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Regeneration of a recombinant infectious bursal disease virus having four amino acid substitutions in VP2 by reverse genetics

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Abstract

Infectious bursal disease virus (IBDV), a virus with a double-stranded, bi-segmented RNA genome, is an economically important pathogen of chickens. Recent understanding of the molecular biology of IBDV has implicated several amino acid residues in the capsid protein VP2 in pathogenicity and tissue culture adaptation. In the present study a recombinant strain of IBDV having four mutations in VP2 (Gln253His, Asp279Asn, Ala284Thr and Ser330Arg) has been generated using reverse genetics. Desired mutations were introduced in the VP2 gene of the cloned cDNA of genome segment A of a very virulent (vv) IBDV by site-directed mutagenesis. Capped RNA transcribed *in vitro* from cloned cDNA of the modified segment A and wild type segment B was co-transfected into chicken embryo fibroblast (CEF) cell culture. The recombinant virus, designated as BD-3tcC, was rescued from the transfected cell culture and characterized *in vitro*. BD-3tcC retained all the four desired mutations and replicated with titres only slightly lower than those of CEF cell-culture-adapted wild-type IBDV. This recombinant strain can be used in future studies for understanding the biological significance of these four amino acid residues in VP2. (*Bangl. vet*. 2014. Vol. 31, No. 1, 12 – 19)

Introduction

Infectious bursal disease (IBD) or Gumboro disease is an economically important disease of poultry worldwide. It is caused by infectious bursal disease virus (IBDV) belonging to the family *Birnaviridae* (Dobos *et al*., 1979; Leong *et al*., 2000; Müller *et al*., 2003). There are two distinct serotypes of IBDV (McFerran *et al*., 1980). Serotype 1 strains are pathogenic and serotype 2 strains are non-pathogenic. The pathogenic serotype 1 field isolates can be further grouped into classical virulent (cv), very virulent (vv) and antigenic variant strains. The vvIBDV can cause up to 70% or even higher mortality in the field (Chettle *et al*., 1989; van den Berg *et al*., 1991). Based on comparison between wild type IBDV strains and their counterparts adapted to cell culture, four amino acid mutations (Gln253His, Asp279Asn, Ala284Thr and Ser330Arg) were identified in the VP2 capsid protein to be associated with tissue

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culture adaptation (Yamaguchi *et al.*, 1996). Subsequently, a vvIBDV was adapted in tissue culture by introducing two mutations (Gln253His and Ala284Thr) in VP2 (Lim *et al.*, 1999). Previously, we also regenerated a recombinant IBDV strain (designated as BD-3tc) by reverse genetics after site-directed mutagenesis of these two amino acids (Gln253His and Ala284Thr) in the backbone of a vvIBDV (Islam *et al.*, 2001b; Raue *et al.*, 2004). The recombinant strain BD-3tc grew in chicken embryo fibroblast (CEF) cell culture and was partially attenuated for chickens, but the point mutations introduced in BD-3tc reverted during infection in chickens (Raue *et al.*, 2004). In the present study, we rescued another recombinant IBDV strain (designated as BD-3tcC), which had the four amino acid substitutions in VP2, as originally suggested to be associated with tissue culture adaptation of IBDV (Yamaguchi *et al.*, 1996). The rescued strain, designated as BD-3tcC, was analysed for the presence of introduced mutations, and growth kinetics were studied *in vitro* in CEF cell culture.

Materials and Methods

Site-directed mutagenesis and re-construction of clones

Previously constructed full-length cDNA clones of segment A and segment B of a wild type vvIBDV (BD-3wt) in pUC19 vector (pVL561 & pVL547) and that of a modified segment A of a recombinant IBDV strain BD-3tc (pVL565) [4, 5] were used as starting materials. All the cloned cDNA had the T7 promoter at the 5' end. BD-3tc had already two amino acid substitutions at positions 253 (Gln→His) and 284 (Ala→Thr) of VP2 gene. In the present study two additional amino acid substitutions at position 279 (Asp→Asn) and 330 (Ser→Arg) were introduced sequentially. The selected sites for mutagenesis with expected amino acid changes (Gln253His, Asp279Asn, Ala284Thr and Ser330Arg) are shown in Table 1.

