Completion of the sequence analysis and comparisons of genome segment 2 (encoding outer capsid protein VP2) from representative isolates of the 24 bluetongue virus serotypes

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Summary

Bluetongue (BT) is a non-contagious, arthropod-transmitted viral disease of domestic and wild ruminants. It is caused by bluetongue virus (BTV), a double-stranded (ds) RNA virus that is classified within the genus *Orbivirus*, family *Reoviridae*. There are at least twenty-four serotypes of BTV worldwide, five of which (1, 2, 4, 9 and 16) have been identified recently in Europe. BTV infects ruminants and its distribution throughout temperate and tropical regions of the world is dependent on the activity and abundance of certain vector-competent species of *Culicoides* midge. The outer capsid protein VP2 of BTV is a major protective antigen and the primary determinant of virus serotype. For the first time, the authors have completed the sequence analysis of full-length VP2 genes from the reference strains of each of the 24 BTV serotypes and their amino acid sequences were deduced. Multiple alignment of the VP2 gene (protein) sequences revealed that the level of nucleotide (amino acid) sequence variation between serotypes ranged from 29% (23%) to 59% (73%), confirming that segment 2/VP2 is the most variable BTV gene/protein. Phylogenetic analysis of VP2 grouped together the BTV types that are known to cross-react serologically. Low identity between types was demonstrated for specific regions within the VP2 amino acid sequences that have been shown to be antigenic and play a role in virus neutralisation. The sequence data represent the completion of an important step in the creation of a comprehensive BTV sequence database, which will support more rapid molecular methods for diagnosis and identification of BTV 'types', as well as continuing molecular epidemiology and surveillance studies of BTV.

Keywords

Bluetongue virus – *Orbivirus* – Phylogenetic analysis – Segment 2 – Sequencing – Serotypes – Viral protein 2.

Introduction

Bluetongue (BT) is an economically important, infectious, non-contagious and arthropod-borne disease of sheep that is caused by members of the bluetongue virus (BTV) species (genus *Orbivirus*, family *Reoviridae*) (9). BTV has a global distribution between latitudes 50°N and 30°S and is transmitted by biting midges (*Culicoides* spp.). Virus transmission and the incidence of disease are therefore dependent on the seasonal and geographical activity, abundance and distribution of adults of vector-competent *Culicoides* species (4, 6).

The BTV genome is composed of ten segments of dsRNA, packaged within a three-layered icosahedral capsid (90 nm in diameter). The outer capsid layer consists of VP2 and VP5 (encoded by segments 2 and 6, respectively), which can elicit a neutralising and protective antibody response in infected mammalian hosts (7, 12). There are twenty-four distinct serotypes of BTV worldwide (2), five of which (1, 2, 4, 9 and 16) have recently been identified in Europe. VP2 (encoded by genome segment 2) is the most variable viral protein and plays a dominant role in determining serotype (5, 8). Sequence information previously available for BTV genome segment 2 included data (many of which were incomplete) from only 14 of the 24 BTV serotypes.

For the first time, the authors report the completion of full-length sequence analyses of genome segment 2/VP2 from at least one representative isolate of each BTV serotype. This has enabled the construction of phylogenetic trees showing the genetic relatedness of different BTV serotypes. These studies also demonstrate that it is possible to identify and distinguish different BTV serotypes by comparisons of segment 2 sequences. This has the potential to considerably reduce the time taken to identify new BTV isolates. The virus/antibody neutralisation assays that are conventionally used for BTV serotype determination require virus isolation and are therefore time-consuming (up to 6-8 weeks), can generate equivocal results and require standardised and therefore expensive serological reagents. The availability on the Web of a complete set of sequence data of the VP2 genes and proteins from each of the 24 South African reference strains of BTV serotypes will be an important step towards the development of a molecular capability for BTV diagnosis and typing.

