions, the BBIX and the degree of damage to the bursae were evaluated, parameters which have been used in many studies of sub-clinical disease (Boot et al., 2000; Brandt et al., 2001; Jackwood et al., 2008; Le Nouen et al., 2006; Liu and Vakharia, 2004; Qi et al., 2009; Raue et al., 2004; Rautenschlein et al., 2003). Atrophy of the bursa was not found in chicken samples from the rGxHT-222 group at 3 d p.i., but rGxHT-222, rGxHT-330, and rGx-F9VP2 did cause obvious histopathological bursal lesions. There is typically a negative correlation between the replication efficiency in cell culture and the virulence of IBDV. However, data from the current study demonstrate that the A222P mutation elevated the viral replication efficiency *in vivo* but did not obviously attenuate rGx-F9VP2. Further studies will be required to uncover the molecular mechanism that underlies this effect.

In conclusion, the current study represents the first demonstration that residue 222 in the P_{BC} loop of VP2 is involved in the replication efficiency of IBDV *in vitro* but does not have a role in its virulence *in vivo*. A mutation at residue 330 in the P_{HI} loop of VP2 did not contribute to the replication and virulence of IBDV. These findings are beneficial to our further understanding of the gene-function of IBDV.

MATERIALS AND METHODS

Viruses, cells, and plasmids

The rGx-F9VP2 strain of IBDV, which shows moderate virulence, was previously rescued and was shown to induce severe bursal lesions in chickens but did not cause mortality (Qi et al., 2009). The rGt virus was previously rescued from the infectious clones of the attenuated strain of Gt and has similar characteristics to the parental strain (Qi et al., 2007). DF-1 cells were cultured in Dulbecco's modified eagle medium (DMEM) (Invitrogen, USA) supplemented with 10% fetal bovine serum at 37°C in a humidified 5% CO_2 incubator. Chicken embryo fibroblast (CEF) cells were prepared from 10-day-old specific-pathogen-free (SPF) chicken embryos. The infectious clones pCmGtAHRT and pCmGtBHRT (containing segments A and B of rGt) (Qi et al., 2007), and the infectious clones pCGxAF9VP2HRT and pCGxBHRT (containing segments A and B of rGx-F9VP2) were constructed previously (Qi et al., 2009).

Animals

SPF chickens were purchased from the Experimental Animal Center of the Harbin Veterinary Research Institute (HVRI), the Chinese Academy of Agricultural Sciences (CAAS), and were housed in negative-pressure-filtered air isolators. All animal experiments were approved by the Animal Ethics Committee of HVRI.

Sequence alignment of the P_{BC} and P_{HI} domains of VP2

To identify interesting amino acid residue differences, the amino acid sequences of the P_{BC} (aa 204 to 237) and P_{HI} (aa 305 to 337) domains of VP2 of virulent and attenuated strains were compared. Virulent strains: D6948 (GenBank accession no. AF240686), HK46 (AF092943), UK661 (X92760), OKYM (D49706), KS (DQ927042), Gx (AY444873), HuB-1(KF569805), SD10LY01 (KF569803), T09 (AY099456). Attenuated strains: CU1 (X16107), D78 (AF499929), P2 (X84034), CT (AJ310185), Gt (DQ-403248). Alignment based on the amino acid sequences was performed using DNAStar (5.01 edition).

Modification of segment A of IBDV using site-directed mutagenesis

To introduce direct mutations into segment A of the rGx-F9VP2 strain, based on the parental pCGxAF9VP2HRT plasmid, PCR for site-directed mutagenesis with specific primer pairs was performed as described previously (Qi et al., 2007). The primer pair GxAG794CU/GxAG794CL or GxAT1120AU/GxAT1120AL (Table 1) was synthesized to introduce a direct mutation G794C or T1120A (resulting in an amino acid mutation A222P or S330R in VP2) into segment A of the rGx-F9VP2 strain. The mutated plasmid was named pCGxATA-G794CHRT and pCGx-ATA-T1120AHRT, respectively (Figure 4A). Similarly, to

Table 1 Primers for genome cloning and mutagenesis of IBDV.

