Virology 377 (2008) 308–318

Contents lists available at ScienceDirect

Virology

journal homepage: www.elsevier.com/locate/yviro

Sequence analysis of bluetongue virus serotype 8 from the Netherlands 2006 and comparison to other European strains

Sushila Maan [a], Narender S. Maan [a], Natalie Ross-smith [a], Carrie A. Batten [a], Andrew E. Shaw [a], Simon J. Anthony [a], Alan R. Samuel [a], Karin E. Darpel [a], Eva Veronesi [a], Chris A.L. Oura [a], Karam P. Singh [a], Kyriaki Nomikou [a], Abraham C. Potgieter [b], Houssam Attoui [a], Eugene van Rooij [c], Piet van Rijn [c], Kris De Clercq [d], Frank Vandenbussche [d], Stéphan Zientara [e], Emmanuel Bréard [e], Corinne Sailleau [e], Martin Beer [f], Bernd Hoffman [f], Philip S. Mellor [a], Peter P.C. Mertens [a,*]

[a] Department of Arbovirology, Institute for Animal Health, Pirbright Laboratory, Ash Road, Woking, Surrey, GU24 0NF (UK)
[b] Virology Division, Onderstepoort Veterinary Institute, 0110 Onderstepoort, South Africa
[c] Central Veterinary Institute of Wageningen UR (CVI) P.O. Box 65, 8200 AB Lelystad, The Netherlands
[d] Department of Virology, Veterinary and Agrochemical Research Centre (VAR), Groeselenberg 99, B-1180 Ukkel, Belgium
[e] UMR 1161 Afssa/INRA/ENVA, Agence Française de Sécurite Sanitaire des Aliments (AFSSA) 22, Rue Pierre Curie, Boite Postal 67, Maisons Alfort Cedex 94703, France
[f] Friedrich-Loeffler-Institut Federal Research Institute for Animal Health Südufer 10, 17493 Greifswald-Insel Riems, Germany

ARTICLE INFO

Article history:
Received 25 January 2008
Returned to author for revision
4 March 2008
Accepted 14 April 2008

Keywords:
Bluetongue virus
BTV
Orbivirus
Reovirus
Full-genome sequence analysis
BTV-8

ABSTRACT

During 2006 the first outbreak of bluetongue ever recorded in northern Europe started in Belgium and the Netherlands, spreading to Luxemburg, Germany and north-east France. The virus overwintered (2006–2007) reappearing during May–June 2007 with greatly increased severity in affected areas, spreading further into Germany and France, reaching Denmark, Switzerland, the Czech Republic and the UK. Infected animals were also imported into Poland, Italy, Spain and the UK. An initial isolate from the Netherlands (NET2006/04) was identified as BTV-8 by RT-PCR assays targeting genome segment 2. The full genome of NET2006/04 was sequenced and compared to selected European isolates, South African vaccine strains and other BTV-8 strains, indicating that it originated in sub-Saharan Africa. Although NET2006/04 showed high levels of nucleotide identity with other 'western' BTV strains, it represents a new introduction and was not derived from the BTV-8 vaccine, although its route of entry into Europe has not been established.

Introduction

'Bluetongue' (BT) is an economically important disease affecting sheep (with case fatality rates that can occasionally exceed 70% Gambles, 1949), some species of deer and to a lesser extent cattle and goats. The bluetongue virus (BTV) is transmitted almost exclusively by adult female hematophagous midges, belonging to certain species of the genus *Culicoides,* and can infect most ruminant or camelid species. BT in sheep is characterised by a loss of condition, muscle degeneration, coronitis, haemorrhages, respiratory distress and swelling of the head and neck, which occasionally leads to cyanosis of the tongue (after which the disease is named). Clinical signs of BT can be particularly severe in naive sheep populations (in areas that are normally free of the disease), or after introduction of an exotic strain into endemic areas (Darpel et al., 2007; MacLachlan 2004; Prasad et al., in press).

A series of BT outbreaks that started in 1998 has spread across much of southern and central Europe, involving eight different virus strains, belonging to five distinct serotypes (BTV-1, 2, 4, 9 and 16). These viruses arrived in Europe via three distinct routes: from the east through Turkey into Greece and Bulgaria; from Algeria or Tunisia into Italy and the eastern Mediterranean islands, or via Morocco into the Iberian Peninsula (Mellor and Wittmann, 2002; Mertens et al., 2007b). The arrival of multiple BTV strains and their persistence in Europe have been linked to the effects of climate change (particularly higher temperatures) and its influence on both the distribution and vector capacity of local *Culicoides* populations (Conte et al., 2003; Purse et al., 2005; Purse and Rogers, in press).

The record temperatures experienced in northern Europe during the summer of 2006 coincided with the first outbreak of bluetongue ever recorded in the region, spreading across the Netherlands, Belgium,

* Corresponding author. Fax: +44 1483 232448.
E-mail address: peter.mertens@bbsrc.ac.uk (P.P.C. Mertens).

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doi:10.1016/j.virol.2008.04.028

Luxemburg, much of Germany and into north-east France (Darpel et al., 2007; OIE, 2006a; Toussaint et al., 2006). During the first year of the outbreak morbidity and mortality levels were low, for example it is estimated that Belgium lost less than 100 animals in total (Elbers et al., 2007). However, the virus overwintered successfully (2006–2007) reappearing in the same regions during May–June 2007, then spreading further across Central France and Germany, arriving in Denmark and the East Anglian region of the UK during September/October 2007, then into Switzerland and the Czech Republic. Most significantly the incidence and severity of the disease dramatically increased in the second year with case fatality levels in sheep approaching 50% in some areas. During the late summer it was estimated that Belgium alone lost >15% of its entire sheep population killed by the disease. There were reports of reduced productivity, loss of milk yield, and low levels of mortality in cattle (<1%), as well as clear indications of vertical transmission (particularly in cattle — Zentis, 2008), and abortion/sterility in both cattle and sheep (Hoogendam, 2007; Wilson and Mellor, 2007). Movement of cattle within Europe resulted in the importation of BTV-8 infected animals and outbreaks in Spain and Italy in 2007–2008. Small numbers of infected animals were also imported into Poland, Romania and the UK (including England, Scotland and Northern Ireland). At the end of 2007 this was already the largest and most economically damaging outbreak caused by a single BTV strain on record. Although there are some plans for mass vaccination, it appears likely that the outbreak will continue to spread and increase in intensity during 2008, particularly in newly affected areas.

Bluetongue virus is the type species of the genus *Orbivirus* within the family *Reoviridae* (Mertens et al., 2005). The virus genome is composed of ten segments of linear double stranded RNA (Verwoerd, 1970), which code for ten distinct viral proteins (Mertens et al., 1984, 1987a,b, 2007c). The inner layers forming the 'core' of the BTV capsid contain VP1, VP3, VP4, VP6 and VP7 (encoded by genome segment (Seg) 1, 3, 4, 9 and 7 respectively). These core proteins and two of the non-structural proteins (NS1 and NS2: encoded by Seg-5 and 8 respectively) are thought to be relatively conserved, and are antigenically cross-reactive between different strains and serotypes of BTV. However, cross-hybridisation and sequencing studies have shown that these genome segments can vary in a manner that reflects the geographic origin of the virus strain (topotype) (Gould and Pritchard, 1990; Mertens et al., 1987b; Pritchard et al., 1995, 2004).