The primers used in site-directed mutagenesis are listed in Table 2. First the mutagenic primer "IBDV-D279N" and a flanking primer "INCO-DC#3" (Islam *et al.*, 2001a) were used on the pVL565 (BD-3tc) template to synthesize a 405 bp megaprimer having the desired mutation (Asp279Asn). Then the synthesized megaprimer and the other flanking primer "INCO-DC#4" were used on the same template to amplify a 677 bp VP2 gene fragment having the desired mutation. The amplified PCR product was cloned in the pCR-2.1 plasmid vector by the TA cloning method and designated as pBAU5. To introduce a further mutation at position 330 (Ser→Arg), a 167 bp megaprimer was synthesized with the mutagenic primer "IBDV-S330R" and the flanking primer "INCO-DC#4" using the plasmid pBAU5 as the template. This megaprimer and the other flanking primer "INCO-DC#3" were then used on the same template to amplify a 677 bp VP2 gene fragment having the four mutations (Gln253His, Asp279Asn, Ala284Thr and Ser330Arg). The amplified PCR product was cloned in the pCR-2.1 plasmid vector by TA cloning and designated as pVL907. For re-construction of a full-length clone of modified segment A having four desired mutations, a 349 bp fragment was cleaved from pVL907 with *Cla*I and *Spe*I and exchanged with the corresponding region of the plasmid pVL561. The sequence of the

re-constructed clone was checked by restriction enzyme analysis and sequencing for the presence of the desired mutations. The modified plasmid was designated as pVL 913.

Regeneration and rescue of mutant virus

The procedure for regeneration and rescuing of mutant IBDV has been described (Mundt and Vakharia, 1996; Raue *et al.*, 2004). Full-length cloned cDNA of the modified segment A (pVL913) and the wild-type vvIBDV segment B (pVL547) (Islam *et al.*, 2001a; 2001b) were used. In brief, cDNA corresponding to genome segments A and B were transcribed *in vitro* using T7 polymerase by run-off transcription method from the plasmids linearised with an appropriate enzyme. The capped RNA corresponding to segments A and B were co-transfected in chicken embryo fibroblast cells in the presence of a liposome. The expression of viral proteins in transfected cells was confirmed by immuno-fluorescence assay using a monoclonal anti-VP2 antibody (1/A6) (Becht *et al.*, 1988) 24 h after transfection. The supernatant from transfected cells was further passaged in fresh CEF cell cultures and observed for the development of virus-specific cytopathic effects (CPE). The rescued recombinant virus was characterized *in vitro* for the presence of the desired mutations by RT-PCR followed by restriction enzyme analysis with appropriate enzymes and sequencing. The recombinant IBDV strain, regenerated and rescued in this study, having four amino acid substitutions (Gln253His, Asp279Asn, Ala284Thr and Ser330Arg) was designated as BD-3tcC.

Table 1. Selected sites for mutations with expected amino acid changes

Nucleotide position in Segment A	Original codon	Mutated codon	Amino acid position in VP2	Original Amino acid	Exchanged Amino acid	Remarks
877-879	CAA	CAT*	253	Gln	His	*Stu*I site disappears *Nco*I site is introduced
965-967	GAC	AAT	279	Asp	Asn	-
980-982	GCC	ACC*	284	Ala	Thr	*Nae*I site disappears
1118-1120	AGT	CGT	330	Ser	Arg	*Eco* 72I/*Pml*I site is introduced

* Previously done in BD-3tc (Islam *et al.*, 2001a)

