

REPORT OF THE COMMITTEE ON BLUETONGUE AND RELATED ORBIVIRUSES

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The Committee met on October 12, 2009 at the Town and Country Hotel, San Diego, Calif., from 1:00 to 4:45 p.m. There were 16 members and 28 guests present. James MacLachlan and William Wilson, Chairperson and Vice-chairperson, respectively, introduced the meeting. There was no discussion of previous committee business or resolutions.

The molecular epidemiology of bluetongue virus infection in Europe: impact of vaccination

Professor Peter P. Mertens, Institute of Animal Health, United Kingdom

The paper in its entirety is included at the end of this report.

Southeastern Wildlife Cooperative Surveillance Activities

Dr. Joseph L. Corn and Ms. Stacey L. Vigil

Southeastern Cooperative Wildlife Disease Study (SCWDS), University of Georgia

An update on surveys for *Culicoides* sp. in the Southeastern United States was provided. These surveys are being conducted as part of a Cooperative Agreement for Exotic Arthropod Surveillance with USDA-APHIS-VS. Surveys are ongoing in Florida, Georgia, Alabama, Mississippi, Louisiana, Arkansas and Texas. Survey sites in Arkansas, Mississippi, Florida and Texas include premises where exotic bluetongue virus or exotic epizootic hemorrhagic disease virus positive animals had previously been detected. Contents of light traps are processed and *Culicoides* sp., identified at SCWDS. During November 2007 – August 2009 traps were set for 2,247 trap nights at 127 premises in 55 counties throughout the Southeastern United States. A total of 1,298,696 insects have been sorted from traps set out during this period; 31,284 of these were *Culicoides* sp. Thirty-two species have been identified to date. Exotic *Culicoides* sp. have not been identified, but identification of most of the insects collected is pending. Possible range expansions of *Culicoides insignis* and *Culicoides alachua* have been detected. Additional field collections and identification of *Culicoides* sp. collected are underway and will continue in 2010.

Epidemiology of bluetongue virus infection in California

Dr. Christie Mayo

School of Veterinary Medicine, University of California

An overview of a recent surveillance program for bluetongue virus that has been initiated in California was provided. This is a collaborative undertaking between the University, the California Department of Food and Agriculture, and the California Animal Health and Food Safety Laboratory, and utilizes some 120 sentinel calves in different regions of the state. Calves are monitored monthly for the presence of viral nucleic acid and/or antibodies. Thus far the study has demonstrated limited perinatal infection of calves as well as seasonal infection from August onwards.

NVSL update, OIE Manual update, and review of OIE meeting in Teramo

Dr. Eileen Ostlund

Bluetongue virus (BTV) and epizootic hemorrhagic disease virus (EHDV) isolations/PCR positives

Calendar year 2008

Bluetongue virus or RNA was detected in 15 samples submitted during calendar year 2008. The positive bluetongue virus isolation and polymerase chain reaction (PCR) test results from submissions to the National Veterinary Services Laboratories (NVSL) in 2008 are listed in Table 1.

Table 1. BT virus isolation (VI) / PCR positives, Calendar year 2008

<i>State</i>	<i>No.</i>	<i>Species</i>	<i>PCR</i>	<i>VI</i>
AR	1	Deer isolate/tissue (received from SCWDS*)	Positive	BTV-3
CA	1	Sheep	Positive	Negative
FL	1	Deer	Positive	BTV-9
KS	1	Cattle	Positive	Negative
OK	1	Deer isolate	Positive	BTV-3
TX	1	Deer isolate/tissue (received from SCWDS*)	Positive	BTV-12
TX	2	Deer isolate (year unknown)	Positive	BTV-17 (one also EHDV-6)
UNK	6	Bovine hemoglobin	Positive	Negative
UNK	1	Bovine Serum Albumin	Positive	Negative

*Southeastern Cooperative Wildlife Disease Study, Athens, GA

During calendar year 2008, 15 samples tested positive for EHDV by virus isolation and/or PCR. The positive EHDV isolation and PCR test results from submissions to the National Veterinary Services Laboratories (NVSL) in 2008 are listed in Table 2.