Name	Sequence	Orientation	Position (nt)
GxAG794CU	CATCACAGTACCAAcCAGGTGGGGTAAC	sense	A: 780–807
GxAG794CL	GTTACCCCACCTGgTTGGTACTGTGATG	antisense	A: 780–807
GxAT1120AU	GTCAGCAAGaGGGAGCCTAGCA	sense	A: 1,111–1,132
GxAT1120AL	TGCTAGGCTCCCtCTTGCTGAC	antisense	A: 1,111–1,132
GtAC794GU	CATCACAGTACCAAgCAGGTGGGGTAAC	sense	A: 780–807
GtAC794GL	GTTACCCCACCTGcTTGGTACTGTGATG	antisense	A: 780–807
GtAA1120TU	GTCGGCAAGtGGGAGCCTAGCA	sense	A: 1,111–1,132
GtAA1120TL	TGCTAGGCTCCCaCTTGCCGAC	antisense	A: 1,111–1,132
GxAU	G*GAATTC*GGATACGATCGGTCTGAC	sense	A: 1–18
GxA1477L	AGGTAGCCCATGTCTGGT	antisense	A: 1,460–1,477
B3P	ACTACCCACTCCTGAACAAA	sense	B: 2,009–2,028
B37	GC*TCTAGA*GGGGGCCCCCGCAGGCGAAGGCCGGGGAT	antisense	B: 2,799–2,827

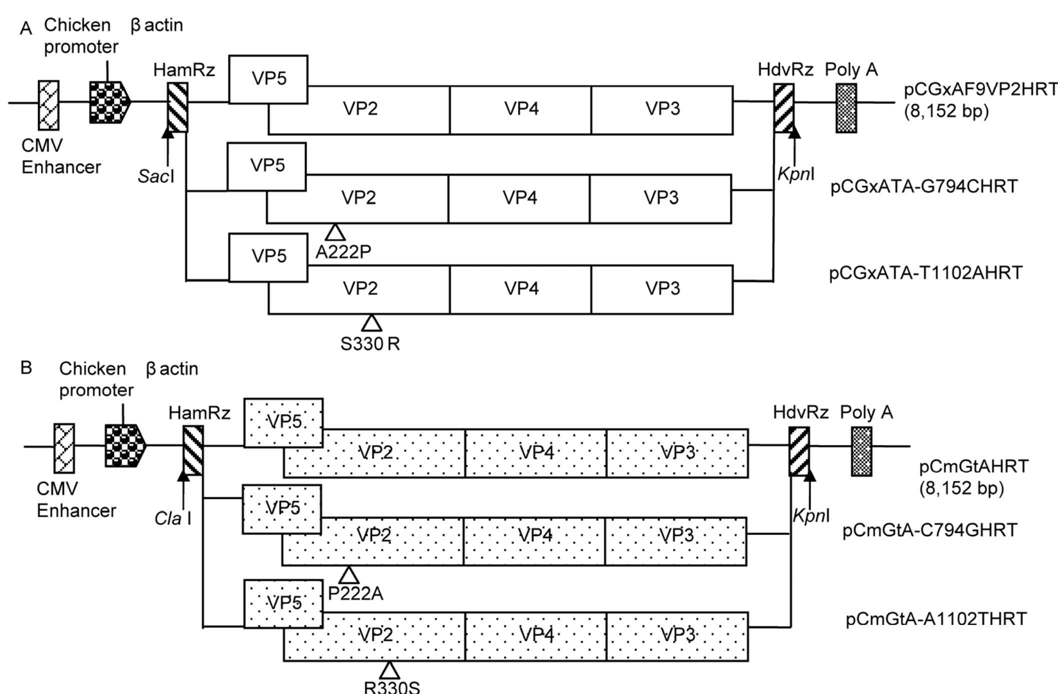

Figure 4 Schematic diagrams of the infectious clones containing the modified cDNAs of segment A of IBDV (not drawn to scale). Mutations were introduced into VP2 of segment A backbone of the virulent strain rGx-F9VP2 (depicted by an open box) (A) or the attenuated strain rGt (depicted by a box with dot) (B). The restriction enzyme sites used for the construction of recombinant vectors are also shown.

