Molecular epidemiology of bluetongue viruses from disease outbreaks in the Mediterranean Basin

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Summary

Bluetongue virus (BTV) serotype is primarily controlled by the variable outer coat protein VP2, encoded by genome segment 2. Phylogenetic analyses of segment 2 show that recent Mediterranean isolates of BTV-2 have a similar genetic lineage to those from sub-Saharan Africa and North America but are distinct from Asian strains. In contrast, isolates of BTV-9, from the eastern Mediterranean, are related to a genetic lineage from Asia. BTV-1 from Greece 2001 is also more closely related to Indian isolates, suggesting (in both cases) virus movement from east to west. Recent BTV-4 field isolates from Greece and Turkey are similar to each other, but differ from the Turkish type 4 vaccine strain. These sequencing studies are being used to establish a database for molecular epidemiological studies which is available on the website of the Institute for Animal Health. This resource will support and improve BTV serotype identification methods, by using sequence comparisons (via the Web) rather than by conventional serological techniques that require standardised (and therefore expensive) serological reagents. Phylogenetic trees for BTV genome segment 2 are available on the website.

Keywords

Bluetongue virus – *Orbivirus* – Phylogenetic analysis – Segment 2 – Serotype – Viral protein 2 – Virus.

Introduction

Prior to 1998, Europe had been largely free of bluetongue (BT) disease, apart from sporadic and limited epizootics caused by single bluetongue virus (BTV) types that were restricted to areas of the Iberian Peninsula (below 40°N), or to some of the Mediterranean islands (27, 28, 29). The distribution of disease outbreaks in southern Europe (both BT and African horse sickness) mirrored the distribution and abundance of the major vector species *Culicoides imicola* (27).

However, since 1998, five BTV types (1, 2, 4, 9 and 16) have caused outbreaks across much of southern Europe, collectively representing the largest epizootic of BT ever recorded. Morbidity and mortality levels are estimated in some areas at 18% and 3.4%, respectively, and overall losses already exceed 500 000 sheep. The virus and disease have spread northwards into new areas of Europe (Bulgaria, Serbia and Croatia) that are beyond the range of *C. imicola*, suggesting the involvement of novel insect vector species (26). Indeed, the virus has recently been isolated in several areas, from both *C. obsoletus* and *C. pulicaris* (species that are abundant across much of northern Europe). These observations, together with the projected effect of global climate change on the distribution and activity of vector species, and the existence of an overwintering mechanism for BTV (33, 34), suggest that the virus will persist and may continue to spread northwards. Additional areas of Europe must therefore also be considered to be 'at-risk' from the disease.

BTV serotype is primarily controlled by the variable outer coat protein VP2 (encoded by segment 2 of the virus genome), which determines the specificity of interactions between neutralising antibodies and the virus particle. Sequence variations in genome segment 2 consequently reflect variation in virus 'type'. The results from sequence analyses of BTV genome segment 2 are being used to establish a

database for molecular epidemiological studies (22, 25). This will provide a resource to support and improve BTV serotype identification by sequence comparisons (via the Web), rather than by conventional serological techniques that require standardised (scarce and expensive) serological reagents. More rapid and accurate BTV typing and strain identification techniques will also provide valuable information concerning the route and direction of virus spread, which cannot be obtained from conventional serological typing assays. It is anticipated that such information will be of considerable importance to achieve a full understanding of the disease and to combat transmission that may occur as a result of 'climate change' and during the increasingly complex movement of animals in the livestock trade.

This study presents the results of a phylogenetic analysis of the five BTV types that have caused outbreaks in the Mediterranean Basin in recent years. Field strains of each type were compared to viruses of the same type, isolated at different times and from different geographical origins.

Materials and methods

Strains used in this study are shown in Table I. More information concerning the strains used and those stored in the reference collection at the Institute for Animal Health in Pirbright can be found on the website (23).

Oligonucleotide primers for sequencing used in the study are shown in Table II. A list of primers (which will be periodically updated) that can be used to amplify genome segment 2 of BTV can also be found on the IAH website (24).

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 (17). An anchor-primer sequence, phosphorylated at the 5´ end, with a C9 spacer connecting two partially complementary halves (S. Rao, manuscript in preparation), was ligated to whole viral dsRNA. The 10 µl ligation reaction had 1 µg of viral dsRNA, 20 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 primer by 1% agarose gel electrophoresis and segments 2 and 3 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 carried out 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 to be 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 analysis were performed using Orf Finder and Clustal X (version 1.81).