Table 2. EHDV isolation (VI)/ PCR positives, Calendar year 2008

<i>State</i>	<i>No.</i>	<i>Species</i>	<i>PCR</i>	<i>VI</i>
IN	2	Deer isolate	Positive	EHDV-2
IA	1	Deer	Positive	EHDV-2
MT	1	Deer	Positive	EHDV-2
OK	1	Deer isolate	Positive	EHDV-2
SD	5	Deer	Positive	EHDV-2
TX	1	Deer isolate	Positive	EHDV-1
TX	2	Deer isolate	Positive	EHDV-2
TX	2	Deer isolate	Positive	EHDV-6 (also BTV-17)

Calendar year 2009 (January 1– October 1)

As of 1 October 2009 bluetongue virus has been identified in a deer isolate sample submitted from TX. Both EHDV-2 and EHDV-6 were isolated from a MO deer submission. Two additional

deer samples from TX were submitted as isolates. One of these contained EHDV-1 and the other contained EHDV-6.

In addition to results reported above, two lots of fetal bovine serum (FBS) from Mexico tested positive for bluetongue virus by sheep inoculation. One of these lots was tested late in 2008 and one lot in early 2009.

Summary of non-endemic bluetongue virus isolates identified at NVSL 1999-2009

In the United States, bluetongue virus types 2, 10, 11, 13 and 17 are considered endemic. Some states are free or seasonally free of bluetongue activity while others experience less seasonality. Of the endemic types, BTV-2 is restricted primarily to Florida and the other types are more widespread. Since 1999, NVSL had identified 25 isolates of non-endemic bluetongue virus from U.S. ruminant species. At least one isolate has occurred in each of 6 southeastern states (AR, FL, LA, MS, OK, TX); the largest number have been identified in samples originating from Florida. A total of 10 previously unrecognized bluetongue serotypes have been identified to date (BTV types 1, 3, 5, 6, 9, 12, 14, 19, 22, 24). Of these, BTV-3 has been the most frequent non-endemic isolate and has been found in 4 states; BTV-3 isolates have occurred in 6 of the past 10 years. None of the non-endemic bluetongue types has caused widespread disease outbreaks. The *Culicoides spp.* vectors responsible for transmission of the non-endemic types are unknown.

Examination of archived Caribbean and Central American isolates of bluetongue virus

In 2008, the NVSL typed 6 bluetongue virus isolates that were obtained in 1990 from water buffalo from Trinidad and Belize. The BTV types found were BTV-3, BTV-13, BTV-17, BTV-18, BTV-19, and BTV-22. Additionally the NVSL typed 5 isolates from Brahman cattle from the Dominican Republic. Three of these were BTV-19 and one each was BTV-10 and BTV-11.

2009 Bluetongue Serology Proficiency Test

Fifty-four laboratories participated in the 2009 bluetongue (BT) proficiency test. The panel consisted of 20 serum samples. The passing score was one or zero samples missed. All 54 laboratories passed the proficiency test with 53 of 54 laboratories agreeing with each other and NVSL on all 20 samples. Laboratories approved to conduct official (export) bluetongue serology are listed on the website:

http://www.aphis.usda.gov/animal_health/lab_info_services/approved_labs.shtml

OIE Bluetongue Network

An international network of OIE bluetongue reference laboratories, OIE collaborating centers, and National Reference Laboratories has been formed to facilitate information and reagent exchange among laboratories. Representatives of the network have met in Italy in 2007 and in 2009. Further information about the OIE bluetongue network can be obtained at www.oiebtnet.izs.it.