In vitro growth curve and plaque morphology of mutant virus

The growth curve and plaque morphology of the newly generated recombinant strain BD-3tcC having four mutations, a previously generated recombinant strain BD-3tc having two mutations (Islam *et al.*, 2001b), and a cell culture-adapted classical IBDV strain Cu-1 (Nick *et al.*, 1976) were studied. Confluent CEF cell monolayers grown in Petri dishes were infected at a multiplicity of infection (m.o.i.) of 0.5. After incubation

for 1 h at 38ºC the supernatant was removed, the cells were washed twice with PBS, and fresh medium was added. At 0, 4, 8, 16, 24 and 48 hr after infection, cell culture supernatants from two Petri dishes each were collected and centrifuged at low speed (400g) for 10 min and the clarified supernatant was preserved at -20°C to determine extracellular virus concentration. Then 2 ml fresh medium was added to the dishes. After 3 cycles of freezing and thawing the cell lysate was centrifuged at 3,000g for 10 min, the supernatant was collected and frozen at -20ºC to determine intracellular virus concentration. Virus titres in the culture supernatant and cell lysate preparation at each time point were determined by plaque assay (Nick *et al.*, 1976; Müller *et al.*, 1986).

Table 2. Primers used in the synthesis of megaprimers and mutated gene fragments by PCR

Primer	Orientation	Sequence	Nucleotide position
INCO-DC#3*	Sense	5′-AACAGCCAACATCAACG-3′	571-587
Mutagenic Primer IBDV-D279N	Anti-sense	5′-AGCCCATTaTtTGCGGCTACAGCT-3′	975-952
Mutagenic Primer IBDV-S330R	Sense	5′-GGTCAGCAcGTGGGAGCCTAGCAG-3′	1110-1133
INCO-DC#4*	Anti-sense	5'-GCTCGAAGTTGCTCACCC-3'	1247-1230

NB. Mutated nucleotides are given in lower case, * Islam *et al.* (2001a)

Results and Discussion

Regeneration and rescue of mutant virus

Site-directed mutagenesis was successfully accomplished to introduce the desired mutations (Table 1) in the cloned full-length cDNA of genome segment A of vvIBDV (BD-3wt). On restriction enzyme analysis of the modified clone pVL913, the disappearance of *Stu*I and *Nae*I sites and introduction of *Nco*I and *Pml*I sites in the insert confirmed the desired mutations (data not shown). PCR amplification and sequencing of the cDNA fragment corresponding to the hyper-variable region of inserted VP2 gene in the plasmid pVL 913 confirmed the desired mutations.

Run-off transcription of the linearised plasmids pVL913 and pVL543 produced RNA transcripts (Fig. 1) corresponding to the modified segment A and wild type segment B of vvIBDV, respectively. Co-transfection of CEF cells with capped RNA transcripts of modified segment A cDNA, having four mutations, and wild type segment B cDNA resulted in the expression of IBDV protein in the cytoplasm as indicated by specific immuno-fluorescence observed at 24 hours after transfection (Fig. 2). The cells showed extensive necrosis at 48 and 72 hours after transfection, but it could not be ascertained whether cell death was due to non-specific effects of transfection or virus-induced cytopathic effects (CPE). However, when the supernatant of the transfected

cells was passaged in fresh CEF cells, a clear CPE was noticed by 48 hours after infection, and RNA isolated from the culture supernatant tested positive in IBDV-specific RT-PCR. On restriction enzyme analysis the disappearance of *Stu*I and *Nae*I sites and introduction of *Nco*I and *Pml*I sites confirmed the desired mutations. Sequencing of the RT-PCR product further confirmed the introduced mutations resulting in desired amino acid substitutions (Gln253His, Asp279Asn, Ala284Thr and Ser330Arg). Alignment of the deduced amino acid sequences of VP2 hyper-variable region of the parental strain BD-3wt, previously generated recombinant strain BD-3tc and the present recombinant strain BD-3tcC are presented in Fig. 3.

