Analysis and typing of BTV isolates from Morocco 2006

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Samples of suspect BTV material, which were sent to the BTV Community Reference Laboratory (CRL) at IAH Pirbright, by Mehdi El Harrak (Biopharma Laboratory, BP4569, Rabbat Akkari, Morocco) arrived on the 20th October 2006. These included two samples of a virus isolate designated 1- OV4 (passage1E3BSR and 1E4BSR), derived from the blood of a sheep showing clinical signs of BT, from a small village called Guenfouda near Oujda in the East of Morocco. The isolation was originally made in embryonated chicken eggs followed by passage in BSR cells (as indicated). These samples were passaged twice in BHK cells and have been entered in the CRL reference collection (see www.iah.bbsrc.ac.uk/dsRNA_virus_proteins/ReoID/btv-1.htm) as MOR2006/06 and MOR2006/07. Initial suspicions were that these isolates are BTV-1.
An EHDV isolate was also received (designated 2-451- passage1E3BSR [2 ampoules]). One sample was passaged twice in BHK cells and has been included in the CRL reference collection as MOR2006/05. The virus was originally isolated in Morocco in embryonated chicken eggs (followed by passage in BSR cells as indicated), from the blood of a bovine showing clinical signs of EHD, from near Ahfir in the East of Morocco.

RNA was extracted directly from the original samples (Qiagen Mini Viral RNA kit) and assayed by BTV group specific RT-PCR using the methods described by Anthony et al (2006). The two suspect BTV samples gave clear positive results, generating cDNA bands of the expected size when analysed by agarose gel; electrophoresis (figure 1). The two samples of the EHDV isolate gave a negative result. This confirmed that samples MOR2006/06 and MOR2006/07 contain BTV, while nor BTV was detected in MOR2006/05 (result achieved with 24 hours of receipt).

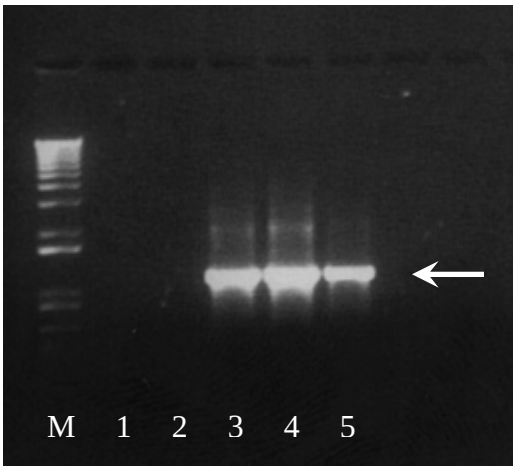

Figure 1 : RNA extracted from the original morocco samples amplified by RT-PCR using BTV serogroup specific primers targeted at genome segment 7 ('BTV-GSP/7/TF' and 'BTV-GSP/7/TR' which generate a product of 1156 bp - Anthony et al 2006): M = DNA marker ladder, lane 1 = EHDV RNA (isolate MOR2006/05, vial 1), lane 2 = EHDV RNA (isolate MOR2006/05, vial 2), lane 3 = RNA extracted from MOR 2006/06, lane 4 = RNA extracted from MOR 2006/07, lane 5 = RNA extracted from tissue cultures

infected with the northern European strain of BTV-8 (NET2006/01). The size of the expected amplification product from BTV SEG-7 is indicated by an arrow. These data confirm that isolates MOR2006/06 & 07 contain BTV.

The same RNA samples were also assayed by RT-PCR on the 20th of October using serotype specific primers (targeting genome segment 2) for the European BTV serotypes (types , 1, 2, 4, 9, 8 and 16) (data not shown). Although minor DNA bands were detected in several of the reactions, the only reactions products that were of the expected sizes were obtained with primers for BTV-type 1. This gave the first indication that MOR2006/06 and 07 contain BTV-1 (results obtained within 24 hours).

A further analysis of the RNA samples was carried out on the 23rd of October using three primer pairs that are specific for BTV serotype 1 (figure 2). Amplified products of the expected sizes were obtained with all three primer sets.