Real-time PCR for the simultaneous detection of all serotypes of EHDV

Dr. Alfonso Clavijo

Texas Veterinary Medical Diagnostic Laboratory

A new real-time RT-PCR for the simultaneous detection of all serotypes of EHDV has been developed. The new assay targets the EHDV NS1 gene that is relatively conserve with the EHDV serogroup and detects all eight serotypes. The assay did not cross-react with US serotypes of BTV. This assay complements the previous published real-time RT-PCR assay that targets the EHDV NS3 gene and is a less complex design. This work was done in collaboration and is complementary to previous work done by the USDA, ARS, Arthropod-Borne Animal Diseases Research Laboratory. Further studies will include this addition of this assay into the multiplex real-time RT-PCR assay that detects and distinguishes between BTV and EHDV.

1. Wilson, W. C., E. S. O'Hearn, C. Tellgren-Roth, D. E. Stallknecht, D. G. Mead and J. O. Mecham.: Detection of all eight serotypes of Epizootic hemorrhagic disease virus by real-time reverse transcription polymerase chain reaction. *J. Vet. Diagn. Invest.*, 21: 220-225, 2009
2. Wilson, W. C., B. J. Hindson, E. S. O'Hearn, S. J. Hall, C. Tellegren-Roth, C. Torres, J. O. Mecham and R. J. Lenhoff.: Development of a Multiplex Bluetongue and Epizootic

Hemorrhagic Disease Real-Time RT-PCR serogroup detection and differentiation assay. J. Vet. Diagn. Invest., 21: xxx-xxx, 2009

Update on bluetongue and epizootic hemorrhagic virus isolations during 2008 and 2009.

Dr. David Stallknecht

Southeastern Cooperative Wildlife Disease Study (SCWDS), University of Georgia

An update on bluetongue and epizootic hemorrhagic virus isolations during 2008 and 2009 was reported. In 2008, isolations were made from wild and captive white-tailed deer in Arkansas (BTV-3), Indiana (EHDV-2), Kansas (EHDV-2, EHDV-6), and Texas ((EHDV-1, EHDV-2, EHDV-6, BTV-12, BTV-17). As of October 9 this year (2009), viruses have been isolated from white-tailed deer in Florida (EHDV-2), Kansas (EHDV-2), Louisiana (EHDV-2), Michigan (EHDV-6), Missouri (EHDV-2), Tennessee (EHDV-2), and Texas (BTV-17). BTV-3, BTV-12, and EHDV-6 all represent viruses that were not known to occur in the United States prior to 1999 (BTV-3), 2006 (EHDV-6), and 2008 (BTV-12). There have been multiple isolations of BTV-3 and EHDV-6 suggesting that these viruses are established.

The Arthropod-borne Animal Diseases Laboratory: research program update and current status

Dr. Barbara S. Drolet

USDA, ARS, Arthropod-borne Animal Diseases Laboratory (ABADRL)

Dr. Drolet presented the Arthropod-borne Animal Diseases Laboratory research program update on behalf of herself, Kristine Bennett, James Mecham, Myrna Miller, and William Wilson. The Arthropod-Borne Animal Diseases Research Laboratory (ABADRL), located in Laramie, Wyoming, currently consists of 26 staff including microbiologists, virologists, entomologists, and veterinarians, as well as staff who support the laboratories, administration and facilities. The Research Leader position has been vacant since August of 2007, with three ABADRL research scientists rotating as Acting Research Leaders. All ABADRL facilities have been officially downgraded to BSL-2 for lab, small animal, insect, and large animal work. The mission of the laboratory is to solve major endemic, emerging, and exotic arthropod-borne disease problems in U.S. livestock. Research emphasizes the molecular biology of pathogens and vectors, vector biology and competence, epidemiology, and animal pathogenesis. Arboviruses, including bluetongue virus (BTV), vesicular stomatitis virus (VSV), and Rift Valley fever virus (RVFV), are the major focus of concern because they were identified during the ARS Animal Health stakeholder workshop as high priority insect-transmitted livestock pathogens.

