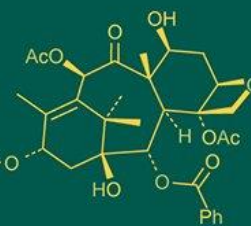
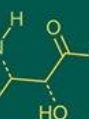
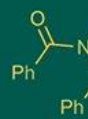


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Molecular amplification and cloning of a truncated VP2 (segment 2) gene fragment of bluetongue virus serotype 10

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Abstract

The outer capsid protein VP2 of bluetongue virus is encoded by genome segment 2 and is a major determinant of virus serotype. The present study was undertaken to recover and clone a truncated VP2 coding-region fragment of bluetongue virus serotype 10 for downstream molecular applications. Bluetongue virus serotype 10 was propagated in BHK-21 cells, after which viral double-stranded RNA was extracted by sequential lithium chloride precipitation and segment 2 was recovered following agarose gel electrophoresis. The purified segment was reverse transcribed with gene-specific primers, and a 1962 bp fragment was amplified by polymerase chain reaction using primers carrying *Bam*HI and *Xho*I restriction sites. The amplicon was purified, directionally ligated into similarly digested pFastBac HT B vector DNA and introduced into chemically competent *Escherichia coli* TOP10F' cells. Recombinant clones were screened by colony polymerase chain reaction and further examined by amplification of isolated plasmid DNA and double restriction digestion. A single prominent approximately 1962 bp amplification product was observed, while restriction analysis of recombinant plasmid produced bands corresponding to the vector backbone labelled approximately 4857 bp and the released 1962 bp insert. These findings support successful recovery of the targeted BTV-10 VP2 fragment and its maintenance in the recombinant plasmid. The study establishes a molecular cloning intermediate that can support subsequent work involving VP2, but it does not by itself demonstrate DNA sequencing, baculovirus generation, protein expression, antigenic characterization or biological function.

Keywords: Bluetongue virus, BTV-10, VP2, segment 2, reverse transcription polymerase chain reaction, molecular cloning

Introduction

Bluetongue is an economically important arboviral disease of ruminants caused by bluetongue virus (BTV), an orbivirus with a non-enveloped particle containing a segmented double-stranded RNA genome. The viral genome is organized into ten segments that encode the structural and non-structural proteins required for particle formation and replication [1, 2]. The segmented genome and the structural organization of the virion have provided a strong foundation for molecular studies of BTV biology, diagnosis and genetic diversity.

Among the viral proteins, VP2 is encoded by genome segment 2 (Seg-2) and forms part of the outer capsid. Comparative molecular studies have shown that VP2 is highly variable among BTV serotypes and contains the principal determinants recognized by neutralizing antibodies; consequently, Seg-2 sequence information has become central to molecular identification and differentiation of BTV serotypes [3, 4, 10]. Earlier work on BTV serotype 10 established the complete nucleotide sequence of the L2/segment-2 RNA and showed that it encodes a VP2 protein of 956 amino acids [5].

The molecular study of BTV-10 VP2 has also included recombinant approaches. Segment 2-derived DNA has previously been placed into baculovirus transfer systems to produce recombinant VP2, and recombinant BTV-10 VP2 has subsequently been investigated for antigenic and cell-entry properties [7, 8]. These studies illustrate the value of obtaining defined VP2-derived DNA constructs for downstream recombinant-virus or protein studies.

However, cloning a target fragment into a transfer vector should be distinguished from expression or functional characterization: the former establishes a molecular construct, whereas the latter requires additional experimental steps.

Reverse transcription polymerase chain reaction targeting Seg-2 is an established approach for recovering BTV VP2 sequences and has been applied to serotype-specific molecular assays [4]. In the present work, a BTV-10 segment-2 fragment was recovered from infected BHK-21 cells, amplified with gene-specific primers carrying restriction sites, and cloned into pFastBac HT B using *Bam*HI and *Xho*I. The objective was to obtain and preliminarily confirm a recombinant plasmid carrying an approximately 1962 bp truncated VP2 coding-region fragment of BTV-10 by PCR and restriction analysis. The work therefore focuses on molecular recovery and cloning of the target fragment and does not extend to sequence validation, baculovirus rescue or protein expression.

Materials and Methods

Virus propagation and cell culture

BTV-10 was revived from cryopreserved virus stocks and propagated in BHK-21 cells in 75 cm² tissue-culture flasks. Cultures were infected at a multiplicity of infection of 0.01. After adsorption for 1 h at 37 °C, the cultures were maintained in GMEM supplemented with 2% fetal bovine serum. Cultures were harvested at 48 h post-infection when cytopathic changes were evident. The source material reported initial cell rounding, enlargement and increased refractility from approximately 24 h post-infection, followed by extensive degeneration, loss of adherence and accumulation of non-viable cells by 48 h post-infection.