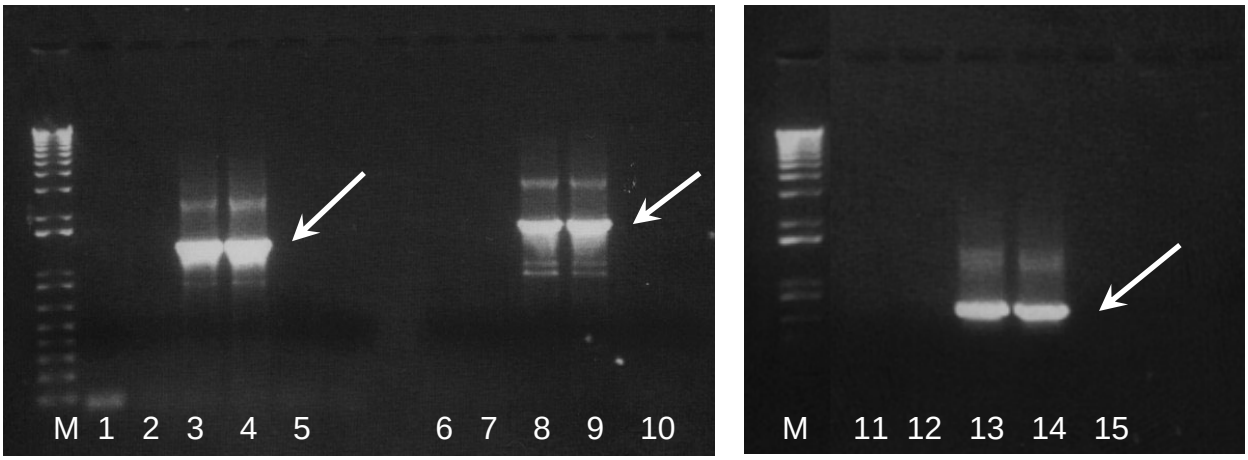

Figure 2: RNA samples from MOR2006/06 and MOR2006/07 were amplified by RT-PCR using primers derived from the consensus nucleotide sequence of BTV -1 genome segment 2. Lane M = DNA marker ladder; Lane 1 and 2 = RNA from the two samples of EHDV (MOR2006/05) amplified with primers 'BTV-1/2/524F' and 'BTV-1/2/968R' generating no product; Lane 3 and 4 = RNA from the two samples of BTV (MOR2006/06 &07) amplified with primers 'BTV-1/2/524F' and 'BTV-1/2/968R' generating a product of =1332bp [indicated by arrow]; Lanes 5 = RNA from the northern European strain of BTV-8 (NET2006/01) amplified with primers 'BTV-1/2/524F' and 'BTV-1/2/968R' generating no product; Lanes 6 and 7 RNA from the two samples of EHDV (MOR2006/05) amplified with primers 'BTV-1/2/406F' and 'BTV-1/2/968R' generating no product; Lanes 8 and 9 = RNA from the two samples of BTV (MOR2006/06 & 07) amplified with primers 'BTV-1/2/406F' and 'BTV-1/2/968R' generating a product of 1586bp [indicated by the arrow]; Lane 10 = RNA from the northern European strain of BTV-8 (NET2006/01) amplified with primers 'BTV-1/2/406F' and 'BTV-1/2/968R' generating no product; Llanes 11 and 12 = RNA from the two samples of EHDV (MOR2006/05) amplified with primers BTV-1/2/335F and BTV-1/2/591R, generating no product; lanes 12 and 13 = RNA from the two samples of BTV (MOR2006/06 &07) amplified with primers BTV-1/2/335F and BTV-1/2/591R, generating a cDNA product of 768bp [indicated by the arrow]; Lane 15 = RNA from the northern European strain of BTV-8 (NET2006/01) amplified with primers BTV-1/2/335F and BTV-1/2/591R generating no product. These results confirm that the samples MOR2006/06 & 07 contain BTV-1. (Results obtained after 2 working days).

On the 25th of October RT-PCR reactions were carried out with the original RNA samples from MOR2006/06 & 07, with type specific primers for all 24 BTV serotypes (figure 3) The only primers that generated cDNA products of the expected sizes were those derived from BTV-1 sequences. These results demonstrate that MOR2006/06 & 07 do not contain any of the other BTV serotypes.