Results

Comparisons of genome segment 2 sequences from different field and vaccine strains belonging to the five BTV types examined (1, 2, 4, 9 and 16) demonstrate that they cluster into five distinct and type-specific groups (Fig. 1). Although significant variation was detected between isolates of the same type within each of these clusters, some isolates also showed significantly closer relationships. For example, within the BTV-1 cluster, the strains that were analysed from India all appear to be closely related, suggesting a recent common ancestry. These isolates were also more closely related to the BTV-1 strains from Australia and Malaysia, than to those from Africa (Nigeria, Sudan and Cameroon), indicating diversity between 'eastern' and 'western' isolates. The BTV-9 isolates from outbreaks in Europe are more closely related to strains from the East (Australia and Indonesia) than to South African reference strains, as are the BTV-16 isolates. BTV-4 isolates from Greece and Turkey are clustered (within the serotype) with African reference strains and are distinct from the published sequence of a Chinese isolate. This probably reflects the western group of viruses. The same effect is noticeable for BTV-2, the European field isolates are more closely related to the Western group represented by isolates from Africa.

Discussion

In October 1998, there were outbreaks of bluetongue (caused by BTV-9) on four Greek islands close to the Anatolian coast of Turkey (Rhodes,

Table I
Details of virus isolates used for sequencing studies of segment 2 of bluetongue virus serotypes 1, 2, 4, 9 and 16

Serotype and country of origin	Species	IAH dsRNA virus reference collection no. *Published data*	Accession No.
BTV-1 South African vaccine strain (OVI)	Not known	RSAvvvv/01	AJ585110
BTV-1 South African reference strain (OVI)	Not known	RSArrrr/01	AJ585122
BTV-1 India (Rajasthan)	Ovine	IND1992/01	AJ585111
BTV-1 India	Not known	IND1992/02	AJ585112
BTV-1 India	Not known	IND1988/01	AJ585113
BTV-1 India (Haryana)	Ovine	IND1999/01	AJ585114
BTV-1 India (Chennai)	Ovine	IND2001/01	AJ585115
BTV-1 Malaysia (Kuala Lumpur)	Not known	MAY1987/01	AJ585116
BTV-1 Sudan	Not known	SUD1987/01	AJ585117
BTV-1 Nigeria	Not known	NIG1982/01	AJ585118
BTV-1 Cameroon	Ovine	CAR1982/01	AJ585119
BTV-1 Australia	Not known	AUS??/01	AJ585120
BTV-1 Australia	Not known	AUS1981/01	AJ585178
BTV-1 Greece	Ovine	GRE2001/01	AJ585121
BTV-1 Australia	*Not known*	*(37)*	*X06464**
BTV-1 Australia	*Not known*	*(20)*	*M21844**
BTV-1 Australia	*Not known*	*(21)*	*X55800**
BTV-1 China	*Not known*	*(18)*	*AF135217**
BTV-2 South African reference strain (OVI)	Not known	RSArrrr/02	AJ585123