Materials and methods

Total RNA was isolated from BTV infected BHK-21 monolayers using Trizol® in accordance with the protocol of the manufacturer; dsRNA was separated from contaminating ssRNA by precipitation in 2M lithium chloride (1). An 'anchor-primer' sequence (S. Rao, manuscript in preparation), phosphorylated at the 5´ end, with a C9 spacer connecting two partially complementary halves, was ligated to whole viral dsRNA (S. Rao, personal communication). The 10 µl ligation reactions, containing 1µg of viral dsRNA, 10 units of RNA ligase (New England Biolabs) and 1.1 µg of anchor-primer (Integrated DNA technology, USA), were incubated at 10°C for 12 h. Ligated product was separated from unligated anchor-primer by 1% agarose gel electrophoresis and segments 2 and 3 were excised and purified using RNaid kit (Bio 101, Vista, California, USA). The RNA was precipitated using the Pellet Paint method. First strand cDNA was synthesised at 37°C for 40 min then at 42°C for 10 min using a 'reverse transcription system' (Promega, UK) without further addition of primers.

For amplification of fragments to be cloned and sequenced, PCRs were performed using a 'Triple Master PCR system' (Eppendorf AG, Cambridge, UK) using primer (5-15-1) which is partially complementary to the anchor spacer (S. Rao, manuscript in preparation). Gel slices containing full-length amplicons of segment 2 were excised, purified and prepared for cloning, or used directly in sequence reactions. PCR products were subjected to cycle sequencing, using a Beckman capillary sequencer/ALF express DNA sequencer (Pharmacia Biotech Sweden). Sequence analysis software, BioEdit (version 5.0.9.1), was used to align all sequences. Ambiguities were resolved by manually checking chromatograms and by re-sequencing from both directions using multiple primers. Sequence and phylogenetic analyses were performed using Orf Finder and ClustalX (version 1.8.1).

Results

Complete sequence analysis and comparison of genome segment 2 from the 24 BTV serotypes showed significant levels of variation, which correlate with virus serotype. The length of BTV genome segment 2 varies from 2 904 bp (BTV-12) to 2 947 bp (BTV-19). The different BTV serotypes also show some variation in the length of the single VP2 open reading frame (ORF) and non-coding regions (Table I). The plus-strand conserved-terminal hexanucleotide sequences of genome segment 2 from each of the 24 BTV serotypes, agree with those previously described for BTV and EHDV by Mertens *et al.* (11).

A neighbour-joining tree was drawn using the deduced amino acid sequences and compiled from multiple alignments of the VP2 amino acid sequences (Fig. 1). This tree demonstrates that the 24 BTV serotypes can all be differentiated from each other. However, there are groups of serotypes which show closer relationships, possibly indicating a more recent common ancestry (e.g. BTV-5 and BTV-9 and similarly BTV-4, BTV-10, BTV-11, BTV-17, BTV-20 and BTV-24). Some BTV serotypes are generally acknowledged to have closer serological relationships (3, 14) (Fig. 2), which are clearly reflected in higher levels of amino acid/nucleotide sequence identity, which therefore suggest closer phylogenetic relationships (Fig. 1). Several discrete groups of highly conserved amino acids were also found during these comparisons.

Genome segment 2 nucleotide sequences were shown to vary by up to 32% within a single serotype (within BTV-9), while VP2 amino acid sequences varied by up to 16% (within BTV-16). The level of nucleotide sequence variation between serotypes ranged from a minimum of 29% (BTV-8 and BTV-18) to a maximum of 59% (BTV-16 and BTV-22) while the level of VP2 amino acid differences between serotypes ranged between 23% minimum (BTV-6 and BTV-14) and 73% maximum (BTV-14 and BTV-15) confirming that segment 2/VP2 as the most variable BTV gene/protein.