The cDNA products derived from MOR2006/06 using the three sets of BTV-1 type specific primers (sizes 1332, 1586 and 768 bp respectively) were purified and used for sequence reactions with BTV-1 specific sequencing primers. The sequences that were generated using sequencing primer BTV-1/2/529F were then used for sequence comparison with other BTV-1 strains (Figure 3). These sequence data were chosen to allow comparisons with the incomplete data set that was already available for segment 2 of the 2006 Algerian isolate of BTV-1 (ALG2006/01). These data show a close similarity to other BTV-1 Seg-2 sequences, which clearly confirm our earlier conclusions that MOR2006/06 & 07 both contain BTV-1 (Maan et al 2006). The sequence comparison also revealed that the 2006 Moroccan and Algerian strains of BTV-1 are related and both are from an African lineage. The sequence data generated for the Algerian strain of BTV-1 are incomplete and our initial results indicate that there may be some differences compared to MOR2006/06. Further sequence analyses of the complete SEG-2 (as well as selected other genome segments) of both viruses are currently underway and will help to clarify the nature of the relationship between these North African field isolates of BTV-1.

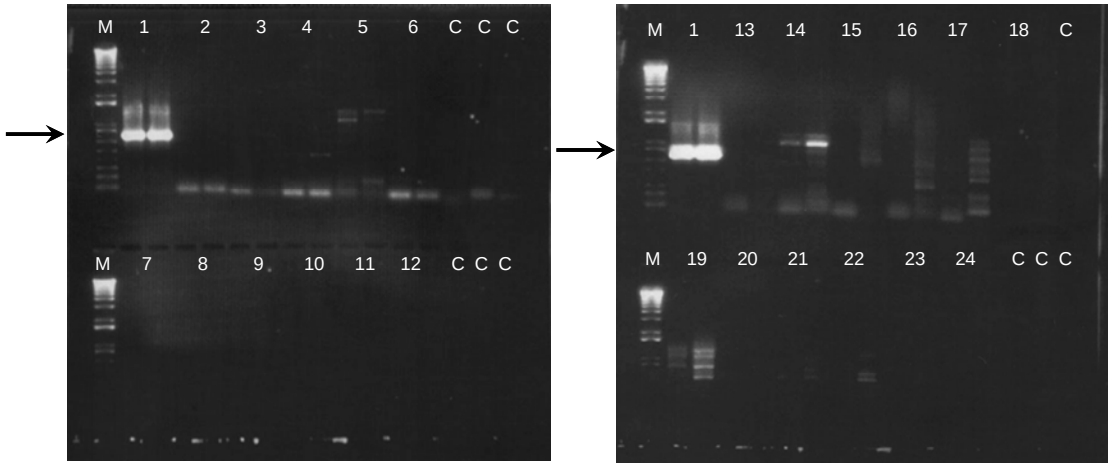

Figure 3: Analysis of RNA samples from MOR2006/06 & 07 by Type specific RT-PCR. M= DNA ladder, The serotype of the primers used to analyse each sample pair, is indicated by the number in each case. Only the primers derived from the sequence of Seg-2 from BTV-1 gave a product of the expected size (indicated by the arrow).