BTV-2 India	Not known	IND1982/01	AJ585152
BTV-2 Nigeria	Not known	NIG1982/02	AJ585153
BTV-2 Corsica	Ovine	FRA2001/03	AJ585154
BTV-2 Sudan	Not known	SUD1985/01	AJ585155
BTV-2 Tunisia	Not known	TUN2000/01	AJ585156
BTV-2 South African vaccine strain (OVI)	Not known	RSAvvvv/02	AJ585157
BTV-2 Sardinia	Ovine	SAD2001/01	AJ585161
BTV-2 Sardinia	Ovine	SAD2001/02	AJ585162
BTV-2 Sicily	*C. pulicaris*	ITL2002/05	AJ585158
BTV-2 Sicily	*C. pulicaris*	ITL2002/06	AJ585159
BTV-2 Sicily	Ovine	ITL2002/07	AJ585160
BTV-2 Zimbabwe (Lone Oak)	*C. imicola*	ZIM2003/01	N/A
BTV-2 Corsica	*Not known*	*Bréard et al. 2002, unpublished*	*AF356601**
BTV-2 USA	*Not known*	*(37)*	*M21946**
BTV-2 South African vaccine strain	*Not known*	*Bréard et al. 2002, unpublished*	*AF481096**
BTV-2 China	*Not known*	*(18)*	*AF135218**
BTV-4 South African reference strain (OVI)	Not known	RSArrrr/04	AJ585125
BTV-4 South African vaccine strain (OVI)	Not known	RSAvvvv/04	AJ585163
BTV-4 Turkey vaccine strain	Not known	TURvvvv/04	AJ585164
BTV-4 Turkey	Not known	TUR1978/01	AJ585165
BTV-4 Sudan	Not known	SUD1983/01	AJ585166
BTV-4 Greece	Not known	GRE2000/01	AJ585167
BTV-4 South African vaccine strain (OVI)	Not known	RSAvvv3/04	AJ585168
BTV-4 Argentina	Bovines	ARG2002/01	AJ585169
BTV-4 Cyprus	Bovine	CYP1969/01	AJ585180
BTV-4 China strain V413	*Not known*	*(18)*	*AF135220**
BTV-9 S. African reference strain (OVI)	Not known	RSArrrr/09	AJ585130
BTV-9 Bulgaria	Not known	BUL1999/01	AJ585170
BTV-9 Greece	Not known	GRE2000/02	AJ585171
BTV-9 Serbia	Ovine	SER2001/01	AJ585172
BTV-9 South African vaccine strain (OVI)	Not known	RSAvvv1/09	AJ585173
BTV-9 Bosnia	Ovine	BOS2002/02	AJ585174
BTV-9 Turkey	Not known	TUR2000/03	AJ585175
BTV-9 Turkey	Not known	TUR2000/04	AJ585176
BTV-9 Turkey	Not known	TUR2000/05	AJ585177
BTV-9 Australia strain DPP836	*Not known*	*(30)*	*L46686 (P)**
BTV-9 Greece (Chalkidiki)	*Ovine*	*S. Zientara personal communication**	*N/A*
BTV-9 Indonesia (ISA88-AA)	*Not known*	*L.I. Pritchard, personal communication***	*P*
BTV-9 Indonesia (ISA91-AB)	*Not known*	*L.I. Pritchard, personal communication***	*P*
BTV-16 S. African reference strain (OVI)	Not known	RSArrrr/16	AJ585137
BTV-16 S. African vaccine strain (OVI)	Not known	RSAvvvv/16	AJ585149
BTV-16 Nigeria	Not known	NIG1982/10	AJ585150
BTV-16 Turkey	Not known	TUR2000/01	AJ585146
BTV-16 Turkey	Not known	TUR2000/02	AJ585147
BTV-16 Turkey	Not known	TUR2000/10	AJ585148
BTV-16 Indonesia	Not known	ISA1991/01	AJ585151
BTV-16 South African strain	*Not known*	*Bréard et al. unpublished*	*AF530067**
BTV-16 China strain SW	*Not known*	*(18)*	*AF135222**
BTV-16 Australian strain DPP965	*Not known*	*(29)*	*L46683**