Extraction of viral double-stranded RNA

Viral double-stranded RNA was extracted from infected BHK-21 cell pellets by sequential lithium chloride precipitation. Cell pellets were treated with proteinase K and lysed in TRI reagent, followed by chloroform extraction and isopropanol precipitation. The nucleic acid pellet was dissolved in TE buffer and subjected sequentially to 4 M and 8 M lithium chloride precipitation to remove cellular RNA and enrich viral double-stranded RNA. The final pellet was washed with 70% ethanol, dissolved in TE buffer and stored at -80 °C.

Agarose gel electrophoresis and recovery of segment 2

Approximately 2 µg of purified BTV double-stranded RNA was resolved on a 1% agarose gel prepared in 1× TAE containing ethidium bromide at 0.5 µg/mL, following the electrophoretic approach described by Pedley *et al.* [6]. Electrophoresis was performed at 60 V for 2 h. The viral RNA segments were visualized under ultraviolet illumination, and segment 2 was excised for downstream analysis. The source document reported recovery of approximately 100 ng/µL purified segment-2 material from each infected 75 cm² culture flask. The excised material was processed using a QIAquick Gel Extraction Kit (Qiagen, Hilden, Germany) according to the source protocol, with the gel dissolved in Buffer QG, followed by isopropanol addition and column purification. After washing with Buffer PE, the material was eluted in 50 µL Buffer EB and stored at -20 °C until reverse transcription.

Reverse transcription of segment 2

Gel-purified segment-2 material from BTV-10 was reverse transcribed in a 20 µL reaction using the RevertAid First Strand cDNA Synthesis Kit (Thermo Fisher Scientific, EU) and gene-specific primers. For the first reaction, 10.0 µL of gel-purified material (approximately 50 ng/µL), 1.0 µL forward primer (10 pmol) and 1.0 µL reverse primer (10 pmol) were mixed, briefly centrifuged, denatured at 95 °C for 5 min and rapidly cooled on ice. The remaining components were then added: 4.0 µL 5× RT reaction buffer, 2.0 µL dNTPs (10 mM), 1.0 µL RiboLock RNase inhibitor (20 U/µL) and 1.0 µL RevertAid reverse transcriptase (200 U/µL). The reaction was incubated at 25 °C for 10 min, 42 °C for 15 min and 45 °C for 45 min, followed by enzyme inactivation at 70 °C for 10 min. The resulting cDNA was snap-cooled and used for PCR.

PCR amplification of the VP2 fragment

The BTV-10 VP2 coding-region fragment was amplified using gene-specific primers carrying terminal *Bam*HI and *Xho*I restriction sites (Table 1). PCR was performed in a 50 µL reaction using GoTaq Long PCR Master Mix 2× (Promega, Madison, WI, USA). The reaction contained 25 µL master mix, 1.0 µL forward primer (10 pmol), 1.0 µL reverse primer (10 pmol), 5 µL cDNA template and 18.0 µL nuclease-free water. A nuclease-free-water non-template control was included. Amplification was performed in a gradient thermal cycler (Takara, Japan) using an initial denaturation at 94 °C for 1 min; 25 cycles of 95 °C for 30 s, 65 °C for 30 s and 65 °C for 2 min; a final extension at 68 °C for 6 min; and a 4 °C hold. PCR products were resolved on a 1% agarose gel at 80 V for 1 h using a GeneRuler 1 kb DNA Ladder (Thermo Fisher Scientific, EU).

Preparation of pFastBac HT B vector DNA

Plasmid DNA of the pFastBac HT B vector was isolated from recombinant *E. coli* using the SureSpin Plasmid Mini Kit (Nucleopore, Genetix Biotech, Asia) according to the manufacturer's instructions. In the present manuscript, pFastBac HT B is described as a cloning/donor vector. Although *E. coli* was used for plasmid propagation, no insect-cell transfection, recombinant baculovirus rescue or protein-expression experiment was reported in the source material.

Restriction digestion of PCR product and vector

Purified BTV-10 PCR product and pFastBac HT B vector DNA were double-digested with *Bam*HI and *Xho*I. For the vector, 5-10 µL of vector DNA corresponding to approximately 1 µg was used; for the PCR product, 10-15 µL corresponding to approximately 0.2 µg was used. Each reaction also contained 2 µL 10× FastDigest Buffer, 1 µL FastDigest *Bam*HI, 1 µL FastDigest *Xho*I and nuclease-free water to a final volume of 20 µL. Reactions were incubated at 37 °C for 30 min to 1 h, followed by heat inactivation at 80 °C for 5 min. Digested products were purified using the GeneJET PCR Purification Kit (Thermo Fisher Scientific, USA).