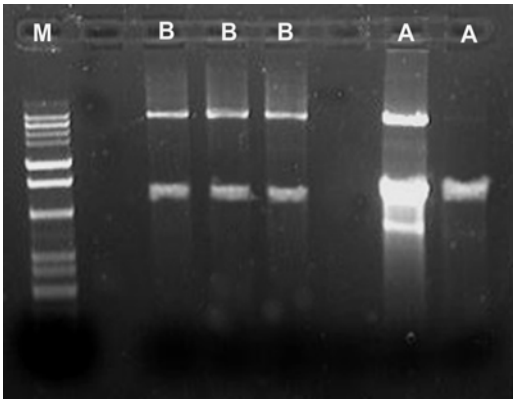


Fig. 1. RNA transcribed *in vitro* from the linearised plasmids with T7 RNA polymerase.
(M = Marker; A = RNA corresponding to IBDV segment A transcribed from pVL543, B = RNA corresponding to IBDV segment B transcribed from pVL913)

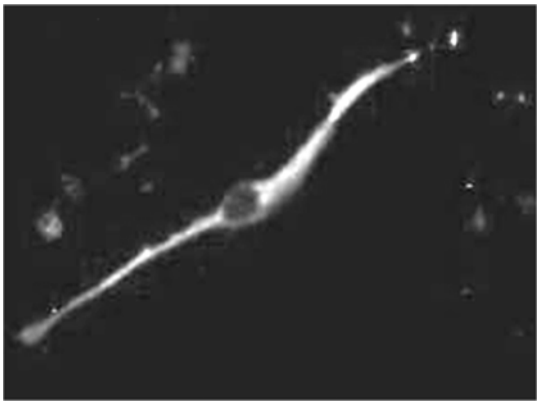


Fig. 2. Positive immuno-flurescence in CEF cells with IBDV-specific antibodies 24 hours after transfection with IBDV RNA

In vitro *growth curve and plaque morphology of mutant virus*

The growth kinetics in CEF cell culture of the recombinant IBDV strain BD-3tcC (having four mutations) is shown in Fig. 4. A previously generated recombinant IBDV strain, BD-3tc (having two mutations), and a tissue-culture-adapted classical IBDV strain Cu-1 were included in the study for comparison. BD-3tc and BD-3tcC were used after second passage in CEF cells, while cu-1 had an unknown passage history.

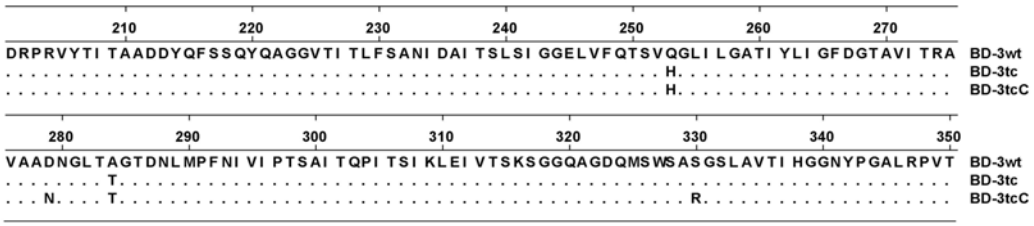


Fig. 3. Alignment of deduced amino acid sequences of VP2 (residues 201-350) of BD-3wt, BD-3tc and BD-3tcC. Residues identical to that of BD-3wt are indicated as dots.