N/A not available
OVI Onderstepoort Veterinary Institute
P partial sequence
* published sequences used for sequence comparisons are shown in italics
** personal communication

Table II
Primers used for sequencing segment 2 of bluetongue virus serotypes 1, 2, 4, 9 and 16

Primer designation	Orientation	Primer sequence (5′- 3′)	Effectiveness for sequencing
BTV-1/2/p368R	Antisense	GGATATGGRTTYGACCACCA	+++
BTV-1/2/p207F	Sense	AGGAGAGATCSGAGTCA	+++
BTV-1/2/p207R	Antisense	TGACTCSGATCTCTCCT	+++
BTV-1/2/p337F	Sense	AATCAACGTAGAGATGARGA	+++
BTV-1/2/p529F	Sense	GCWCCRATGTTYAAYGCTAA	+++
BTV-1/2/p620F	Sense	AAGAAACCCTGYCCGACTGT	+++
BTV-1/2/p797F	Sense	GAATAYATGGTTTTCTTTCC	+++
BTV-1/2/T-F	Sense	GTTAAAATAGTRKCGCGATGGATGAG	+++
BTV-1/2/T-R	Antisense	GTAAGTMTRATAGYGCGCGGA	+++
BTV-1/2/iF-413F	Sense	TATATGCCTTATGARAARTCAAGGAGAACAAG	+++
BTV-1/2/iR-614R	Antisense	GTGGAYTGTGCCTGTTGCCGCGAAAGCGCCTG	+++
BTV-2/2/p200R	Antisense	ACGCTCTCTCYGAATGCACTATTAA	+++
BTV-2/2/p302R	Antisense	CTTCATCDACBGGTTCCGC	+++
BTV-2/2/p302F	Sense	GCGGAACCBGTDGATGAAG	+++
BTV-2/2/p558R	Antisense	TCATYAGTCATCGGTTTRAG	+++
BTV-2/2/p558F	Sense	CTYAAACCGATGACTRATGA	+++
BTV-2/2/p845R	Antisense	GGTKGAAACAACRTTMAAATT	+++
BTV-2/2/p845F	Sense	AATTTKAAYGTTGTTTCMACC	+++
BTV-2in/p883F	Sense	GATTGGTATCCTACCTAGAG	+++
BTV-2/2/T-F	Sense	GTTAAAACAGGATCGCGATGGATG	+++
BTV-2/2/T-R	Antisense	GTAAGTTGAACAGATCGCGGACCTG	+++
BTV-2/2/iF-200F	Sense	TTAATAGTGCATTCRGAGAGAGCGT	+++
BTV-2/2/iR-600R	Antisense	CCTTCGCGTCAAATGTTGATTGAGT	+++
BTV-4/2/Fpp106R	Antisense	TTCATCCACTTAGCATCCGTC	+++
BTV-4/2/p106F	Sense	GACGGATGCTAAGTGGATGAA	+++
BTV-4/2/p233F	Sense	GGTCTATCATATAACAGATT	+++
BTV-4/2/Fpp876F	Sense	GATAAGCTCGACCCGCA	+++
BTV-4/2/p876R	Antisense	TGCGGGTCGAGCTTATC	+++
BTV-4/2/p474F	Sense	GTGTAAGATAGATGATGAG	+++
BTV-4/2/p841R	Antisense	AACTTGGACGTCACAACAGG	+++
BTV-9/eup245F	Sense	ATAGATATGAGCGATCCGG	+++
BTV-9/2/eup638R	Antisense	CTGGTCCACATGGTAGATC	+++
BTV-9/2/Eup864R	Antisense	CATCCGATAGACCGCCAC	+++
BTV-9/2/euFp864F	Sense	GTGGCGGTCTATCGGATG	+++
BTV-9/2/Fpp50R	Antisense	TCACCATCTGGCACATC	+++
BTV-9/2/p50F	Sense	GATGTGCCWGATGGTGA	+++
BTV-16/2/Fpp151R	Antisense	ATCGTATGATATGACGTGG	+++
BTV-16/2/p151F	Sense	CCACGTCATATCATACGAT	+++
BTV-16/2/p393F	Sense	GACTGCAAGCTCGGTGACG	+++
BTV-16/2/p745R	Antisense	GCGAGTCCGACACCTCAAG	+++
BTV-16/2/Fpp745F	Sense	CTTGAGGTGTCGGACTCGC	+++