Genome segment 10 (encoding non-structural proteins NS3/N3a of BTV) is more variable than the majority of the genome segments encoding the other non-structural or core proteins. The significance of variations in NS3/NS3a is not fully understood, although it is clear that these non-structural proteins do not determine virus serotype. It has been suggested that variations in Seg-10 might relate to transmission of the virus by different insect vector populations and species (Balasuriya et al., 2008; Bonneau et al., 1999; Nikolakaki et al., 2005; Wilson et al., 2007). Seg-7 of BTV, encoding the outer core protein VP7, also shows significant variations (Wilson et al., 2000), despite the role of VP7 as the major serogroup-specific antigen. It has been suggested that these variations could also relate to the insect populations that act as vectors for different virus strains in different geographic areas (Bonneau et al., 2000; Wilson et al., 2000).

The BTV outer capsid proteins VP2 and VP5 (encoded by Seg-2 and Seg-6 respectively) determine the specificity of interactions between the virus particle and the neutralising antibodies generated during infection of the mammalian host (Cowley and Gorman, 1989; Huismans et al., 1985; Mertens et al., 1989). Genome segments 2 and 6 show high levels of sequence variation that correlate with virus serotype (particularly in VP2/segment 2 — Maan et al., 2007a; Mertens et al., 2007g; Singh et al., 2004). However, these genome segments also show variations within each serotype that correlate with the geographic origin of the virus strain (Seg-2 and 6 'topotypes') (Bonneau et al., 1999; Gould and Pritchard 1990; Maan et al., 2007a; Mertens et al., 2007g; Singh et al., 2004; Zhang et al., 1999).

Bluetongue virus was isolated from the blood of sheep showing severe clinical signs of disease in the Netherlands during August 2006 (IAH reference collection number NET2006/04). We report the first identification of this virus as BTV-8, the cause of the northern European BT outbreak in 2006. We also report the complete nucleotide sequence of the virus genome, and a comparison of each genome segment to other European field strains, and the BTV vaccine strains that have been used in the region. Comparisons were also made to representative 'eastern' and 'western' bluetongue viruses from other parts of the world (including other strains of BTV-8), in an attempt to clarify the origins of the outbreak virus.

Results

Virus isolation and propagation in cell culture

Blood samples from sheep showing clinical signs of bluetongue (sample number A83/06-6) were sent from the Netherlands to the Community Reference Laboratory (CRL) at IAH Pirbright. Initial attempts to isolate virus directly into BHK-21 cells failed, even though the sample was positive by ELISA and real-time RT-PCR. No virus (or CPE) was detected when material passaged in embryonated chicken eggs (BTV8-E1) was titrated on BHK cells, although BTV8-E1 was positive for BTV RNA by real-time RT-PCR (CT 18.05). However, supernatant from *Culicoides sonorensis* cell cultures (KC cells), 7 days after inoculation with RBC from sample number A83/06-6, did cause 100% CPE in BHK-21 cells at 4 days post infection. This virus isolate was added to the IAH reference collection as NET2006/04 — (passage level KC1/BHK1 — Mertens et al., 2007d).

Identification and typing of NET2006/04

The northern European virus was identified as BTV by RT-PCR assays targeting Seg-7 (Anthony et al., 2007), generating an amplicon of the expected size (1156 bp) from EDTA treated blood samples, infected egg material and infected cell-culture supernatants (Fig. 1—Panel D). Real-time RT-PCR assays targeting Seg-1 (Shaw et al., 2007) confirmed this result and showed that the virus belongs to a 'western' lineage, indicating that it originated from Africa or America.

Serotype specific RT-PCR assays targeting Seg-2 only generated amplicons of the expected size with the primers for BTV-8 (Mertens et al., 2007b, and in preparation) excluding the other 23 BTV serotypes (Fig. 1—Panels A–C). This represents the first positive identification of BTV-8 in Europe and was confirmed using multiple additional primer pairs and sequencing of Seg-2. Sequence comparisons with Seg-2 of reference strains for the 24 BTV serotypes (Maan et al., 2007a), showed 93.4% nt sequence identity with the BTV-8 reference strain (RSArrrr/08).

Full-length sequence analyses and comparison of the NET2006/04 genome

Genome segments 1 to 10 of NET2006/04 range in size from 3944 (Seg-1) to 822 base pairs (bp) (Seg-10), encoding proteins from 1302 (VP1) to 229/216 amino acids (aa) (NS3/NS3A) respectively. Details of individual genome segments are given in Table 1.

Genome segment 1 of BTV-8 NET2006/04 was compared to available data for other 'western' viruses, and to BTV-2 from Taiwan (Table 1 — supplementary data). In each case Seg-1 is 3944 bp long, encoding the RNA polymerase protein (Pol) of 1302 aa. The 5′ non-coding regions (NCR) is 11 bases long and is 100% conserved, while the 3′ NCR is 24 nucleotides (nt) long and is >91.6% conserved, for the BTV strains analysed (Table 1). The full-length sequence of Seg-1 is also highly 'conserved', showing >78.7% identity overall, with NET2006/04 most closely related (94.5% identity) to BTV-2 from Corsica 2002 (Ac. No. AY154458). The African and American isolates of BTV-2 (L20508); BTV-10 (X12819); BTV-11 (L20445); BTV-13 (L20446); and BTV-17 (L20447)

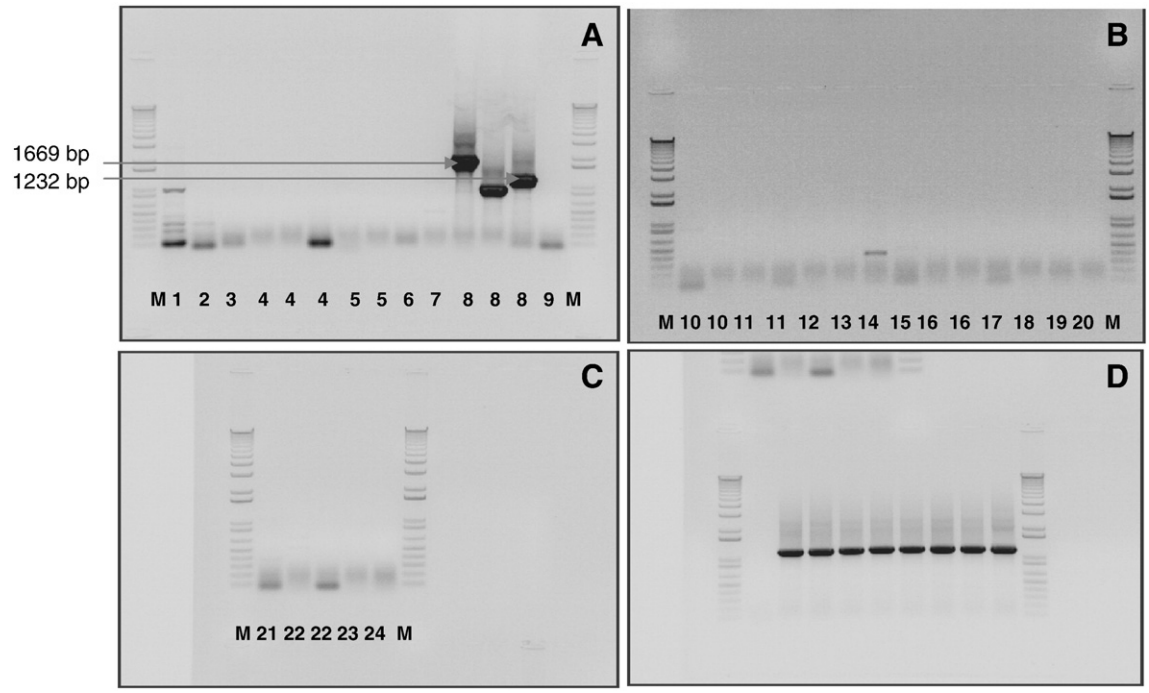

Fig. 1. Electrophoretic analysis of cDNA products from Seg-2 and Seg-7 of BTV-8 NET2006/04 isolate using the type specific primer pairs against 24 BTV types and generic primers for Seg-7. PCR amplicons were generated from cDNA of BTV-8 isolate NET2006/04 with three type 8 specific primer pairs (lane 8) (Mertens et al., 2007b) as indicated in panel A. No amplification was seen with primer pairs of other serotypes (Lanes 1 to 7 and 9-24-Panel A–C). Panel D showed the amplification of BTV RNA with Seg-7 specific primers (Anthony et al., 2007). Lane M: 1 kb marker.

also showed high levels of identity to BTV-2 from Corsica and BTV-8 from the Netherlands (91% identity), but only 78.7–80.1% identity to BTV-2 Taiwan (AY493686), indicating a clear separation into eastern and western groups (Fig. 1 — supplementary data).