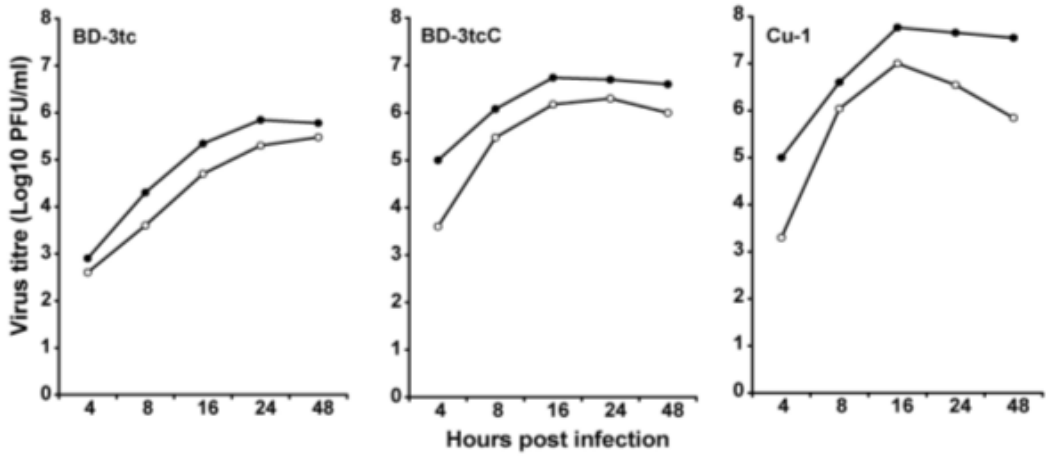
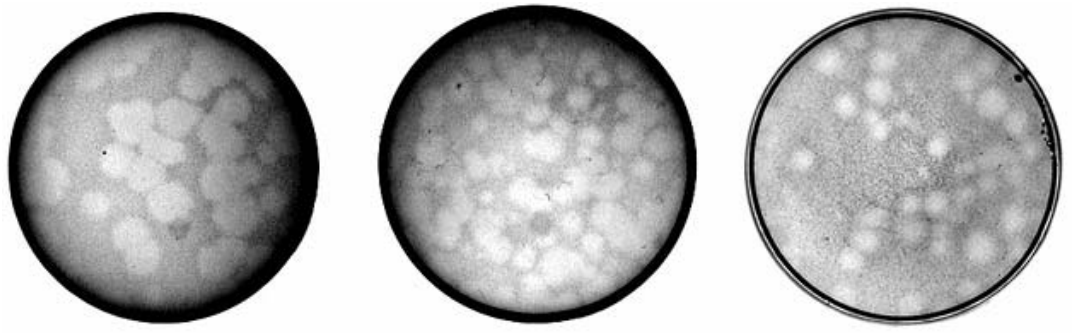


Fig. 4. Growth curves of BD-3tc, BD-3tcC and Cu-1, established as the concentration of free virus (line with closed circles) and cell-associated virus (line with open circles) after infection of CEF cells. The titres given as plaque forming units (PFU) per ml represent the mean of two independent experiments

All three virus strains grew in cell culture. Although the growth curve was quite similar, Cu-1 yielded the highest amount of virus followed by BD-3tcC and BD-3tc. In each case the amount of virus in the culture supernatants was higher than in the cells. Virus replication reached its peak by 16 to 24 hours and the growth was fastest in Cu-1 and slowest in BD-3tc.

The plaque morphology of BD-3tc and BD-3tcC was similar to that of conventional cell-culture-adapted classical virulent strain Cu-1. However, the plaque size of BD-3tc and BD-3tcC was slightly smaller than that of Cu-1 (Fig. 5).



Cu-1	BD-3tc	BD-3tcC

Fig. 5. Plaques in CEF cell culture produced by BD-3tc, BD-3tcC, and tissue-culture-adapted classical virulent IBDV strain Cu-1

Reverse genetics procedure for IBDV was first described by Mundt and Vakharia (1996). Subsequently, reverse genetics and site-directed mutagenesis procedures were

applied for generating recombinant strains of IBDV with one or two defined mutations (Lim *et al.*, 1999; Mundt, 1999). Previously we generated a recombinant strain BD-3tc having two mutations (Gln253His and Ala284Thr) from the wild type vvIBDV BD-3wt (Islam *et al.*, 2001b; Raue *et al.*, 2004). In the present study a recombinant strain of IBDV, BD-3tcC, having four mutations in the capsid protein VP2 has been generated. As observed in the growth curve assay, BD-3tcC replicated in CEF cell culture similarly to that of classical cell-culture adapted-strain like Cu-1 but more slowly, as indicated by the lower titre and smaller plaque size of BD-3tcC. The new recombinant strain BD-3tcC having four mutations appeared to be more adapted to grow in CEF cell culture than its predecessor BD-3tc having two mutations.

Conclusions

The recombinant IBDV strain BD-3tcC, in the backbone of very virulent genotype and having four defined mutations in the outer capsid protein VP2, would be a valuable asset for future investigation on the biological significance of these mutations. The protocol outlined in this paper could also be used to adapt available vaccine viruses to the current field situation through site-directed mutagenesis and reverse genetics to overcome the problems of vaccination failure due to antigenic drift in field isolates.

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