Table of primers used to identify the 24 BTV serotypes

Serotype of primers	Forward Primer		Reverse Primer	
1	BT1-TF	GTTAAAATAGTRKCGCGATGGATGAG	BT1-TR	GTAAGTMTRATAGYGCGCGGA
2	BT2-TFi	GTTAAAACAGGATCGCGATGGATG	BT2-TRi	GTAAGTTGAACAGATCGCGGACCTG
2	BT2/302/F	GCGGAACCBGTDGATGAAG	BT2/845/R	GGTKGAAACAACRTTMAAATT
3	BT3/156/F	CACCACATCGTACCACGC	BT3/772/R	GCGTATAATATTATTGG
4	BT4/56/F *	GGTTGATGTGCCTAAACTAG	BT4/675/R	TCACGGAAGGATGTACATACG
4	BT4/67/F	GCTTAACTATAAACCAACGAGG	BT4/775/R	GTATCAACCTGACCGCGTCG
5	BT5/191/F	GTGGCGCAAGAGTGTGCGT	BT5/562/R	GATGTCCTACAGCTCTCAG
5	5NIG/123/F	TACGGATCATAAATGGATGG	5NIG/791/R	TATATGCTCACAGATATCT
6	BT6/301/F	GGTGGTATGTATAGAGGAAG	BT6/853/R	CAAAGGGAACCTCGCGCGTAATC
7	BT7/83/F	GATGTATCCATAGCGGCAT	BT7/853/R	GATAGATAAGCGAATCGAG
8	BT8/134/F	GCCCACACGCGCTCAAGG	BT8/765/R	CCACTGTCTATGTGCGTAA
8	BT8/280/F	GAGAACAATGGATCGCTCG	BT8/623/R	GTTGCTTAGAGAGAGCGCGG
8	BT8/101/F	GAATGATGGTCATAGCGAG	BT8/500/R	CCGATGTGCGCATTCCTCTC
9	BT9/287/F	GCACACTGCTCTCGCGGATAG	BT9/852/R	CCACATARTGAYGAATGATAGAT
10	BT10/400/F	TGCGTCCAGAATATGGT	BT10/665/R	GGTGCATGTATGTCTCATG
10	BT10/183/F	GATGTTCCACATCTTACAG	BT10/803/R	GTGAAACCTAATGAAATTG
11	BT11/147/F	TGTATTGTTAAGGCTAGG	BT11/770/R	GTCATCGTATAGTATCAT
12	BT12/226/F	GCTGGCGTTACGCGCGGCG	BT12/500/R	CATAATAATACGGCATAAC
12	BT12/111/F	GGATCACAATATAGATGTG	BT12/776/R	CTACGATCATATGATAACTC
13	BT13/157/F	CGAGGAAAGCGGATACCAC	BT13/800/R	GAACATCATCAATTCCAGAATG
14	BT14/121/F	GAAGGTTAGCTTAGGTTTG	BT14/805/R	CTCCGCTTCATCCAGCTC
15	BT15/343/F	GTGGCAGAACGCAGAGGCAG	BT15/806/R	GTGAGACATATAATGTTCAAG
15	BT15/103/F	GAGACCGACTGACCATGACG	BT15/793/R	GTATGCGTGCCAATCGCCTAG
16	BT16/165/F	GTGAGTGTCGTCATGTACG	BT16/832/R	GATAGCGCCTGCGCACGCAAG
17	BT17/146/F	GCGAATGCTGCCAACGCTG	BT17/800/R T	GAAACCGTGTAAAGTTCAT
18	BT18/160/F	GTCTTATCATATAGAGCCAG	BT18/828/R	GTACTCAGATAATAGTCGAG
19	BT19/190/F	TGTGCTCAAGCAAGCGCGTAT	BT19/841/R	GTCATCGTCGAGTGCGTG
20	BT20/135/F	CATTACTCGATAGATTACC	BT20/812/R	GTACGTCGTCAGCAATCTG
21	BT21/116/F	GAGGAATGGCTGAACTGG	BT21/754/R	GCCTCCACACAGCGAAGACAG
22	BTV-22/311F	GACGCGTTGGATAGGATAT	BTV-22/682R	GCTTATACCTCGCATCATCG
23	BT23/459/F	GCTTAGACCTGGCGATAAG	BT23/653/R	GTTTAACATGCATACTCAG
24	BT24/313/F	TGGATTTATCTACACGATT	BT24/783/R	CATAAGCTCCAACTTCAAC

*(will work with Balaeric isolates and might not work with any other BTV-4 field or vaccine strain)