Genome segment 2 encodes VP2, the outermost component of the virus capsid and the most variable of the BTV proteins (Maan et al., 2007a). Although Seg-2 can vary in length between different BTV types, it usually has a uniform length within each serotype, or Seg-2 nucleotype (Maan et al., 2007a). Seg-2 of BTV-8 (including NET2006/04) is conserved at 2939 bp, encoding a protein of 961 aa (Table 1). It has upstream and downstream NCRs of 17 bp and 36 bp, which show 100% and 97.2% identity respectively to Seg-2 NCRs of the other BTV-8 strains analyzed.

The full-length sequence of Seg-2 from NET2006/04 did not group closely with any of the other European strains analysed (Fig. 2A). It clustered closely with the reference strain of BTV-8 (93.4% nt identity), confirming its initial identification by RT-PCR. In contrast it showed only 45% to 71.1% nt identity to reference strains of the other 23 BTV serotypes. NET2006/04 showed some similarity to reference strains of BTV-18 (71.1%) and BTV-23 (69.8%), which have previously been grouped with BTV-8 within 'Seg-2, nucleotype D' (Maan et al., 2007a).

Seg-2 of the different BTV-8 isolates analysed showed 92.4–99.9% nt identity to the BTV-8 reference strain (RSArrrr/08). Seg-2 of the BTV-8 vaccine strain had only 9 nt differences from RSArrrr/08 (99.7% nt identity) from which it was derived (isolated in South Africa in 1937: reviewed by Alpar et al., in press). Although Seg-2 of BTV types 1, 2, 9 and 16 all showed evidence for separation into east–west topotypes, within each serotype (Fig. 2A), the BTV-4 and 8 strains analyzed were all from western origins; consequently there was no evidence for separation of Seg-2 into eastern and western groups within these two serotypes. NET2006/04 was most closely related (97% identity) to BTV-8 from Nigeria (NIG1982/07) but showed less similarity (93.4%) to the BTV-8 vaccine strain, indicating a more distant relationship. The recent South African isolates of BTV-8 (RSA1998/01, RSA1992/01 and RSA1987/01) are closely related to each other, with 97.8% to 99.6% nt identity (based on ~1000 bp region at the downstream end of Seg-2). However, they are more distantly related to NET2006/04 (94.1–94.7% nt identity) and the earlier South African strains (92.4–93.6% nt identity — Figs. 2A and B). Sequence data for Seg-2 of multiple northern European isolates of BTV-8 (2006–2008) showed that they are all closely grouped with NET2006/04 (>99.8% identity — including France (FRA2006/01) and the UK (UKG2007/05)).

Genome segment 3 encodes the highly conserved BTV sub-core-shell protein, VP3(T2). Seg-3 of the different European BTV strains is conserved at 2772 nt, encoding 901 aa, with upstream and downstream NCRs of 17 and 49 bases respectively (Table 1). Seg-3 of NET2006/04 showed >95% nt similarity to other 'western' group viruses, but only 80.9%–81.1% similarity to the 'eastern' European isolates analysed, which form a distinct cluster with >90.7% identity (Fig. 2 — supplementary data).

Genome Segment 4 encodes the highly conserved BTV capping and transmethylase enzyme — VP4(CaP). Seg-4 was 1981 nt long (regardless of BTV strain) encoding 644 aa, with a fully conserved 8 bp upstream NCR (Table 1). In contrast the 38 bp downstream NCR was more variable, showing 97.3% identity between the western strains, which show only 92% identity with BTV-2 from Taiwan (Table 1 — supplementary data). The full-length nt sequence of Seg-4 from NET2006/04 showed >78.6% nt identity to other BTV strains, grouping it with the other 'western' isolates (which showed 88.8–99.7% nt identity — Fig. 3— supplementary data). In contrast, BTV-2 from Taiwan (Ac. No. AY493689) showed only 78.6 to 80.1% to the 'western' viruses. The western group contained two subgroups, the larger of which included prototype strains of serotype 10, 11, 13 and 17 from the USA. NET2006/04 shows 80.1% nt identity to BTV-2 Taiwan, and is included in the 2nd subgroup (along with BTV-2 strains from the USA (Ac. No. L08637), Corsica (Ac. No. AY129085) and South Africa (vaccine strain — Ac. No. AY134477)), which shows a slightly closer relationship to BTV-2 Taiwan. Within this subgroup NET2006/04 is most closely related to the Corsican and South Africa (vaccine) strains (95.7% and 94.8% identity respectively).

Table 1
Characteristics of dsRNA genome segments (cDNA copy) and proteins of the bluetongue virus serotype 8 Netherland (NET2006/04)

†Genome segment (size: bp)	ORFs Bp (including stop codon)	Protein nomenclature (§: protein structure/ function)	Number of amino acids (Da)	Location	Accession numbers	5′ Terminal sequences of the positive strand	3′ Terminal sequences of the positive strand
1 (3944)	12–3920	VP1 (Pol)	1302 (149,834)	Within the sub-core at the 5 fold axis	AM498051	5′-GTTAAAATGCAATGGTCGCA	TGAGAGCACGCGCCGCATTACCCTTAC-3′
2 (2939)	18–2903	VP2	961 (111,317)	Outer capsid	AM498052	5′-GTTAAAATAGCGTCGCGATG	TAGCTCTCGTGACTGAGAGCTCGCGCGCTATCA ACTTAC-3′
3 (2772)	18–2723	VP3 (T2)	901 (103,265)	Sub-core capsid layer (T = 2 symmetry)	AM498053	5′-GTTAAATTTCCGTAGCCATG	TAGATGTGCGACCAATCTATGCACTTGGTAGCG GCAGCGGGAACACACTTAC-3′
4 (1981)	9–1943	VP4 (Cap)	644 (75,147)	Within the sub-core at the 5 fold axis	AM498054	5′-GTTAAAACATGCCTGAGCCA	TAATGCGTGACTGCTAGGTAAGGGGGGCCTTTA CAACTTAC-3′
5 (1776)	35–1693	NS1 (TuP)	552 (64,446)	Cytoplasm forms tubules	AM498055	5′-GTTAAAAAAGTTCTCTAGTTGGCAACCACCAAACATG	TAGTTACTGACTTCTGTTTTCTGTTTTTTCATTC TTCTTTCTACTTCTATTTTCTCTTAGCACTCTACT AGAACTTTTCAACTTAC-3′
6 (1637)	28–1608	VP5	526 (59,339)	Outer capsid	AM498056	5′-GTTAAAAAAGCGATCGCTCTCGCGAAGATG	TGAGCGCAGCGGAGCCACCGCTTTCCACTTAC 3′
7 (1156)	18–1067	VP7 (T13)	349 (38,558)	Outer core (T = 13 symmetry)	AM498057	5′-GTTAAAAATCTATAGAGATG	TAGTCCACTTTGCACGGGTGTGGGTTACATATG CGGTGTGTCGGTTGTGGGATATATGTAACCCAT TCAAACGTCTCTTAGATTACACTTAC-3′
8 (1125)	20–1084	NS2 (ViP)	354 (40,639)	Cytoplasm, viral inclusion bodies (VIB)	AM498058	5′-GTTAAAAAATCCTTGAGTCATG	TAGGCGCTTGTGACCGCGTGGTTGGGGGGGGA TTTTACACTTAC-3′
9 (1049)	16–1005	VP6 (Hel)	329 (35,568)	Within the sub-core at the 5 fold axis	AM498059	5′-GTTAAAAAATCGCATATGTCA	TAAAGGGTCCAGGGTACTCTCCTGACGTAGGGC GATTTACATCTTAC-3′
10 (822)	20–709	NS3	229 (25,514)	Cell membrane	AM498060	5′-GTTAAAAAGTGTCGCTGCCATG	TGAGGACAGTAGGTAGAGTGGCGCCCCGAGGT TTACGTCGTGCAGGGTGGTTGACCTCGCGGCGT AGACTCCCACTGCTGTATAACGGGGGAGGGTG CGCGACACTACACACTTAC-3′
	59–709	NS3a	216 (23,953)			5′ - GTTAAAAAGTGTCGCTGCCATGCTATCCGGGCTGATCCA AAGGTTCGAAGAAGAAAAAATG	TGAGGACAGTAGGTAGAGTGGCGCCCCGAGGT TTACGTCGTGCAGGGTGGTTGACCTCGCGGCGT AGACTCCCACTGCTGTATAACGGGGGAGGGTG CGCGACACTACACACTTAC-3′

