# Phylogenetic analysis of bluetongue virus genome segment 6 (encoding VP5) from different serotypes

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## Summary

Full-length cDNAs were sequenced for genome segment 6 from representative isolates of all 24 bluetongue virus (BTV) serotypes. Segment 6 is 1635 to 1645 nucleotides in length with a single open reading frame in all isolates. The deduced VP5 protein sequence is 526 amino acids long for most BTVs, except BTV-15 (isolate number RSArrrr/15), which is 527 amino acids long, and BTV-12 (isolate number RSArrrr/12) and BTV-22 (isolate number Nig1982/11), which were 529 amino acids long. Sequence comparisons have revealed the level of genetic diversity in segment 6, between different BTV serotypes and between isolates within a single serotype. The resulting sequences can be used to design RT-PCR primers for amplification and identification of segment 6 from new BTV isolates, providing potentially valuable diagnostic and research tools. These data are available on the international databases and accession numbers are listed on the website of the Institute for Animal Health (iah.bbsrc.ac.uk/dsRNA_virus_proteins/btv_sequences.htm).

## Keywords

Bluetongue – Genome – *Orbivirus* – Phylogenetic analysis – Segment 6 – Serotype – Viral protein 5 – Virus.

## Introduction

Bluetongue virus (BTV) is an arbovirus (transmitted by certain species of *Culicoides* biting midges), which infects both domesticated and wild ruminants. The virus causes clinical disease in sheep, particularly in the improved European breeds used for wool or mutton, but it is usually mild or inapparent in cattle and goats. BTV is the prototype species of the genus *Orbivirus*, within the family *Reoviridae*. Twenty-four distinct serotypes of BTV have been recognised, which can be distinguished by serum neutralisation (SN) assays. BTV is currently prevalent in the Mediterranean region, causing outbreaks of disease involving serotypes 1, 2, 4, 9 and 16, with strong evidence that the disease is gradually penetrating further north (12). The virus is also endemic in the Indian sub-continent involving as many as 21 different serotypes.

The BTV particle is icosahedral, non-enveloped and has three concentric capsid layers (4, 10). The BTV core contains the 10 dsRNA segments of the virus genome and the three minor protein components of the transcriptase complex, VP1(Pol), VP4(Cap) and VP6 (Hel) (6, 9). Cores also have two major protein components, VP3 (T2) (which forms the innermost subcore shell) and VP7 (T13) (forming the outer core-surface layer) (4). In the intact virus particles the core is surrounded by an outer capsid layer, composed of two additional major proteins (VP2 and VP5). These proteins, which are more variable than the core proteins, or the three non-structural virus proteins (NS1, NS2 and NS3) (9), are involved in cell attachment and penetration during initiation of infection and contain epitopes that bind neutralising antibodies (10). The specificity of interactions between the outer capsid proteins (particularly VP2) and neutralising antibodies can be evaluated in SN assays, thereby distinguishing the different virus serotypes (7, 14). Although VP2 contains the majority of the neutralising epitopes and plays a greater role in the generation and specificity of neutralising antibodies, VP5 is also involved in the determination of virus serotype, possibly by influencing the conformation of the VP2 molecules in the outer capsid layer (11, 17). The segmented nature of the BTV genome makes it possible for

different strains of the virus that infect the same cell, to exchange genome segments by a process called reassortment. This may play an important role in the emergence of novel virus strains with different combinations of proteins and consequently different serological and/or biological properties.

The present study was undertaken to analyse the nucleotide sequence of genome segment 6 (which encodes VP5) from representative isolates of all 24 BTV serotypes. These studies are providing more information on sequence variation in this gene and its correlation with virus serotype, as well as data that will help (in combination with data for genome segment 2) (8) to assess the relative frequency of genome reassortment that occurs in the field.