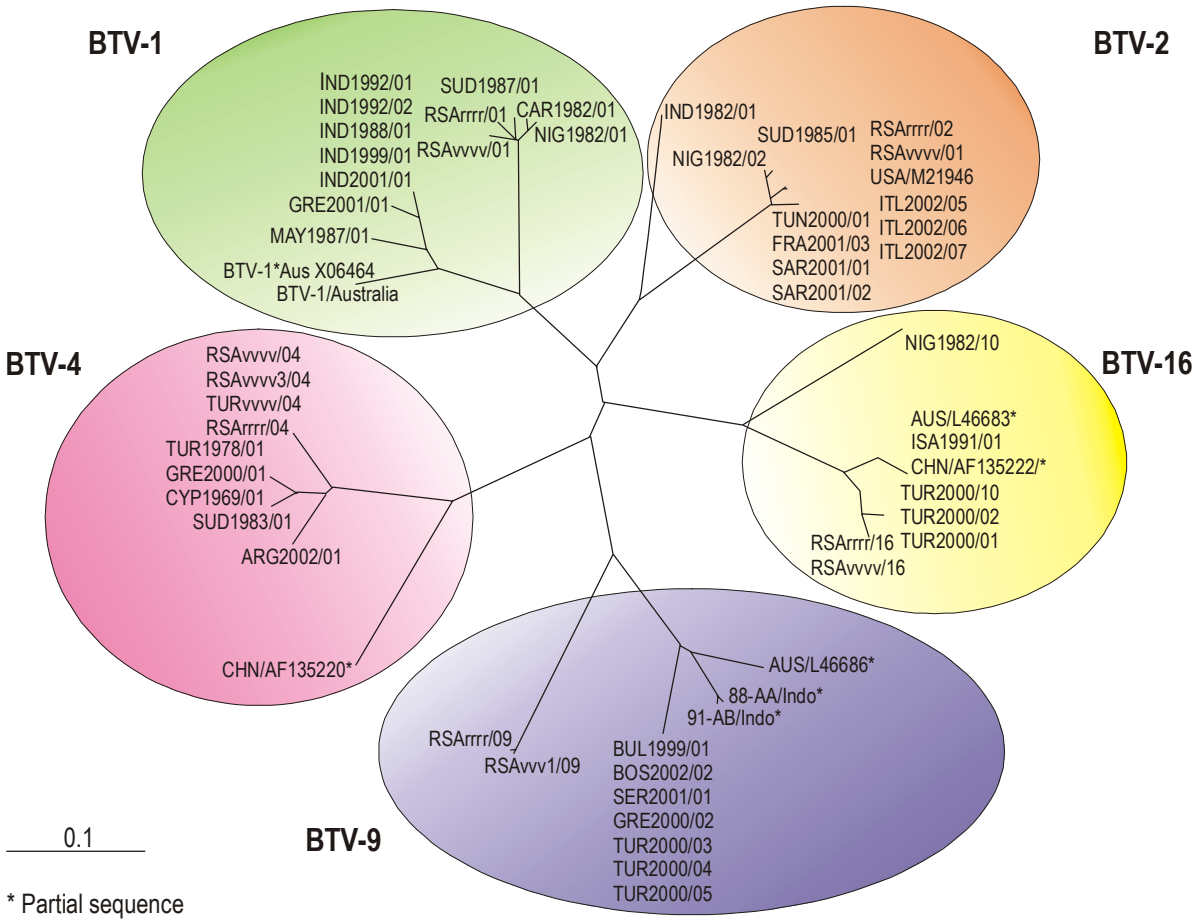

Figure 1
Neighbour-joining (unrooted) tree showing relationships between full length genome segment 2 (VP2 gene) of the five European serotypes of bluetongue virus
The tree was generated using Clustal X (version 1.81) with the default parameters
Published sequences used for sequence comparisons are shown in italics in Table I

Leros, Kos and Samos) (1, 2). This was the first time that BTV-9 had been recorded in Europe, although serological evidence had previously identified the serotype in Turkey (35). After December 1998, no further outbreaks of BT were recorded until June 1999 when BTV-9 was again identified as the cause of outbreaks in south-east Bulgaria, which persisted until October 1999 (3, 4). However, in July 1999 the Turkish authorities also reported a BTV incursion into European Turkey involving animals in those provinces bordering mainland Greece and Bulgaria (4). The Turkish veterinary authorities vaccinated some 60 000 sheep with a BTV-4 vaccine (19) although it was later confirmed that the outbreaks were caused by BTV-9. In August 2000, BTV-16 was also reported from outbreaks in the Province of Izmir, Turkey (8).

In August 1999 the Greek authorities reported BTV on mainland Greece, initially adjacent to the Bulgarian and Turkish borders, although the virus subsequently spread in a westerly direction across northern Greece, as far as Thessaloniki and Larisa (4). Outbreaks were reported until December 1999 and again in the summer of 2000. Three serotypes were found to be responsible for the Greek outbreaks of 1999-2000, BTV-4, BTV-9 and BTV-16. BTV-4 was initially reported near the Turkish border but was later recorded more widely. Initially the BTV-4 outbreaks were viewed with some suspicion because of the use of the live-attenuated BTV-4 vaccine in Turkey. However, BTV-4 has previously been recorded in a number of countries further to the east (e.g. Syria, Jordan Israel and Turkey) (35) and sequence comparisons indicate that the Turkish vaccine strain was not the cause of the European outbreak. In September 2001, BT was again confirmed in north-western Greece, close to the Albanian border and subsequently in central Greece (16). There were also reports form Serbia, Montenegro, Kosovo, Macedonia, Bulgaria and Croatia (10, 13, 14, 15, 16).

Outbreaks of bluetongue were also reported from the south-western Mediterranean Basin during 2000. Tunisia reported BT for the first time in January

2000 (6). Further outbreaks were reported in Tunisia in June and in Algeria during July of 2000 (7, 9). Most of the Algerian outbreaks were close to the Tunisian border, until September 2000, when BTV was confirmed in areas as far as 250 km further west (8). In late 2000, BTV-specific antibodies were found in samples from animals in Algiers and northern Morocco (27). In late August 2000, BTV-2 was found on the Italian island of Sardinia (8) and by October, it had been confirmed on Sicily and in Calabria (southern mainland Italy) (5). The BT outbreaks in Sardinia were severe and 90 000 sheep died, either as a result of disease or control measures (5). BTV-2 was reported on the French island of Corsica during October 2000 and the Spanish islands of Menorca and Mallorca up until December 2000 (8, 11). In September 2001 BT was reported in Corsica, Sardinia and Calabria (southern Italy) (12) and also from north of Rome in Lazio and Tuscany. The outbreaks in Corsica were caused by BTV-2 but those in Calabria were BTV-9.