Pol = RNA polymerase; Cap = capping enzyme (guanylyltransferase); Hel = helicase enzyme [42]; T2 = protein with T = 2 symmetry; T13 = Protein with T = 13 symmetry; ViP = viral inclusion body matrix protein; TuP = tubule protein. Letters in bold blue are the 5′ and 3′ terminal conserved sequences in genome segments of BTV-8 NET2006/04. Letters in red are the substitution of C for A at sixth position from 3′ end in genome segment 1 in NET2006/06, and T for A in genome segment 9, in NET2006/06 and six other western viruses (out of 41 strains analysed).

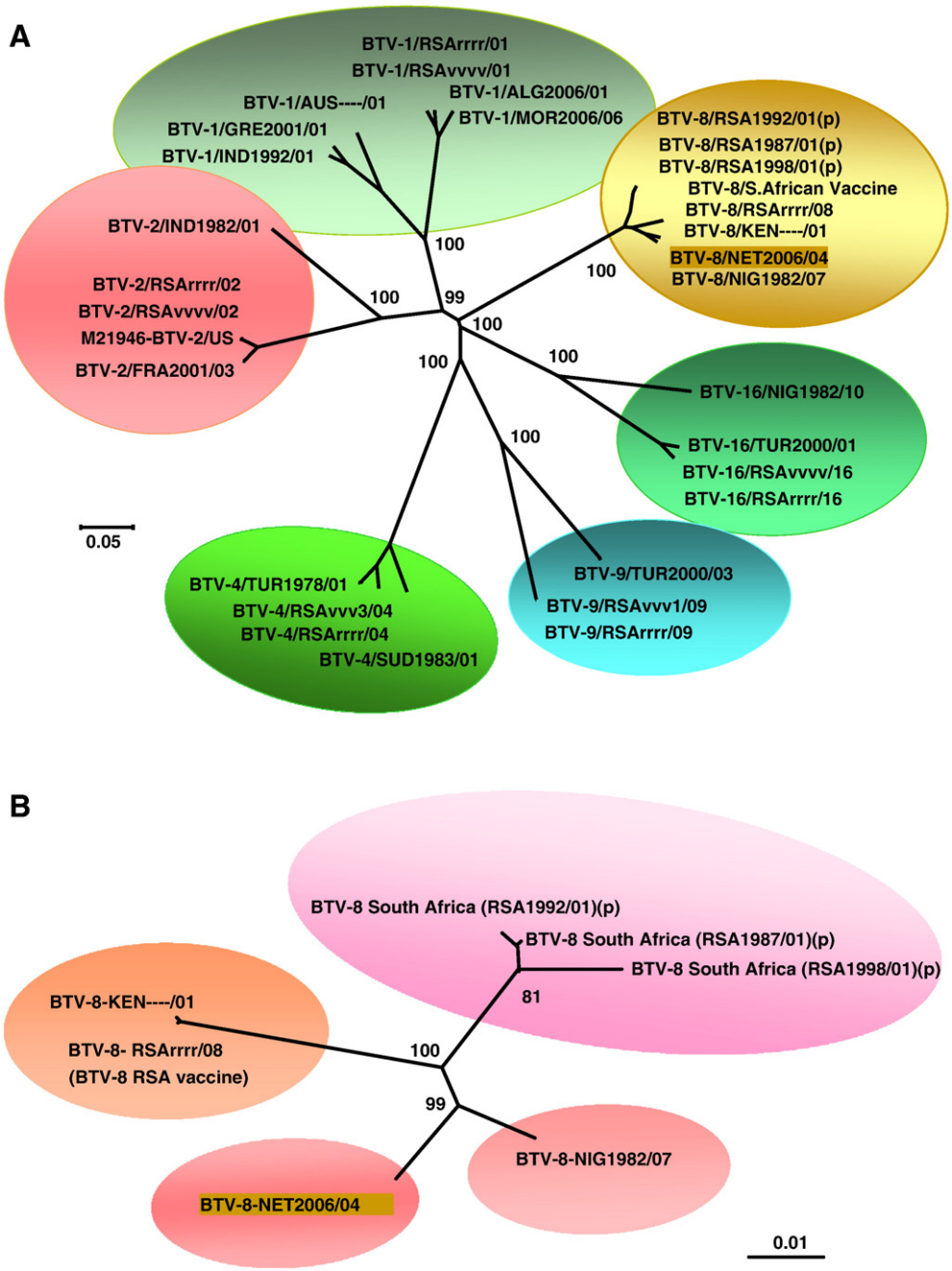

Fig. 2. A: Neighbour-joining tree showing the relationships between nucleotide sequences of Seg-2 from European strains of BTV serotype 1, 2, 4, 8, 9 and 16. The tree was constructed using distance matrices, generated using the p-distance determination algorithm in MEGA 3 (2000 bootstrap replicates) (Kumar et al., 2004). The numbers indicate bootstrap confidence values after 2000 replications. p indicates partial sequences. The trees shown in Figs. 2B–6 were drawn using same parameters. B: Neighbour-joining tree showing relationships between nucleotide sequences of Seg-2 from strains of BTV-8.

Genome segment 5 of NET2006/04 was compared with European field strains, and the South African BTV-vaccine strains (Table 1 and Fig. 4 — supplementary data). Seg-5 of NET2006/04 is 1776 nt long, encoding NS1(TuP) at 552 aa, with upstream and downstream NCRs of 34 and 83 bp in length (Table 1). Seg-5 from the different BTV isolates formed two distinct clusters, representing eastern and western lineages with 82.2–83.5% identity between members of these different groups. The eastern group included European field strains of BTV-1 (from Greece), 9 and 16, as well as the vaccine strain of BTV-16. However, NET2006/04 clusters with the 'western' viruses, including European field strains of BTV-2 and 4, and the South African vaccine strains of types 1, 2, 4 and 9.