## Materials and methods

The viruses used in this study were grown in BHK-21 cells and their dsRNA was extracted using Trizol™ according to the protocol of the manufacturer (Life Technologies), followed by precipitation with 2M LiCl to remove ssRNA. An anchor-oligo was then ligated to the dsRNA segments at 4°C for 12 h (16). For amplification of genome segment 6, ligated dsRNA segments were separated by 1% agarose gel electrophoresis (AGE). Genome segments 4, 5 and 6 were excised together in one piece of gel and purified using RNaid Kit-BIO 101. Using avian myeloblastosis virus (AMV) enzyme (Promega), dsRNA was heat denatured and reverse transcribed. Amplification of cDNA was then performed using primer 5-15-1 (5′ GAGGGATCCAGTTTAGAATCCTCAGAGG TC 3′) and Triple-Master PCR system (Eppendorf) for 30 cycles. The amplified cDNA was analysed by AGE, then segment 6 (1.6 kb) was excised and purified using GeneClean kit-Bio101. The purified PCR products, representing full-length gene segment 6 were cloned in pGEM-T Easy vector (Promega) and transformed into *Escherichia coli* (ElectroMAX DH10B™) cells. Positive clones were screened by PCR using anchor specific primer 5-15-1 and restriction endonuclease digestion using BamH1 and Apa1. Plasmid DNA was prepared from positive clones using Gen-Elute Plasmid minipreps kit (Sigma). The termini of the cDNA insert were sequenced with M-13/T-7 forward and SP-6 reverse primers using the Cycle Sequencing Ready Reaction kit (CEQ DTCS Beckman Coulter) on a Beckman capillary sequencer. Internal forward and reverse primers were designed from the resulting terminal sequence data, making it possible to generate full-length sequences of gene segment 6 in both directions. The cDNA of segment 6 was also sequenced directly using BTV RNA-termini specific and gene-specific primers. The sequence data were aligned and analysed using the Bio Edit sequence alignment editor. The segment 6 sequence data from computerised databases were also analysed and compared using the Blast software program (NCBI: National Center for Biotechnology Information).

## Results and discussion

BTV genome segments 4, 5 & 6 were amplified together by PCR from representative isolates of all 24 serotypes (Fig. 1). Gel purified PCR amplicons of genome segment 6 from different serotypes could readily be cloned, as full-length copies, in the T/A cloning vector pGEM-T. Positive colonies containing full-length inserts were screened and identified by PCR using the 5-15-1 primer, followed by restriction endonuclease digestion (Fig. 2).

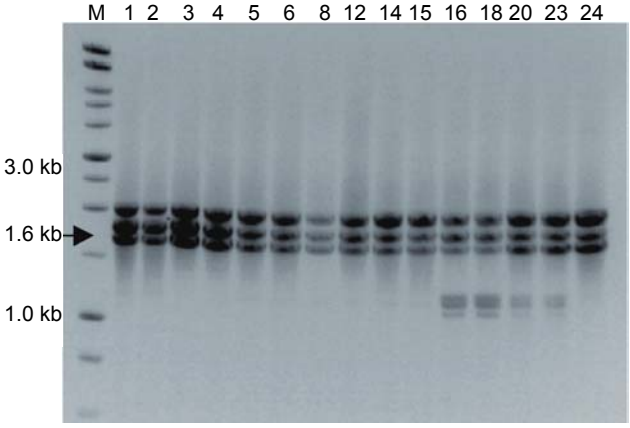


Figure 1
Polymerase chain reaction amplification of cDNA of bluetongue virus segments 4, 5 and 6, together
Lane M is 1.0 kb DNA marker
Each lane marked by Arabic numerals indicates the different BTV serotypes

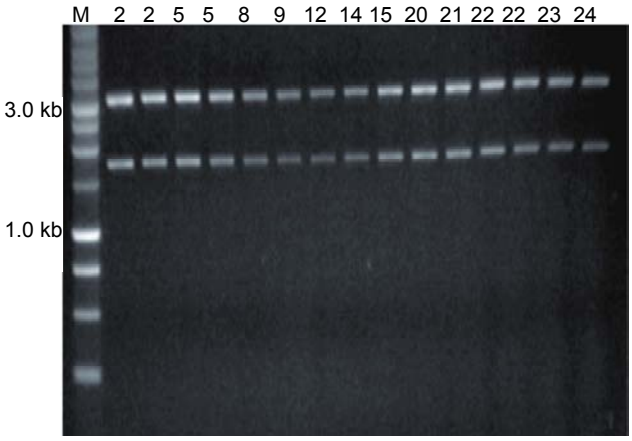


Figure 2
Bluetongue virus clones of different serotypes digested by Bam H1, giving right sized product of segment 6 (1.6 kb) and plasmid DNA (3.0 kb)
Lane M is 1.0 kb DNA marker
Each lane marked by Arabic numerals indicates the different BTV serotypes