Table I
Characteristics of segment 2 and VP2 protein of the reference strains of 24 serotypes of bluetongue virus

BTV serotype	IAH dsRNA virus reference collection number*	Accession number	Segment length (bp)	Open reading frame (nt)	G+C content (%)	A+T content (%)	Size of protein (aa)	Predicted protein molecular mass (Da)	5′ terminal sequences	3′ terminal sequences
BTV-1	RSArrrr/01	AJ585122	2940	18-2900	42.76	57.24	961	111907.6	5′-GTTAAAATAGTAGCGCGATG	CCGCGCACTATTAGACTTAC-3′
BTV-2	RSArrrr/02	AJ585123	2943	18-2903	41.11	58.89	962	111897.5	5′-GTTAAAACAGGATCGCGATG	CCGCGATCTGTTCAACTTAC-3′
BTV-3	RSArrrr/03	AJ585124	2935	22-2898	42.18	57.82	959	112328.0	5′-GTTAAAAACGCTGTCCCGAG	TTGGGCACGTTTTCACTTAC-3′
BTV-4	RSArrrr/04	AJ585125	2926	20-2887	42.89	57.11	956	110732.3	5′-GTTAAAAGAGTGTCCCACAA	CTGTGGCCTCTTACACTTAC-3′
BTV-5	RSArrrr/05	AJ585126	2921	18-2882	41.83	58.17	955	109861.9	5′-GTTAAAAGCTTCTCAGGATG	CTCCTGGAGCATACACTTAC-3′
BTV-6	RSArrrr/06	AJ585127	2922	18-2882	42.78	57.22	955	110263.8	5′-GTTAAATTAGTTTCGTGATG	TCACGAACTAATCAACTTAC-3′
BTV-7	RSArrrr/07	AJ585128	2936	22-2898	43.19	56.81	959	111158.9	5′-GTTAAAAAGGACCTCGCCAG	ATTGGCGGCCTTAAACTTAC-3′
BTV-8	RSArrrr/08	AJ585129	2939	18-2900	42.40	57.60	961	111275.4	5′-GTTAAAATAGCGTCGCGATG	CTCGCGCGCAATCAACTTAC-3′
BTV-9	RSArrrr/09	AJ585130	2921	18-2882	43.65	56.35	955	110036.4	5′-GTTAAAAGCTTTTCACGATG	CTCCTGAAGCTTACACTTAC-3′
BTV-10	RSArrrr/10	AJ585131	2926	20-2887	41.87	58.13	956	111155.5	5′-GTTAAAAGAGTGTTCTACCA	CGGTAGCCTCTTACACTTAC-3′
BTV-11	RSArrrr/11	AJ585132	2926	20-2887	42.96	57.04	956	110540.9	5′-GTTAAAAGAGTGTCCCATCA	CGATGGCCTCTTACACTTAC-3′
BTV-12	RSArrrr/12	AJ585133	2904	17-2866	43.53	56.47	950	109905.3	5′-GTTAAAAGTTGCGAGGATGG	CCCTCGCGGCTTCAACTTAC-3′
BTV-13	RSArrrr/13	AJ585134	2935	22-2898	42.11	57.89	959	112383.3	5′-GTTAAAAACGCTAGCCCGAG	TTGGGCACGTTTACACTTAC-3′
BTV-14	RSArrrr/14	AJ585135	2922	18-2882	42.47	57.53	955	110305.8	5′-GTTAAATTAGTTTCGTGATG	GCACGAACTAATCAACTTAC-3’
BTV-15	RSArrrr/15	AJ585136	2909	17-2872	43.14	56.86	952	110751.3	5′-GTTAAAAGTTGCCGGGATGG	TCACCCGCAACTAAACTTAC-3′
BTV-16	RSArrrr/16	AJ585137	2935	22-2898	42.25	57.75	959	112447.4	5′-GTTAAAAACGTTAGCCTAGA	TTGGGCACGTTTACACTTAC-3′
BTV-17	RSArrrr/17	AJ585138	2923	20-2884	41.77	58.23	955	110520.1	5′-GTTAAAAGAGTGATCCACCA	CGATGGCCTCTTACACTTAC-3′
BTV-18	RSArrrr/18	AJ585139	2927	18-2888	43.76	56.24	957	110369.2	5′-GTTAAAATAGCGTCGTGATG	CGCACGCGCTATCGACTTAC-3′
BTV-19	RSArrrr/19	AJ585140	2947	22-2907	43.50	56.50	962	111160.6	5′-GTTAAAAAGGATTTCGCCAC	GCAGGCGACATTCAACTTAC-3′
BTV-20	RSArrrr/20	AJ585141	2925	20-2887	42.70	57.30	956	110783.4	5′-GTTAAAAGAGCTTCCCACAA	CTGTGGCCTCTTACACTTAC-3′
BTV-21	RSArrrr/21	AJ585142	2922	18-2882	42.44	57.56	955	110741.1	5′-GTTAAATTAGTCTCGTGATG	GCACGGACTAATCAACTTAC-3′
BTV-22	RSArrrr/22	AJ585143	2907	17-2869	43.07	56.93	951	110621.2	5′-GTTAAAAGTTGCCAGGATGG	CCCTAGCAACTTCAACTTAC-3′
BTV-23	RSArrrr/23	AJ585144	2927	18-2888	42.23	57.77	957	110612.5	5′-GTTAAAATAGCGTTGCGATG	TTCGCACGTTATACACTTAC-3′
BTV-24	RSArrrr/24	AJ585145	2924	21-2885	41.96	58.04	955	110762.1	5′-GTTAAAAGAGTGACCCACGA	CTGTGGCCTCTTACGCTTAC-3′