Seg-5 of NET2006/04 was most closely related to BTV-4 from Greece (GRE1999/15 — 97.5% identity) providing the first sequence data indicating that the northern European outbreak in 2006 was caused by a 'western' virus. Within the eastern group, the Sardinian field isolates of BTV-16 (SAD2004/03 and SAD2004/04) were almost identical to the BTV-16 South African vaccine strain, with 99.9% nt similarity, indicating a very recent common ancestry. Within the western group, European field strains of BTV-2 from Spain (SPA2005/01) and BTV-16 from Italy (ITL2002 — Ac. No. DQ017960) were almost identical (99.9–100%) to Seg-5 of the South African BTV-2 vaccine strain (RSAvvvv/02-Ac. No. AM773684 and AY138895), indicating that a reassortment event has taken place between vaccine strains from different serotypes.

Genome segment 6 encodes VP5 — the smaller outer capsid component and second most variable of the BTV proteins. Seg-6 of the different BTV-8 isolates is 1637 bp long showing >94.7% nt identity, and encoding 526 aa (Table 1). The upstream and downstream NCRs are 27 and 29 bp long, with 96.2% and 100% identity respectively. Comparisons of Seg-6 from the European strains of BTV-1, 2, 4, 8, 9 and 16, identified seven main lineages, which primarily correlate with virus serotype (Fig. 3). However, the BTV-1 strains from the Mediterranean region included two distinct lineages, represented by an eastern strain (GRE2001/01) and a western strain (ALG2006/01 and MOR2006/06) (Fig. 3), reflecting two distinct Seg-6 topotypes and introductions of BTV-1 from different geographic origins. The Seg-6 sequence of GRE2001/01 showed a relatively large separation (79% similarity) from the BTV-1 vaccine strain (RSAvvvv/01), reflecting their derivation from eastern and western groups of viruses respectively. In contrast ALG2006/01 and MOR2006/06 showed 95% nt similarity to the vaccine strain, reflecting a closer geographical origin.

Seg-6 from different BTV-9 isolates also formed two distinct groups, containing 'eastern' strains from Europe 2000 to 2002, and the 'western' vaccine strain, respectively. However, these two groups are much more widely separated (30.4% variation) in a manner similar to Seg-6 from different BTV serotypes (Fig. 3). This not only indicates that Seg-2/VP2 sequences (and not Seg-6/VP5) determine the identity of BTV-9, but also that one or other of these lineages has acquired segment 6 by reassortment with a distinct virus type.

Seg-6 of NET2006/04 grouped with other BTV-8 isolates from Nigeria, Kenya and S. Africa (reference strain) with >94.8% similarity overall. As already noted for Seg-2 (encoding VP2), the closest strain to NET2006/04 was BTV-8 from Nigeria isolated during 1982 (NIG1982/07), with 97.7% nt similarity.

Genome segment 7 of NET2006/04 is 1156 bp long, encoding the 349 aa of the major BTV serogroup-specific antigen and core surface protein — VP7. The upstream and downstream NCRs are 17 and 89 bases long respectively (Table 1). The aa sequence of VP7 is significantly more conserved (>80.2% identity) than suggested by the nt sequence of Seg-7 of NET2006/04 which shows 75.5%–97% nt identity to the other BTV strains. This reflects large numbers of synonymous mutations in the third base position.

Unlike majority of the BTV genome segments, Seg-7 formed six distinct clusters. Three of these are primarily from a western origin (with the single exception of the Chinese strain of BTV-12) and three from an eastern origin (Fig. 4). Two other Mediterranean BTV strains (BTV-2 Tunisia — Ac. No. AF469115 and BTV-2 Corsica — Ac. No. AY079124), belonging to western group 2) were included in these comparisons, showing 79.3% and 80.1% identity with NET2006/04 respectively. Within western group 1, NET2006/04 clusters closely (97% identity) with BTV-1 Honduras (Ac. No. AF188670) and the South African BTV-1 reference strain (Ac. No. AF188669) (Fig. 4).

Genome segment 8 of BTV is conserved at 1125 bp, encoding the 354 aa of the viral inclusion body (VIB) matrix protein — NS2. The upstream and downstream NCRs (19 and 41 bp respectively) are fully conserved in the viruses compared (Table 1). Seg-8 of the European BTV isolates showed clear separation into eastern and western groups, with NET2006/04, vaccine strains of BTV-2, 4 and 9 and European field strains of BTV-2 and 4, in the western group (Fig. 5). Overall nt identities of 76.1% to 80.4% were detected between the eastern and western groups. Preliminary sequence data for Seg-8 of BTV-1 from Greece (GRE2001/01) and BTV-16 from Turkey (TUR2000/01) indicate that they both group within the eastern cluster. Seg-8 of NET2006/04 showed 99.8% similarity to a distinct isolate from the same outbreak in the Netherlands, but from a different animal (NET2006/01). It also showed relatively high levels of nt similarity to the South African vaccine strains of BTV-2 (Ac. No. AY138896 — 96.6%), BTV-4 (Ac. No. AY857502 — 95.5%) and BTV-9 (RSArrr1/09 -Ac. No. AM900374 — 95.6%), as well as to the Corsican field strains of BTV-4 (Ac. No. AY857499 — 96%) and BTV-2 (Ac. No. AY124372 — 92.8%).

Genome segment 9 codes for minor core protein VP6, the core associated helicase of BTV. The Seg-9 nt sequences were divided into eastern and western groups (Fig. 5— supplementary data). Seg-9 of the eastern BTV isolates is 1052 bp, encoding a protein 330 aa in length, while Seg-9 from the western lineage is 1049 bp (329 aa) (Table 1). The upstream NCR is 14–15 bp (W–E), while the downstream NCR is 44 bp. Seg-9 of NET2006/04 belongs to the western group and is closely related to the Corsican isolate of BTV-2 (Ac. No. AY124373 — 97.1% identity) and the South African BTV-2 vaccine strain (Ac. No. AF530066 — 94.2% identity), but is more distantly

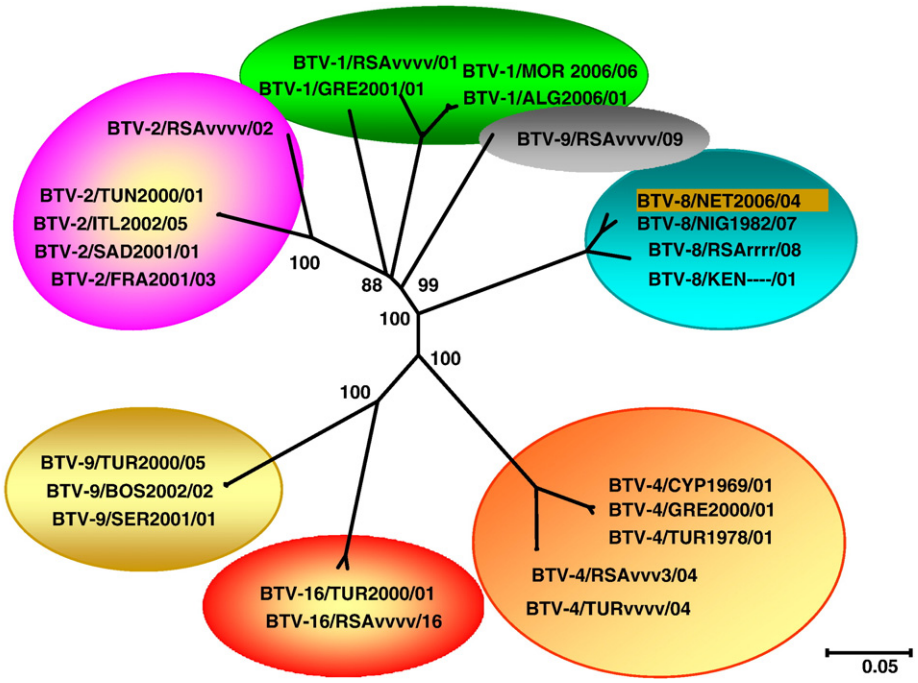

Fig. 3. Neighbour-joining tree showing relationships between nucleotide sequences of Seg-6 from European strains of BTV serotype 1, 2, 4, 8, 9 and 16.