Ligation and transformation

The restriction-digested BTV-10 PCR product was ligated to the similarly digested pFastBac HT B vector using T4 DNA ligase (Promega, Madison, WI, USA). The 20 µL ligation reaction contained approximately 50-100 ng

digested PCR product (5-7 μ L), approximately 50 ng digested pFastBac HT B plasmid (1.0 μ L), 1.0 μ L T4 DNA ligase, 2.0 μ L 10 $\times$ T4 DNA ligase buffer and nuclease-free water to 20 μ L. The reaction was incubated overnight at 4 $^{\circ}$ C. The chemically competent *E. coli* TOP10F' cells were prepared by the calcium chloride method. Approximately 5 μ L ligation mixture was added to 100 μ L competent cells, incubated on ice for 30 min, heat-shocked at 42 $^{\circ}$ C for 60 s and returned to ice for 2 min. SOC medium (900 μ L) was then added, followed by incubation at 37 $^{\circ}$ C with shaking at 180 rpm for 2 h. The recovered cells were plated on LB agar containing ampicillin (100 μ g/mL) and incubated overnight at 37 $^{\circ}$ C.

Screening of recombinant clones

Putative recombinant colonies were screened by colony PCR using the same BTV-10 gene-specific primer pair and the previously described PCR conditions with minor modifications. Plasmid DNA from PCR-positive colonies was isolated using the previously described plasmid preparation protocol. Approximately 50 ng of recombinant plasmid DNA was subsequently used as the PCR template, and the products were analysed by 1% agarose gel electrophoresis to confirm the presence of the target insert. Positive recombinant plasmids were further subjected to double digestion with *Bam*HI and *Xho*I. Each 20 μ L restriction reaction contained approximately 1 μ g recombinant plasmid DNA (5-7 μ L), 2.0 μ L 10 $\times$ Fast Digest Buffer, 1.0 μ L Fast Digest *Bam*HI, 1.0 μ L Fast Digest *Xho*I and 11.0 μ L nuclease-free water. Digestion was performed at 37 $^{\circ}$ C for 30 min to 1 h and the products were resolved by 1% agarose gel electrophoresis.

Results

Propagation of BTV-10 and recovery of viral RNA

BTV-10 was successfully revived from cryopreserved stocks and propagated in BHK-21 cells in 75 cm² tissue-culture flasks. Progressive cytopathic changes were observed following infection, beginning with cell rounding, enlargement and increased refractility at approximately 24 h post-infection. By 48 h post-infection, extensive cellular degeneration, loss of adherence and accumulation of non-viable cells in the culture medium were evident. Viral double-stranded RNA was subsequently recovered from infected cultures following TRI reagent extraction and differential lithium chloride precipitation. The source material reported recovery of approximately 100 ng/ μ L of purified segment-2 material per infected 75 cm² flask for downstream molecular work.

Amplification of the BTV-10 VP2 fragment

Reverse-transcribed BTV-10 segment 2 was amplified using the BTV-10-specific primer pair listed in Table 1. Agarose gel electrophoresis showed a single prominent band at approximately 1962 bp in the amplification lane, with no visible smear or prominent additional product in the source image (Figure 1). The amplified fragment was excised and purified for cloning. The approximately 1962 bp size was consistent with the intended truncated VP2 coding-region fragment targeted by the primers.

Construction and confirmation of the recombinant plasmid

The purified 1962 bp amplicon and pFastBac HT B vector

were digested with *Bam*HI and *Xho*I, purified and ligated. The ligation mixture was introduced into chemically competent *Escherichia coli* TOP10F' cells, and ampicillin-resistant colonies were recovered. In the source study, one to two colonies per construct were reported to yield amplicons of the expected size during the initial colony-PCR screen. Plasmid DNA obtained from PCR-positive colonies was re-amplified with the same primer pair and again produced the expected insert-sized product.

Double restriction analysis of recombinant plasmid DNA produced the clearest visual evidence of the construct (Figure 2). The two bands in the digested sample were consistent with release of an insert of the expected size together with the vector backbone. The result supports the presence of the BTV-10 VP2 fragment in the recombinant plasmid. Because both *Bam*HI and *Xho*I were used for cloning, the strategy is directional; however, the double-digestion gel itself establishes insert presence and release by size rather than independently demonstrating orientation.