In 2002, outbreaks continued around the Mediterranean Basin. During the period from June to August there were outbreaks on mainland Italy attributable to BTV-2, BTV-9 and BTV-16. Sicily had outbreaks of BTV-2 and BTV-9. Sardinia had outbreaks due to BTV-2 and Calabria had BTV-16. In the Balkan states between August and December, there were outbreaks of BTV-9 in Bosnia, Bulgaria, Montenegro, Yugoslavia and Albania and an unconfirmed report of BTV in Kosovo. During October 2003, BTV-4 was confirmed in Sardinia then suspected in Corsica, and confirmed in the Balearic islands.

Phylogenetic analysis of genome segment 2 from several different isolates of BTV types, that were currently causing disease within Europe, demonstrated that they could be separated into five distinct groups, which accurately reflect virus serotype (Fig. 1). Significant but lower levels of sequence variation were also apparent between individual isolates of the same type, within each of these groups. These differences were greatest between isolates from geographically distant locations, and there was clear evidence of an East-West divide, particularly for isolates of types 1, 2 and 9. The BTV-1 isolate from Greece (GRE2001/01) was shown to be more closely related to a group of eastern BTV-1 strains, particularly those from India, indicating that it may have eastern origins. It is considered unlikely that this European BTV-1 was introduced directly from India, and although there was no evidence of BTV-1 infection in Turkey during a serological survey in the early 1980s (35), it is considered likely that it originated from eastern Turkey or the Middle East.

The cluster of BTV-4 isolates contained strains representing Africa, South America, Turkey and China. The overall diversity between most of these BTV-4 isolates was quite low, suggesting that they represent a western grouping and may even reflect the spread of a strain related to, but not identical to, the South African reference or vaccine strain. However, the Chinese isolate showed higher levels of diversity, again suggesting that it belongs to a distinct Far Eastern group.

Sequence comparisons of S2 of BTV-9 strains showed that all of the isolates from recent BTV-9 outbreaks in Europe are grouped together and are almost identical but quite diverse from South African reference or vaccine strains. They belong to the same genetic lineage as those from Australia and Indonesia suggesting an 'eastern' origin. BTV-9 have been reported in Anatolian Turkey, Syria, Jordan and Israel over a number of years (35). It seems likely that these strains have spread in a westerly direction moving into Europe, causing the current outbreaks of BTV-9. However, sequence data for genome segment 2 of other viruses from this and other regions, would be required to fully verify this hypothesis.

The studies of BTV-16 did not include a sufficiently wide selection of virus isolates to exhibit the same phenomena (an east-west split). The BTV-16 isolates from Turkey form a closely related group, as would be expected from isolates from the same year that may all be directly related to each other. They also show a very close relationship to the South African reference and vaccine strains. The strain from Nigeria is less closely related to other isolates of BTV-16, showing a level of divergence similar to that observed between the eastern/western strains of BTV-1, 2 and 9. However, due to the small numbers of isolates available for study, the significance of this difference is still to be confirmed.

Nucleotide sequence comparisons show that BTV-2 isolates from recent outbreaks in the Mediterranean Basin are more closely related to viruses from Nigeria, Sudan and South Africa and this may reflect a sub-Saharan origin. Although the Sahara represents an effective physical barrier to the movement of both insect and mammalian species, and therefore the spread of disease, foot and mouth disease virus (FMDV) isolated form outbreaks of disease in Algeria, Morocco and Tunisia during 1999, was shown to have a sub-Saharan origin (32). It was suggested that transportation of FMDV-infected Zebu cattle across the Sahara in late 1999 may have provided an entry route to North Africa. BTV appeared in Tunisia at around the same time and it is possible that it was transported as a sub-clinical

infection of the same cattle. However, FMD epidemics have also been caused in North Africa by movement of sheep from the Middle East, during periods of religious festivals such as Aid-el-Kebir (31, 35) types 2, 4, 6, 9 and 13 in Syria and Jordan and types 2, 4, 9, and 13 were recorded in Turkey during the early 1980s. Although BTV isolates of Middle Eastern origin were not available for this study, it is also possible that BTV-2 could have spread from the Middle East (Syria, Jordan, Israel) across North Africa to Tunisia. Previous studies have also recorded the movement of BTV as the result of wind-borne spread of infected midges. It is not possible to rule out this route as an important factor in the origin and spread of the current European outbreaks.