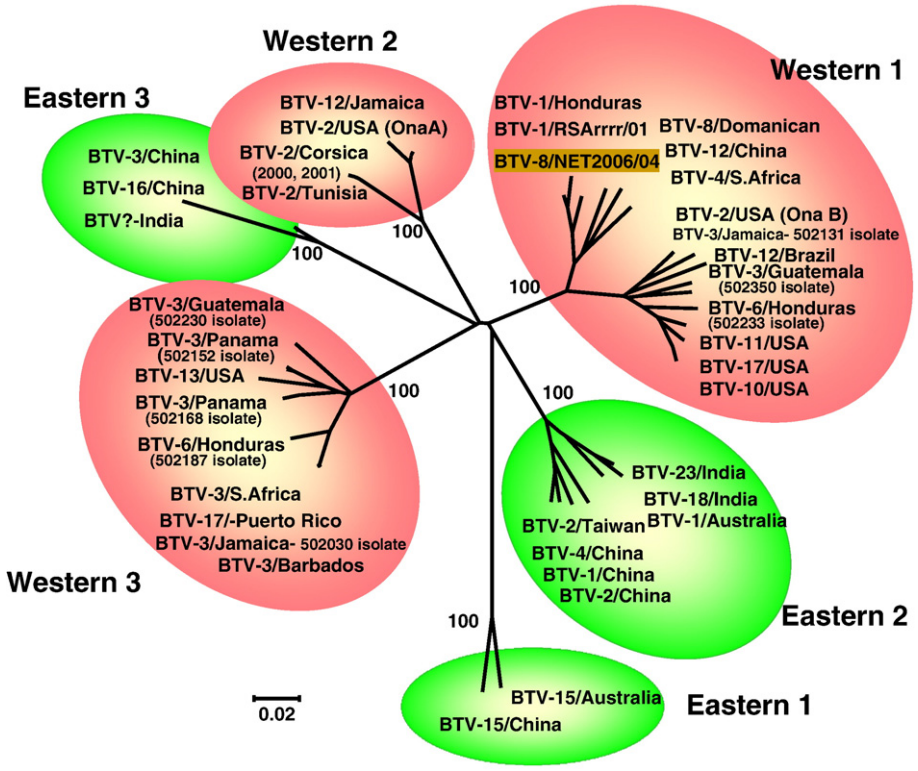

Fig. 4. Neighbour-joining tree showing relationships between nucleotide sequences of Seg-7 from different BTV serotypes.

related to the eastern strains of BTV-1-GRE2001/01 (76.3% identity), BTV-9-BOS2002/02 and BTV-16-TUR2000/01 (76.6% identity). Overall Seg-9 showed >75.8% nt similarity across the eastern and western strains of BTV.

Genome segment 10 of NET2006/04 is 822 bp long, coding for the small non-structural proteins NS3 (229 aa) and NS3a (216 aa) (Table 1).

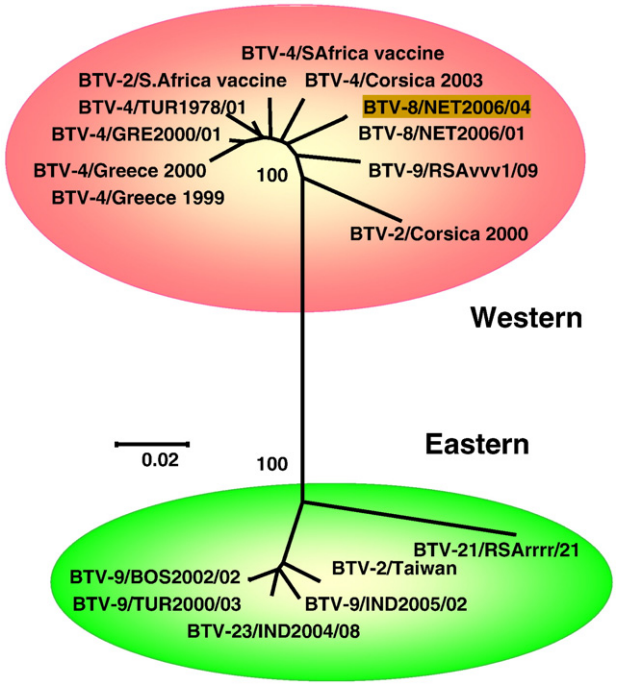

Fig. 5. Neighbour-joining tree showing relationships between nucleotide sequences of Seg-8 from BTV isolates from Europe and around the world.

The upstream and downstream NCRs are 19 and 113 bp long respectively. Seg-10 which is >82% conserved, across all of the isolates compared, can be divided into four groups, two from eastern and two from western origins. Seg-10 from European isolates of BTV-1, 9 and 16 cluster together in eastern group 1 (equivalent to clade A1 of Seg-10 — reported by Balasuriya et al., 2008), while the European isolates of BTV-2 and 4, and South African reference strain of type 1 cluster within western group 2 (equivalent to sub-clade A3 within clade A2 — Balasuriya et al., 2008) (Fig. 6). Seg-10 of the South African vaccine strains of BTV-1, 2, 3, and 4, cluster within western group 1 (equivalent to clade B2 — Balasuriya et al., 2008). NET2006/04, is included in this group and was most closely related to the vaccine strains of BTV-2 (Ac. No. AF481094— 96.2% identity), BTV-4 (Ac. No. AF512908 — 93.8% identity), BTV-3 (Ac. No. AF512918 — 92.3% identity), and BTV-1 (Ac. No. AF512910 — 92.2% identity). But was more distantly related (<82.9% identity) to the European BTV isolates in eastern group 1 or western group 2.

Conserved terminal sequences and stop codons

The upstream and downstream terminal hexanucleotides (Mertens et al., 2007e) are maintained for the majority of the BTV genome segments included in these analyses (Table 1). However, a substitution of C to A was detected at the sixth position from the 3′ end of Seg-1 from NET2006/04. Another substitution of T for A was detected at the sixth position from the downstream end of Seg-9 from NET2006/06 and six of the other western viruses (out of 41 strains analysed).

The stop codon of Seg-1 is …UGA…, while Seg-4 and 9 use UAA, and Seg- 3, 5, 7 and 8 use UAG. The stop codon of Seg-6 is conserved as UGA across isolates of all 24 BTV serotypes. In contrast stop codons are not conserved in Seg-2 between different BTV types, and include … UGA…, …UAG… and …UAA… (Maan et al., 2007a), although all of the BTV-8 strains use …UAG….. Seg-10 of NET2006/04, BTV-1 and BTV-2 vaccine strains, (all of which are in western group 2) use …UGA… The other field strains and the BTV-8 vaccine strain (that are included in