Discussion

The present study established a stepwise molecular workflow for recovery and cloning of an approximately 1962 bp fragment from segment 2 of BTV-10. The overall approach-virus propagation, recovery of genomic double-stranded RNA, separation of segment 2, reverse transcription, target amplification, restriction digestion, ligation and recombinant-plasmid screening-is consistent with established molecular approaches used for BTV Seg-2 analysis [4-6]. The major experimental outcome was the recovery of a discrete 1962 bp PCR product followed by detection of a corresponding insert in recombinant plasmid DNA by double restriction analysis.

VP2 is particularly suitable for this type of molecular investigation because it is the major outer-capsid protein and a principal determinant of BTV serotype [3, 4]. Full-length sequencing of BTV-10 segment 2 showed that the RNA segment is 2926 bp long and encodes a 956-amino-acid VP2 protein [5]. The approximately 1962 bp product observed here is therefore most appropriately interpreted as a partial or truncated VP2 coding-region fragment rather than a complete segment-2 clone. This distinction is important because the present data do not include the complete segment-2 sequence and do not establish the sequence identity of the amplified fragment by sequencing.

The single prominent band observed in Figure 1 provides evidence that the primer pair yielded a major product of the intended approximate size under the reported PCR conditions. The absence of obvious smearing in the supplied image also supports a relatively clean amplification product. Nevertheless, agarose electrophoresis establishes size compatibility rather than nucleotide identity. The use of a non-template control was reported in the methods, but the supplied figure does not provide a lane explicitly identified as the non-template control; therefore, the results are not used to claim visual verification of the control in Figure 1.

The restriction analysis in Figure 2 provides complementary evidence for recombinant plasmid construction. The digested recombinant plasmid showed a band labelled approximately 4857 bp together with a band labelled 1962 bp, whereas the undigested plasmid lane showed a high-molecular-weight plasmid species. The release of an approximately 1962 bp fragment after *Bam*HI/*Xho*I digestion is consistent with the expected insertion strategy and with retention of the target fragment in the plasmid. Similar restriction-based confirmation has long been used as

a practical screening step in recombinant cloning workflows. However, the interpretation should remain limited to what the gel demonstrates: the expected-size insert is present and can be released by the chosen enzymes. The use of *Bam*HI at the forward primer and *Xho*I at the reverse primer is compatible with directional cloning because the two ends of the insert are generated with different restriction overhangs. Directional cloning is useful for placing a target fragment into a defined vector orientation, particularly when the construct is intended for later transfer into an expression system. Nevertheless, direct *Bam*HI/*Xho*I digestion of an isolated recombinant plasmid does not by itself distinguish the intended orientation from an alternative orientation when the insert carries the same two restriction sites. Orientation is therefore inferred from the cloning design rather than experimentally proven by the supplied digestion pattern. This distinction avoids overstating the evidence.

The choice of pFastBac HT B is also relevant to the interpretation of the work. pFastBac HT B is used as a donor/transfer vector in baculovirus-based recombinant expression workflows, while *E. coli* provides the bacterial host used for plasmid propagation and screening. Previous BTV-10 VP2 studies used baculovirus transfer systems to achieve recombinant expression and then investigated the resulting protein for antigenic and biological properties^[7, 8]. The findings are also relevant to later molecular characterization. Seg-2 sequence analysis is widely used for BTV typing, phylogenetic comparison and identification of topotypic relationships^[3, 4, 9]. The construct can also provide a defined DNA template for subsequent sequencing, subcloning or transfer into a baculovirus-based workflow. The study demonstrates a coherent and practically useful molecular sequence from BTV-10 propagation to recombinant plasmid confirmation. This construct can support downstream work, but additional sequencing and expression-related experiments would be required before biological or functional conclusions could be drawn.

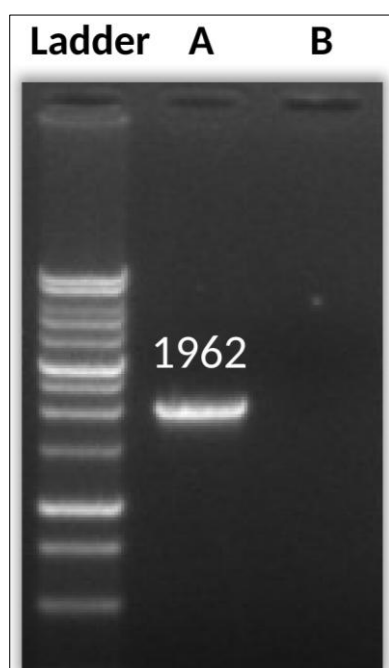


Fig 1: Agarose gel electrophoresis of the amplified BTV-10 VP2 fragment. Lane “Ladder”, DNA molecular weight ladder; lane A, BTV-10 amplification product showing an approximately 1962 bp band; Lane B, non-template control