The asterisk after the isolate number indicates the IAH dsRNA-virus reference-collection number. Additional information is provided on the dsRNA virus website (iah.bbsrc.ac.uk/dsRNA_virus_proteins/ReoID/viruses-at-iah.htm).

Segment 6 was found to be 1 635 base pairs long in isolates of BTV serotype 1-Greece (isolate number* GRE2001/01), 2-India (isolate number* IND1982/01), and 23-India (isolate number* IND1997/01); 1 637 base pairs in 3-Zimbabwe (isolate number* Zim2002/01), 5-Cameroon (isolate number* CAR1982/02), 6-South Africa (isolate number* RSArrrr/06), 7-SA (isolate number* RSArrrr/07), 8-Nigeria (isolate number* NIG1982/07), 9-Serbia (isolate number* SER2001/01), 13-SA (isolate number* RSArrrr/13), 14-Cameroon (isolate number* CAR1982/04), 16-Indonesia (isolate number* ISA1991/01), 18-SA (isolate number* RSArrrr/18), 19-SA (isolate number* RSArrrr/19) and 21-SA (isolate number* RSArrrr/21); 1 638 base pairs in 4-Turkey (isolate number* TUR1978/01), 10-SA (isolate number* RSArrrr/10), 11-SA (isolate number* RSArrrr/11), 17-SA (isolate number* RSArrrr/17), 20-SA (isolate number* RSArrrr/20) and 24-SA (isolate number* RSArrrr/24), 1639 base pairs in 15-SA (isolate number* RSArrrr/15); 1645 base pairs in 12-SA (isolate number* RSArrrr/12), and 22-Nigeria (isolate number* NIG1982/11). Similar nucleotide lengths were previously reported for segment 6 of BTV-10-USA strain (15); BTV-11-USA strain (1); BTV-11 and BTV-17-USA strains (19); BTV-13-USA strain (13); BTV-1-Aust. (2) and BTV-2-USA strain (5); BTV-1-South Africa (18). The deduced length of VP5 for isolates of all 24 BTV serotypes was 526 aa, except for BTV-15-SA (isolate number* RSArrrr/15), which was 527 aa; BTV-12-SA (isolate number* RSArrrr/12) and 22-Nigeria (isolate number* Nig1982/11), which were 529 aa.

Phylogenetic comparisons of the segment 6 nucleotide sequences identified two major clusters (Fig. 3), related to BTV-3 (BTV serotypes 3, 5, 6, 9, 13, 14, 16, and 21) or BTV-4 (BTV serotypes 4, 10, 11, 17, 20 and 24), and four minor clusters, related to BTV-7 (BTV serotypes 7 and 19); BTV-8 (BTV serotypess 8 and 18); BTV-12 (BTV serotypes 12 and 22); BTV-1 (BTV serotypes 1, 2 and 23) and BTV-15. Similar groupings of BTV-3 and BTV-4 have previously been reported by Gould and Hyatt, based on segment 2 gene sequences (3). They noted that BTV-15-Australia was also quite divergent (in genome segments 2 and 3) from other BTV serotypes included in their study, which holds true for BTV-15-SA in our study, although it did show some similarity to BTV-12 and BTV-22. Similarities apparent at both the VP6 gene and amino acid level,