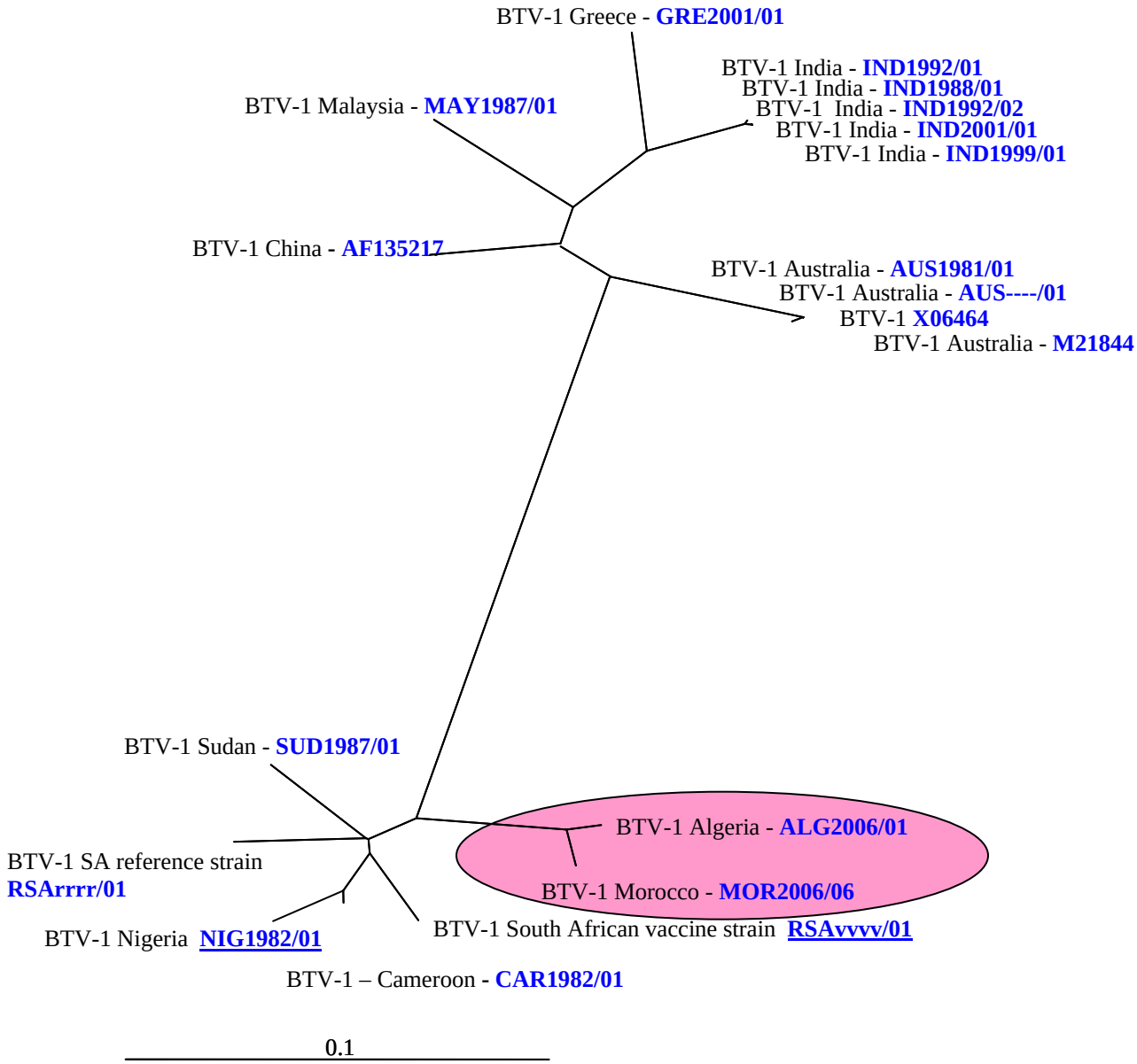

Figure 1: Unrooted neighbour-joining tree showing relationships between partial nucleotide sequences of Seg-2 of BTV-1: Data Title: VP2 partial: 350 nt (nt 1620-1970) Data Type: Nucleotide (coding)

References

Anthony S. , Jones H., Darpel K.E, Elliott H., Maan S., Samuel A., Mellor P. S. and Mertens P. P. C.. (2006) A duplex RT-PCR assay for detection of genome segment 7 (VP7 gene) from 24 BTV serotypes. *Journal of Virological Method*s (Submitted).

Maan S., Maan N.S, Samuel A.R., Rao S, Attoui, H., & Mertens P.P.C (2006) Analysis and Phylogenetic Comparisons of Full-Length VP2 Genes of the Twenty-Four Bluetongue Virus Serotypes. *Journal of General Virology* **88**, 621-630.