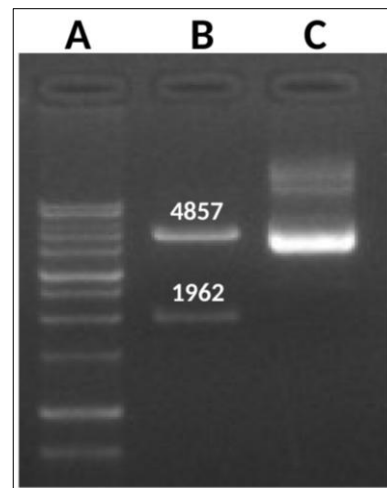


Fig 2: Double restriction analysis of BTV-10 recombinant plasmid. Lane A, GeneRuler 1 kb DNA Ladder (Thermo Scientific); lane B, *Bam*HI/*Xho*I-digested recombinant plasmid showing bands labelled approximately 4857 bp and 1962 bp, corresponding to the vector backbone and released BTV-10 insert, respectively; lane C, undigested recombinant plasmid

Table 1: Primer used for amplification of the BTV-10 VP2 fragment

Target	Primer	Sequence (5'–3')	Expected product
BTV-10/VP2	F	cgCGATCCACGCTACGCGCTTATGA T	~1962 bp
BTV-10/VP2	R	gtgCTCGAGCACATGCAGATCGCATA TAC	~1962 bp

Table 2: Reverse-transcription reaction composition

Reaction	Component	Volume (μL)
A	Gel-purified segment-2 material (approximately 50 ng/μL)	10.0
A	Forward primer (10 pmol)	1.0
A	Reverse primer (10 pmol)	1.0
B	5× RT reaction buffer	4.0
B	dNTPs (10 mM)	2.0
B	RiboLock RNase inhibitor (20 U/μL)	1.0
B	RevertAid reverse transcriptase (200 U/μL)	1.0
	Total	20.0

Table 3: PCR reaction composition and thermal cycling conditions

PCR component	Volume (μL)
GoTaq Long PCR Master Mix 2×	25
Forward primer (10 pmol)	1.0
Reverse primer (10 pmol)	1.0
cDNA template	5
Nuclease-free water	18.0
Total	50

Table 4: PCR thermal cycling conditions

Step	Temperature	Time	Cycles
Initial denaturation	94 °C	1 min	1
Denaturation	95 °C	30 s	25
Annealing	65 °C	30 s	25
Extension	65 °C	2 min	25
Final extension	68 °C	6 min	1
Hold	4 °C	-	-

Table 5: Double restriction digestion reaction for pFastBac HT B vector and PCR product

Component	Volume (μL)
pFastBac HT B vector (~1 μg) / PCR product (~0.2 μg)	5–10 / 10–15
10× FastDigest Buffer	2
FastDigest BamHI	1
FastDigest XhoI	1
Nuclease-free water	Up to 20

Table 6: Ligation reaction for the BTV-10 VP2 fragment and pFastBac HT B vector

Component	Volume (μL)
Digested PCR product (~50–100 ng)	5–7
Digested pFastBac HT B plasmid (~50 ng)	1.0
T4 DNA ligase	1.0
10× T4 DNA ligase buffer	2.0
Nuclease-free water	Up to 20

Table 7: Double restriction digestion reaction for recombinant plasmid confirmation

Component	Volume (μL)
Recombinant plasmid DNA (~1 μg)	5–7.0
10× FastDigest Buffer	2.0
FastDigest BamHI	1.0
FastDigest XhoI	1.0
Nuclease-free water	11.0
Total	20.0

Conclusion

An approximately 1962 bp truncated VP2 coding-region fragment of BTV-10 was successfully amplified from segment-2-derived cDNA and cloned into the pFastBac HT B vector using *Bam*HI and *Xho*I restriction sites. Recovery of the expected amplicon and release of a correspondingly sized insert from recombinant plasmid DNA supported successful preliminary construct confirmation. The study provides a defined recombinant DNA intermediate for future work on BTV-10 VP2. Sequencing, baculovirus rescue, protein expression and functional characterization were not part of the reported experiments and therefore remain necessary for further biological interpretation.

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