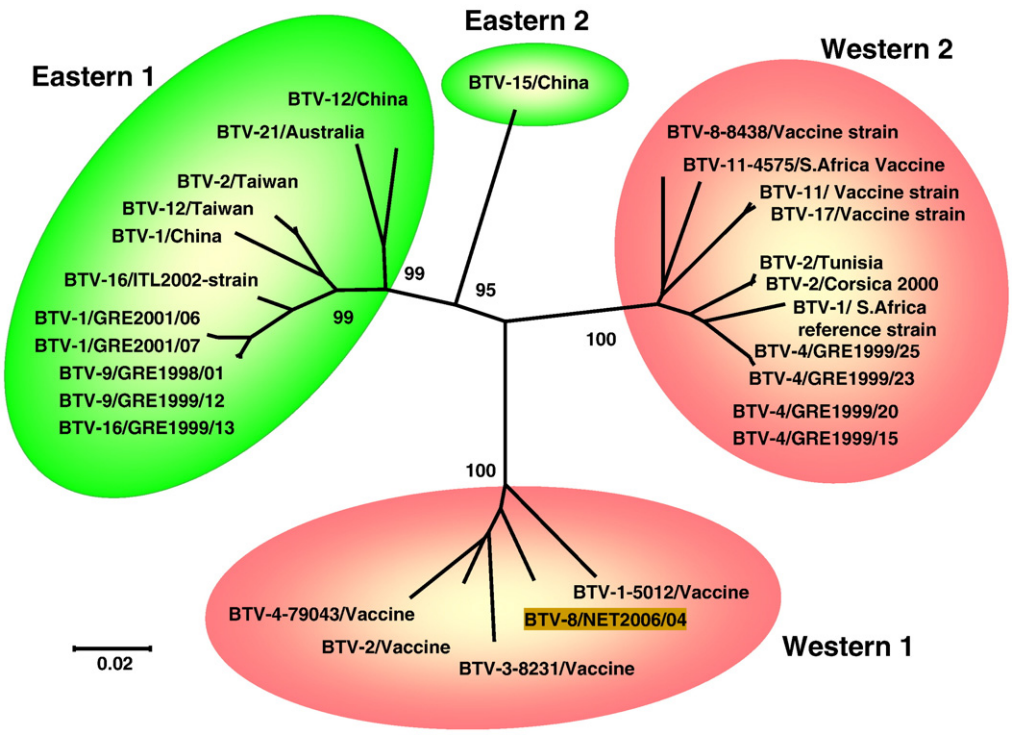

Fig. 6. Neighbour-joining tree showing relationships between nucleotide sequences of Seg-10 from BTV isolates from Europe and around the world.

western group 1), together with both of the eastern subgroups, all use …UAA….

Discussion

In August 2006, sheep in the Maastricht region of the Netherlands started showing severe clinical signs that were typical of 'bluetongue' disease. Blood samples were sent to the Central Institute for Animal Disease Control (CIDC) at Lelystad in the Netherlands, where BTV-specific antibodies were detected by ELISA, and the presence of BTV was confirmed by RT-PCR targeting Seg-10 (Toussaint et al., 2007; Vandenbussche et al., in press). This was the first time that an outbreak of bluetongue had ever been identified in northern Europe. Further blood samples were sent to the CRL at IAH Pirbright in the UK, where competition ELISA confirmed that these samples were positive for BTV-specific antibodies (Batten et al., 2008; Vandenbussche et al., in press). A *C. sonorensis* cell line (KC cells) and BHK cells were used to isolate virus (IAH reference collection isolate number NET2006/04), which was subsequently identified as BTV by conventional and real-time RT-PCR assays targeting Seg-7 and Seg-1 respectively (Anthony et al., 2007; Shaw et al., 2007).

We report the first identification of the northern European strain (NET2006/04) as BTV-8, by conventional RT-PCR assays using serotype-specific primers targeting Seg-2 (Mertens et al., 2007f, g), together with a complete nucleotide sequence for the genome of NET2006/04. Phylogenetic analyses showed that all of the genome segments of this isolate contain significant numbers of nucleotide changes, compared to segments (where published) of the BTV vaccine or field strains that were used, or already circulating in southern Europe. This supports serological evidence that NET2006/04 represents the novel introduction of an exotic BTV strain/serotype into Europe, although details of its entry route to the region remain unknown. These analyses indicate that NET2006/04 is not derived from the BTV-8 vaccine strain, which was used as part of the South African 'Group B' multivalent live attenuated vaccine (containing BTV-3, 8, 9, 10 and 11) in Bulgaria during 2000 (Panagiotatos, 2004; Savini et al., in press). However, there was some serological evidence indicating BTV-8 infections of sentinel animals in Bulgaria during 2006, even though attempts to isolate or detect the virus by RT-PCR were unsuccessful (OIE, 2006b).

The situation that has occurred in Europe since 1998 is unique, with the introduction of multiple field strains from western (BTV-1, 2, 4, 8) and eastern (BTV-1, 9, 16) lineages. Live attenuated monovalent 'vaccine' strains of BTV-2, 4, 8, 9 (western group) and BTV-16 (eastern group) have also been used in the Mediterranean region, in attempts to minimise virus circulation. The release of these vaccine strains, some of which (including BTV-2 and 16) have persisted in the field, has added further genetic diversity generating an unprecedented mix of field and vaccine strain viruses, from both eastern and western lineages within southern Europe. This has provided multiple opportunities for the exchange/reassortment of genome segments between different strains, as already reported between strains of BTV-2 and 16 (Batten et al., in press; Monaco et al., 2005).

Genome segments from European and (selected) other BTV strains from around the world were compared. Variations were detected in each of the segments that correlate with their geographic origins, dividing them into eastern and western topotypes/groups (Fig. 6 — Supplementary data). All of the genome segments of NET2006/04 grouped with other 'western' viruses. The east/west grouping appears to be a dominant characteristic of the *Orbivirus* phylogeny, supporting observations previously made for Seg-3 of BTV and EHDV (Cheney et al., 1995; Gould and Pritchard 1991; Pritchard et al., 1995).

The BTV outer capsid proteins VP2 and to a lesser extent VP5 (encoded by genome segments 2 and 6) interact with neutralising antibodies and are the most variable of the BTV proteins/genome segments (Fig. 6 — Supplementary data) (Cowley and Gorman 1989; Huismans et al., 1987; Maan et al., 2007a; Singh et al., 2004). Sequencing studies and phylogenetic comparisons of Seg-2 from different BTV isolates can be used to identify the 24 BTV serotypes (Maan et al., 2007a) and provide a basis for serotype-specific RT-PCR assays (Mertens et al., 2007f, 2007g). These methods were used successfully to identify different BTV types

that have invaded Europe and the USA over the last 10 years (Johnson et al., 2000, 2007; Mertens et al., 2007f,g; Potgieter et al., 2005; Zientara et al., 2006). In the studies described here, only Seg-2 and Seg-6 showed variations that correlate with virus serotype, confirming the initial identification (by RT-PCR) of NET2006/04 as BTV-8. BTV-8 has previously been isolated from Kenya, Nigeria, South Africa, South and Central America (Mo et al., 1994), and India (Daniels et al., 2004; Prasad et al., in press). Seg-2 of NET2006/04 was most closely related to BTV-8 from Nigeria in 1982 (NIG1982/07), indicating that the northern European strain originated in sub-Saharan Africa. It also showed a close relationship in the more conserved genome segments with the Corsican strains of BTV-2 or BTV-4 (2000 onwards), which were also recently derived from an African lineage.

Seg-3 showed up to 99.8% nt similarity between the BTV-2 strains from Corsica and mainland Italy, with BTV-4 strains from Morocco and Spain indicating that (despite belonging to different serotypes) these strains all share Seg-3 from a recent common ancestor, providing evidence of genome segment exchange/reassortment. Despite also belonging to different serotypes, Seg-5 from the 1999 strain of BTV-16 from Greece (GRE1999/13) is also very similar to BTV-9 from Bulgaria (99.9%) indicating that they have also been involved in a recent reassortment event.

The clear separation of the majority of the BTV genome segments into eastern and western groups indicates that the viruses in these different regions have been separated and have acquired point mutations over a relatively long period of time, with little or no mixing or exchange between them. The existence of distinct eastern and western topotypes of Seg-2 and Seg-6, within individual BTV serotypes, also suggests that these different 'types' diverged and became genetically distinct as an initial step. Individual strains or lineages of each type subsequently became separated (geographically), allowing them to accumulate point mutations, and generating distinct topotypes within the each serotype.