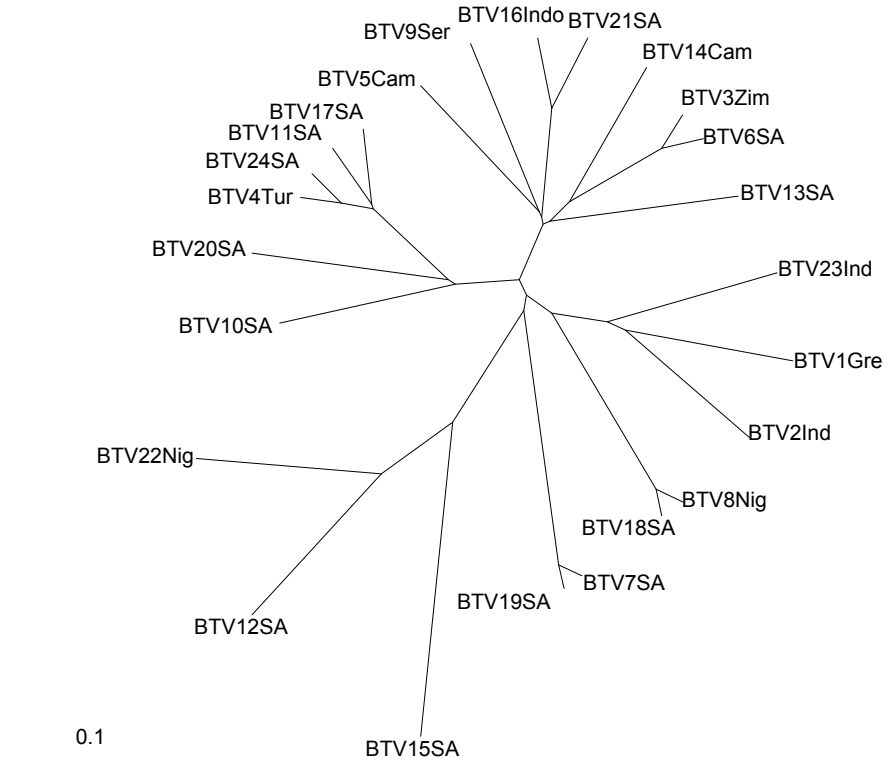


Figure 3
Radial tree of alignment of all bluetongue virus-24 serotypes based on the nucleotide sequences of segment 6

demonstrate that some related serotypes have sequences in common, indicating a relatively closer common ancestry. These relationships do not appear to depend on the geographical origin of individual isolates or serotypes.

In spite of their different geographical origins, comparative analysis of deduced amino acid sequences of all the 24 BTV serotypes showed a high level of conservation (70% sequence identity) between different VP5 proteins. Three variable regions were identified and mapped between residues 120-180, 285-345 and 420-475 (Fig. 4). Gould and Pritchard also reported three non-homologous regions in VP5 of BTV-1-Aust strain at amino acid 137-189, 280-333, 410-475 (2). Oldfield *et al.* observed two variable regions in VP5 of BTV-13-USA strain between residues 130-190, 270-340 (13). Similarly, Yang and Li mapped three variable regions in VP5 of BTV-11 and BTV-17-USA strains between residues 120-180, 273-345 and 435-481 (19).


Figure 4
Variable regions in VP5 (526 aa)

The results of this study have revealed the level of genetic diversity in genome segment 6, between different BTV serotypes, as well as between isolates of the same serotype. The BTV-1 isolate from Greece is genetically closely related in genome segment 6, to strains from India and Australia, but is quite distinct from those from Africa. In contrast, BTV-2 from India is quite different from the European and African isolates, which are themselves closely related (data not shown). These data indicate the origins of the different European BTV strains and have highlighted differences between the vaccine and field isolates of serotypes 1, 2 and 4, which may be sufficient to design RT-PCR primers to distinguish them. Sequence data for BTV genome segment 6 will also help to determine the origins of other virus strains, increasing our understanding of BTV epidemiology and transmission.

This database can also be used to facilitate primer design and RT-PCR conditions suitable for the amplification of segment 6 from new BTV isolates. These methods (together with those that have also been generated for BTV genome segment 2) (8), are being used as the basis for serotype-specific RT-PCR assays, to improve the speed and reliability of BTV serotype determination. The sequences that have been generated have been added to those already available for the segments of different BTV serotypes in the international databases and accession numbers are listed on the website of the Institute for Animal Health (6).

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