Once an arthropod-borne disease, like BT, is established within the mammalian host-population and competent vector species are present (as in the Mediterranean Basin) outbreaks may occur in successive years. This is particularly true, if a mechanism exists for virus survival from one 'vector-season' to the next (BTV-overwintering) (33, 36). At present, only live-attenuated vaccines are commercially available for disease control measures. A monovalent, live-attenuated BTV-2 vaccine was used successfully in Corsica and the Balearic islands. However, pentavalent vaccines were also used in Bulgaria but did not stop the spread of BTV-9. In Italy, a BTV-2 monovalent vaccine and subsequently bivalent BTV-2 and BTV-9 vaccines have so far failed to prevent re-occurrence of the disease. The use of live vaccines poses an as yet unquantified risk of transmission of the vaccine strain. Vaccination in the face of an outbreak may also allow genome segment reassortment to occur between the vaccine and field virus, increasing the genetic diversity within the virus population, and potentially generate a virus strain that has novel biological properties.

The introduction of five serotypes of BTV into the Mediterranean region and the occurrence of outbreaks at latitudes further north than previously described, is a stark reminder that the epidemiology of exotic diseases needs to be closely monitored. The absence of the major European vector species (*C. imicola*) from the Balkan region indicates the involvement of novel vector species, possibly *C. obsoletus* and/or *C. pulicaris*. If future control measures for BTV and other related viruses are to be effective it is essential that the way in which they enter the region is full understood. Phylogenetic analysis of sequence data is a powerful tool that can be used to identify virus strains accurately and pinpoint where these introductions come from. Access to an adequate collection of contemporary virus isolates from different regions and an open exchange of data is essential for the continuation and success of these studies. Completed sequence data will therefore be added to the international sequence databases as soon as possible. Other data will be disseminated via the dsRNA virus website of the IAH.

References

1. Anon. (1998). – Bluetongue in Greece: confirmation of diagnosis. *Dis. Info.*, **11**, 166-167.
2. Anon. (1998). – Disease outbreaks reported to the OIE in November-December 1998. *Bull. Off. Int. Épiz.*, **110**, 506.
3. Anon. (1999). – Bluetongue in Bulgaria: additional information. *Dis. Info.*, **12**,122.
4. Anon. (1999). – Disease outbreaks reported to the OIE July-August 1999. *Bull. Off. Int. Épiz.*, **111**, 379.
5. Anon. (2000). – Bluetongue in Italy: follow-up report no.1. *Dis. Info.*, **13**, 209-210.
6. Anon. (2000). – Disease outbreaks reported to the OIE January-February 2000. *Bull. Off. Int. Épiz.*, **112**, 16.
7. Anon. (2000). – Disease outbreaks reported to the OIE July-August 2000. *Bull. Off. Int. Épiz.*, **112**, 687-688.
8. Anon. (2000). – Disease outbreaks reported to the OIE September-October 2000. *Bull. Off. Int. Épiz.*, **112**, 552.
9. Anon. (2000). – Disease outbreaks reported to the OIE July-August 2000. *Bull. Off. Int. Épiz.*, **112**, 449.
10. Anon. (2001). – Bluetongue in Croatia: suspected outbreak. *Dis. Info.*, **14**, 262-265.
11. Anon. (2000). – Bluetongue in France: in the island of Corsica. *Dis. Info.*, **13**, 195-197.
12. Anon. (2001). – Bluetongue in Greece and Italy. *Dis. Info.*, **14**, 215-218.
13. Anon. (2001). – Bluetongue in Kosovo (FRY), territory under United Nations interim administration. *Dis. Info.*, **14**, 242-243.
14. Anon. (2001). – Bluetongue in the Former Yugoslav Republic of Macedonia: follow-up report no.1. *Dis. Info.*, **14**, 265-268.
15. Anon. (2001). – Bluetongue in Yugoslavia. *Dis. Info.*, **14**, 280-281.
16. Anon. (2001). – Bluetongue in Greece: follow-up report no. 2. *Dis. Info.*, **14**, 262-265.
17. Attoui H., Billoir F., Cantaloube J.F., Biagini P., de Micco P. & de Lamballerie X. (2000). – Strategies for the sequence determination of viral dsRNA genomes. *J. Virol. Methods*, **89**, 147-158.
18. Bonneau K.R., Zhang N., Zhu J. , Zhang F., Li Z., Zhang K., Xiao L., Xiang W. & MacLachlan N.J. (1999). – Sequence comparison of the L2 and S10 genes of bluetongue viruses from the United States

and the People's Republic of China. *Virus Res.*, **61**, 153-160.