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BTV bluetongue virus

* More details on virus isolates can be obtained from iah.bbsrc.ac.uk/dsRNA_virus_proteins/ReoID/viruses-at-iah.htm

Letters in blue are the 5′ and 3′ terminal conserved sequences in genome segment 2 of BTV
Letter in red is the substitution of G for A at sixth position from 3′ end in genome segment

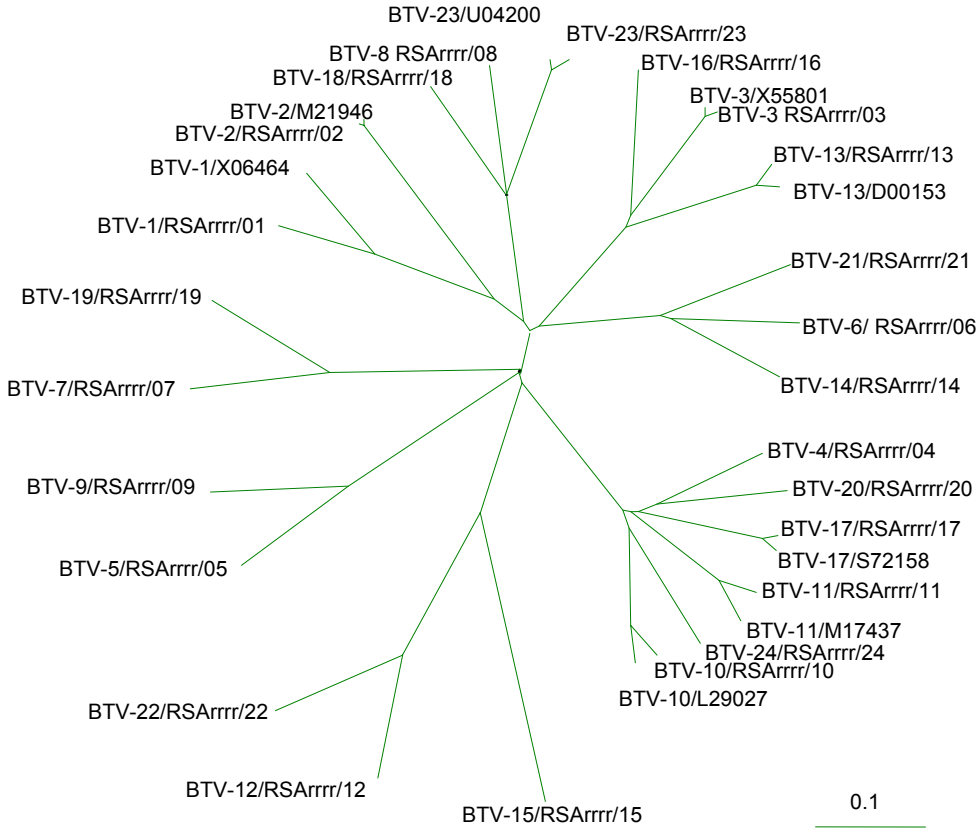

Figure 1
Phylogenetic tree (unrooted neighbour-joining) constructed (using ClustalX version 1.8.1) from amino acid sequence data of the full-length VP2 gene of 24 reference strains of bluetongue virus
The branch lengths reflect genetic distances
Sequences obtained from GenBank (Accession Nos X06464, M21946, U04200, X55801, D00153, S72158, M17437, L29027)