Genome Seg-7 and Seg-10 also showed major variations that do not simply reflect the geographic origins of the virus isolate (Fig. 6 — Supplementary data). Seg-10 is the fourth most variable segment of the virus strains compared here, which separated into 4 distinct groups, two eastern and two western (although one of the eastern groups is represented by only a single strain of BTV-15 from China — Nikolakaki et al., 2005). The significance of these variations in Seg-10 remains uncertain, however they may relate to the role of NS3/NS3a in the release of progeny virus particles from the host cell (Hyatt et al., 1989). In a recent extensive study of sequence variations in BTV Seg-10, Balasuriya et al. (2008) identified two main groups (A and B) with a number of sub-clades. Some of these groups (clades A4, B1 and B3) containing viruses from the Americas, and the BTV-9 vaccine strain, were not included in the study presented here. However, sub-clades A1, A2–A3 and B2 appear to be equivalent to eastern group1, western group 2 and western group 1 respectively. The Chinese isolate of BTV-15, which forms the eastern group 2 identified here, was not included in the study by Balasuriya et al. (2008), although it appears likely that it would form a further distinct clade or sub-clade.

Wilson et al. (2000) compared BTV Seg-7 sequences, primarily from western isolates, showing several distinct clades that appear to form three distinct groups. These authors also identified two eastern strains that did not fit within these western groups. The sequences compared here indicate that Seg-7 is the third most variable of the BTV genome segments (Fig. 6 — Supplementary data). Seg-7 separated into six distinct groups, three of which fit with the groups described by Wilson et al. (2000), containing mainly western viruses (with the exception of BTV-12 from China, which grouped along with NET2006/04 within western group 1). The three other groups all contained eastern viruses. However, unlike Seg-7, Seg-10 of BTV-12 from China groups with other eastern strains, suggesting that this virus acquired a 'western' Seg-7 by reassortment, possibly from an exotic strain of the virus introduced into the region.

The data presented here provide the first complete sequence (segments 1–10) of the northern European strain of BTV-8. Although subsequent data for other isolates from the European outbreak in 2006 to 2008 have detected small numbers of nucleotide changes in several genome segments, they confirm that the strains were all closely related, representing a single virus lineage. Comparisons of NET2006/04 with the sequences of subsequent isolates will be used to track virus movements at different stages (years) of the outbreak. These comparisons will also help to identify any subsequent reassortment events involving this virus strain, and help to identify other related strains from different locations.

Materials and methods

Virus isolates, virus isolation and propagation in cell culture

Virus isolates from the reference collection at IAH Pirbright are referred to by their reference collection number, with the generic format 'three letter country code' year of isolation/isolate number for that year. Table 1 (supplementary data) gives details concerning the country of origin, year of isolation and EMBL/GenBank accession numbers for the isolates used in this study.

Blood samples (containing EDTA) taken from pyrexic animals at the start of the 2006 Netherlands outbreak of BT, were provided by Dr. Eugène van Rooij — CIDC-Lelystad, Netherlands. A 3.0 ml aliquot of each EDTA blood sample was washed three times with 10 ml of sterile phosphate-buffered saline (PBS). The red blood cells (RBC) were centrifuged at 3000 ×*g* for 5 min at 4 °C and the supernatant discarded. After the final wash, the RBCs were resuspended in 3.0 ml of PBS. RNA was extracted from 1.0 ml of the washed RBC for RT-PCR. 1.0 ml of the washed RBC was injected into embryonated chicken eggs. The harvested material was designated BTV8-E1. The remainder of the washed RBC were used for virus isolation in *C. sonorensis* (KC) cells (originally provided by colleagues at the USDA lab in Laramie, Wyoming). At 7 dpi KC culture supernatants (inoculated with sample number A83/06-6, from 'sheep number 6') was used to infect BHK-21 cells. The resulting virus isolate (NET2006/04–KC1/BHK1) was used for sequencing studies. Further details concerning the origins and passage history of individual virus isolates can be found at Mertens et al. (2007d).

Isolation and purification of nucleic acids

RNA was extracted from cell-free supernatants, or EDTA treated blood samples, using the QIAamp Viral RNA Mini Kit (QIAGEN) as per manufacturer's protocol, The RNA required for synthesis of full-length cDNA copies of genome segments, for sequencing (Attoui et al., 2000; Maan et al., 2007a,b), was purified from infected monolayers using Trizol® (Invitrogen), in accordance with manufacturer's instructions.

Identification and typing of the Netherland isolate (NET2006/04)

RNA from cell-free supernatants or EDTA treated blood samples, were used in RT-PCR assays. Serogroup-specific real-time RT-PCR assays targeting Seg-1 were carried out as described by Shaw et al. (2007). Conventional serogroup-specific RT-PCR assays targeting Seg-7 (Anthony et al., 2007), and serotype-specific assays (targeting Seg-2: Mertens et al., 2007f,g), were performed using a single tube RT-PCR method following the "One-Step RT-PCR kit" protocol (Qiagen, Courtaboeuf, France). Five microlitres of each RT-PCR was analyzed by electrophoresis on a 1% agarose gel.

Full length cDNA synthesis and amplification by PCR

Purified viral dsRNA was used for cDNA synthesis as described by Maan et al. (2007a,b). Briefly, ~400 ng of dsRNAs were ligated to a single-stranded anchor-primer using T4 RNA ligase (NEB) at 4 °C for

12 h. The oligo-ligated dsRNAs of BTV-8 isolate (NET2006/04) were converted to full length cDNAs using the AMV reverse transcription system (Promega) at 37 °C for 1 h. The DNA segments were then purified by agarose gel electrophoresis (AGE) and recovered from the gel as four pools: large genome segments (Seg-1, tube 1; Seg-2 and Seg-3, tube 2); medium (Seg-4 to 6, tube 3) and small (Seg-7 to10, tube 4). PCRs were performed on the cDNAs using the 'Triple Master PCR system' (Eppendorf) with a single primer complementary to the anchor-primer: denaturing at 95 °C for 30 s, then annealing and extension at 68 °C for 3 min, for 30 cycles.

Sequence determination of genome segments

Full length cDNA amplicons were generated (in four separate reactions: tubes 1 to 4) for all 10 genome segments of NET2006/04. Each cDNA was purified by 1% TAE agarose gel electrophoresis (AGE), excised from the gel and purified using a GFX™ PCR DNA and gel band purification kit' (Amersham Pharmacia Biotech, Inc) as per the manufacturer's instructions. The purified cDNA of each amplicon was used for direct sequencing, in both directions, using BTV-specific 'phased-primers' (Maan et al., 2007b) and '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, generating full-length contiguous sequences of all 10 genome segments.

Sequence analysis

Consensus sequences from each segment were assembled and analyzed using SeqMan Software (DNAStar Inc.). All sequence alignments were performed with the CLUSTAL W software (Thompson et al., 1994). Phylogenetic trees were constructed by neighbour-joining using distance matrices generated by the p-distance determination algorithm in MEGA 3 (2000 bootstrap replicates) (Kumar et al., 2004). Sequence relatedness is reported as percentage identity. The sequences obtained for each genome segment has been submitted to EMBL and the accession numbers obtained are AM498051 to AM498060 (Table 1). For accession numbers of previously published sequences see: Mertens et al. (2007a) and Table 1 — supplementary data.

Acknowledgments

The authors are very grateful to Kasia Bachanek-Bankowska, Abid Bin-Tarif and Lydia Kgosana from the CRL for their help with ELISA and virus isolation. The authors also wish to thank Camille Szmaragd and Anthony Wilson for information concerning the impact of the outbreak and useful discussion. Financial support was provided by DEFRA, the European Commission, The Wellcome Trust, The Commonwealth Commission and BBSRC. The authors thank international colleagues who provided virus isolates for analyses and comparisons.

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.virol.2008.04.028.

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