19. European Commission (2000). – Possible use of vaccination against bluetongue in Europe: report of the Scientific Committee on animal health and animal welfare (V. Moennig, chairman) (Sanco/C3/AH/R19 /2000). European Commission, Brussels, 27 pp.
20. Gould A.R. (1988). – Conserved and non-conserved regions of the outer coat protein, VP2, of the Australian bluetongue serotype 1 virus, revealed by sequence comparison to the VP2 of North American BTV serotype 10. *Virus Res.,* **9**, 145-158.
21. Gould A.R. & Pritchard L.I. (1990). – Relationships amongst bluetongue viruses revealed by comparisons of capsid and outer coat protein nucleotide sequences. *Virus Res.*, **17**, 31-52.
22. Institute for Animal Health (IAH) (2004). – The RNAs and proteins of dsRNA viruses (P.P.C. Mertens & D.H. Bamford, eds). IAH, Compton (iah.bbsrc.ac.uk/dsRNAvirusproteins/ orbivirus-accession-numbers.htm accessed on 11 July 2004).
23. Institute for Animal Health (IAH) (2004). – ReoID. Phylogenetic sequence analysis and improved assay systems for viruses of the family *Reoviridae*. IAH, Compton (iah.bbsrc.ac.uk/ReoID/viruses-at-iah.htm accessed on 11July 2004).
24. Institute for Animal Health (IAH) (2004). – ReoID. Phylogenetic sequence analysis and improved assay systems for viruses of the family *Reoviridae*. BTV RT-PCR and sequencing primers. IAH, Compton (iah.bbsrc.ac.uk/ReoID/btv-S2-primers.htm accessed on 11 July 2004).
25. Maan S., Maan N.S., Samuel A.R., O'Hara R., Meyer A.J., Rao S. & P.P.C. Mertens (2004). – 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 *In* Bluetongue, Part II (N.J. MacLachlan & J.E. Pearson, eds). Proc. Third International Symposium, Taormina, 26-29 October 2003. *Vet. Ital.*, **40** (4), 484-488.
26. Mellor P.S. (2004). – Infection of the vectors and bluetongue epidemiology in Europe. *In* Bluetongue, Part I (N.J. MacLachlan & J.E. Pearson, eds). Proc.Third International Symposium, Taormina, 26-29 October 2003. *Vet. Ital.*, **40** (3), 167-174.
27. Mellor P.S. & Wittmann E.J. (2002). – Bluetongue virus in the Mediterranean Basin, 1998-2001. *Vet. J.*, **164**, 20-37.
28. Mertens P.P.C. (2002). – Bluetongue viruses. *In* Encyclopedia of life sciences, Vol. 13. Nature Publishing Group, London, 533-547.
29. Mertens P.P.C. & Mellor P.S. (2003). – Bluetongue. *State Vet. J.*, **13**,18-25.
30. Pritchard L.I. & Gould A.R. (1995). – Phylogenetic comparison of the serotype-specific VP2 protein of bluetongue and related orbiviruses. *Virus Res.*, **39**, 207-220.
31. Samuel A.R. & Knowles N.J. (2001). – Foot-and-mouth disease type O viruses exhibit genetically and geographically distinct evolutionary lineages (topotypes). *J. Gen. Virol.*, **82**, 609-621.
32. Samuel A.R., Knowles N.J. & Mackay D.K.J. (1999). – Genetic analysis of type O viruses responsible for epidemics of foot-and-mouth disease in North Africa. *Epidemiol. Infec.*, **122**, 529-538.
33. Takamatsu H.H., Mellor P.S., P.P.C. Mertens, Kirkham P.A., Burroughs J.N. & Parkhouse R.M.E. (2003). – A possible overwintering mechanism for bluetongue virus in the absence of the insect vector. *J. Gen. Virol.*, **84**, 227-235.
34. Takamatsu H.-H., Mellor P.S. & Mertens P.P.C. (2004). – A potential overwintering mechanism for bluetongue virus – recent findings *In* Bluetongue, Part II (N.J. MacLachlan & J.E. Pearson, eds). Proc. Third International Symposium, Taormina, 26-29 October 2003. *Vet. Ital.*, **40** (4), 456-461.
35. Taylor W.P. & Mellor P.S. (1994). – Distribution of bluetongue virus in Turkey, 1978-81. *Epidemiol. Infec.*, **112**, 623-633.
36. White D.M., Wilson W.C., Blair C.D. & Beaty B.J. (2004). – Possible overwintering mechanism of bluetongue virus in vectors. *In* Bluetongue, Part I (N.J. MacLachlan & J.E. Pearson, eds). Proc. Third International Symposium, Taormina, 26-29 October 2003. *Vet. Ital.*, **40** (3), 513-519.
37. Yamaguchi S., Fukusho A. & Roy P. (1988). – Complete sequence of VP2 gene of the bluetongue virus Australian serotype 1 (BTV-1). *Nucleic Acids Res.*, **16**, 2725.