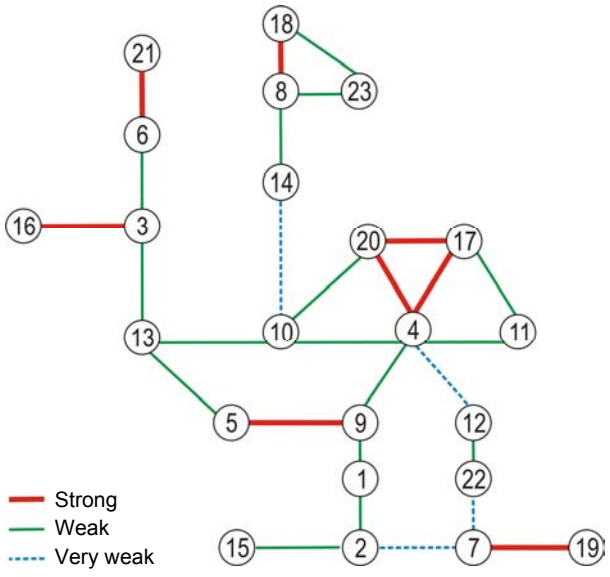

Figure 2
Schematic illustration of the inter-relationships between bluetongue virus serotypes
Adapted from Erasmus (3)

Discussion

The analysis of full-length sequence data of VP2 genes from all 24 serotypes of BTV is described. This represents the first report of the sequence analysis of a single BTV genome segment and protein from all 24 serotypes. These data suggest that BTV serotypes originated from a common ancestor but have evolved differently, some as closely related groups, others independently. The 24 BTV serotypes are currently identified by their ability to cross-neutralise in serological tests, a process that involves virus isolation, tissue culture passage and serological assays, which may take up to eight weeks. This study was conducted to develop molecular methods for BTV serotype identification that are more reliable and rapid than the current serological assays (potentially within 24 h). These techniques will not only support more rapid design and implementation of appropriate vaccination strategies, they also provide epidemiological information that cannot be generated by the conventional serotyping assays. These data will help identify the origins of individual strains and help

understand the mechanisms and routes by which they have spread. This will facilitate the design and refinement of appropriate disease control strategies.

Although sequences of more isolates will inevitably generate a clearer picture of inter-relationships, the strains of different BTV serotypes that were compared to generate the amino acid tree appear to cluster as ten evolutionary lineages. Earlier studies of reassortant BTV viruses that were generated by co-infections using parental strains of two different types, demonstrated that both VP2 and VP5 help to determine BTV serotype. These sequencing studies indicate that some BTV 'types' are serological variants within a single genetic lineage of genome segment 2/VP2. However, this may reflect variations in genome segment 6 (encoding the smaller outer coat protein VP5) and its usually minor role in determination of serotype (10). It has previously been observed that some serotypes cross-react as indicated in Figure 2, although it is again uncertain in each case what contribution, if any, VP5 makes to this cross-reactivity. Further sequencing studies of VP5 (13), combined with serological analyses using a panel of high quality sera against all 24 BTV serotypes may help to resolve these questions.

Many serotypes analysed are represented by only single isolates and many of the isolates are from Africa. A current evaluation of the genetic diversity and relationships within and between the serotypes may be biased by the isolates that are available. However, the authors believe that this database of segment 2/VP2 sequences represents a significant resource for the comparison and identification of BTV types that will be strengthened by addition of further data for additional BTV isolates.

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