introduce a reverse mutation into segment A of the rGt strain, based on the parental plasmid pCmGtAHRT, a primer pair GtAC794GU/GtAC794GL or GtAA-1120TU/GtAA1120TL (Table 1) was used to introduce a nucleotide mutation C794G or A1120T (resulting in an amino acid mutation P222A or R330S in VP2) into segment A of rGt to obtain the modified plasmids pCmGtA-C794GHRT and pCmGtA-A1120THRT, respectively (Figure 4B).

Rescue and identification of modified IBDV

Using the reverse genetics system directed by RNA polymerase II, virus rescue was performed as previously described (Qi et al., 2007). The purified plasmids with the rGx-F9VP2 backbone (pCGxATA-G794CHRT or pCGx-ATA-T1120AHRT) were co-transfected with pCGxBHRT into DF-1 cells. The purified plasmids with the rGt backbone (pCmGtA-C794GHRT or pCmGtA-A1120THRT) were co-transfected with pCmGtBHRT into DF-1 cells. Three days post-transfection, the cell cultures were freeze-thawed three times and the supernatants were transferred to fresh CEF cells. The viruses were harvested from the infected cell cultures when a visible cytopathic effect (CPE) was apparent. The modified viruses were blind-passaged six times in CEF cells prior to subsequent experiments. To characterize the modified viruses, an indirect immunofluorescence assay (IFA) with an anti-VP2 mAb, an electron microscopy assay, RT-PCR, and sequencing using the primer pairs GxAU/GxA1477L and B3P/B37 (Table 1) were performed as previously described (Qi et al., 2007).

Replication of modified IBDV *in vitro*

To assess the replication abilities of the modified viruses and the control strain rGx-F9VP2 or rGt, confluent secondary CEF cells in 60 mm culture plates (approximately 10^6 cells/plate) were infected with each virus strain at a 50% cell culture infective dose ($TCID_{50}$) of 1×10^4 and were subsequently harvested at 24, 36, 48, 60, and 72 h p.i.. The titer of the infectious viral progeny was determined as $TCID_{50}$ per milliliter using the Reed-Muench formula (Reed and Muench, 1938). The mean values and standard deviations of the data obtained from three independent experiments were calculated.

Animal experiments

Three-week-old SPF chickens were randomly divided into four groups (eighteen chickens per group). Chickens were inoculated via ocular and intranasal routes with $10^{5.8}$ $TCID_{50}$ of rGxHT-222, rGxHT-330, and rGX-F9VP2. DMEM was used as a negative control. Chickens were observed daily for signs of clinical symptoms. At 2, 3, 5, 7, 10, and 14 d p.i., three chickens were randomly selected from each group, euthanized for necropsy, and examined for signs of pathological changes. The bursa:body-weight index (BBIX) was calculated with standard deviation (BBIX=(bursa∶body-weight ratios)/(bursa∶body-weight ratios in the negative group)). The mean values and standard deviations of the data obtained from three independent chicken samples were calculated. Bursa with a BBIX lower than 0.7 were considered atrophied (Lucio and Hitchner, 1979). Each bursa was then divided into two parts, one for a histopathological assay and the other for detecting the viral gene.

Histopathology

Bursae from each group isolated on different days p.i. were fixed immediately after necropsy in 10% neutral buffered formalin and were stained with hematoxylin and eosin for histopathological examination, as described previously (Qi et al., 2009). The severity of bursal follicular necrosis was recorded using the average histopathologic bursa lesion score (HBLS) system, as described earlier (Schroder et al., 2000).

Statistical analyses

One-way ANOVA was used to evaluate the significant differences among the different groups. $P<0.05$ was considered a significant difference.

Compliance and ethics *The author(s) declare that they have no conflict of interest.*

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