To accomplish their continuing BSL-3 inclusive research mission, the ABADRL is contracting work out, as well as establishing more national and international collaborations with scientists who have access to BSL-3 facilities and/or reside where the BSL-3 agents are endemic. Significant amounts of time and budget resources are being used to travel to collaborator locations to conduct research. These locations include: Colorado State University, Fort Collins, CO; USDA, APHIS National Wildlife Research Center, Fort Collins, CO; U.S. Army Medical Research Institute for Infectious Diseases (USAMRIID), Fort Detrick, MD; Canadian Food Inspection Agency (CFIA), Winnipeg, Canada; Kenya Centers for Disease Control; Kenya Medical Research Institute (KEMRI), Kenya Agriculture Research Institute (KARI), Kenya Central Veterinary Laboratory; Onderstepoort Veterinary Institute (OVI), South Africa, Onderstepoort Biological Products (OBP), South Africa; Animal Health Research Institute of Egypt; and the Department of Veterinary Services of Yemen.

The ABADRL has three 5-year project plans under two ARS National Research Programs. One project plan under the Animal Health National Program is entitled "Countermeasures to control and eradicate Rift Valley fever". Research objectives in this plan are 1) to determine the vector competence of North American mosquito species for both wild type and vaccine strains of RVFV; 2) to develop vaccine and diagnostic expression and delivery systems for RVFV; and 3) to develop operator-safe, sensitive diagnostic tests for the early detection of RVFV, including assays to distinguish infected from vaccinated animals. Research progress to date includes vector competence studies for wild type virus, animal infection model studies for both wild type and

vaccine strains, production of BSL-2 anti-RVFP antisera derived from expressed viral proteins, and the development of BSL-2 diagnostic assays including ELISA and immunohistochemistry.

A second project plan under the Animal Health National Program is entitled "Virus-vector-host interactions of arboviral diseases of livestock" and focuses primarily on BTV and VSV. Research objectives in this plan are 1) to identify biological determinants of disease susceptibility associated with arboviral infections; and 2) to determine the host-range specificity of exotic bluetongue viruses, namely the susceptibility of North American sheep and white-tailed deer to the European strain of BTV type 8 (EU-BTV-8). Research progress to date includes *in vitro* studies to determine the role of *Culicoides* salivary proteins on establishment of VSV infection and interferon production in porcine cells, molecular analysis of bovine membrane proteins, and the cloning and expression of BTV attachment proteins. Additionally, white-tailed deer are being hand reared and weaned in preparation of the EU-BTV-8 infection study, but researchers are still waiting for permit approvals for importation of the virus from The Netherlands to our CSU collaborator.

The project plan under the Veterinary, Medical, and Urban Entomology National Program is entitled "Vector competence and protection of US livestock and wildlife from arthropod-borne diseases". This includes research on important vector insect species such as mosquitoes, midges, and sand flies and important arboviruses such as exotic BTV and RVFP, as well as insecticide resistance mechanisms and molecular mosquito taxonomy tools. Research objectives in this plan are 1) To assess the risk of endemic arthropod vectors to transmit introduced exotic arboviruses in North America, and 2) to identify targets and evaluate tools for vector control and interruption of transmission cycles to protect livestock and humans from vector-borne pathogens. This past year the research plan was written and approved by ARS National Program Leaders as well as an Office of Scientific Quality and Review Panel and given an official start date of October 1, 2009.

The current facilities at Laramie cannot support the high containment research mission of the lab and funds are not available to replace the current facility. The President's FY09 budget called for the relocation of the ABADRL from Laramie, WY to Ames, IA for consolidation with USDA, ARS, National Animal Disease Center. The response of the US senate to these recommendations was a request for more information regarding the research mission and current facilities of the ABADRL, and an assessment of no fewer than two relocation sites. A study of four possible relocation sites (Fort Collins, CO; Moscow, ID; Manhattan, KS; and Ames, IA), conducted by ARS Headquarter, Area Office, and Location staff, addressed each location as to facilities, capacity, expertise, and scientific synergy with the ABADRL mission. The Agriculture Appropriations Committee reviewed the site visit report and voted unanimously for ABADRL to be relocated to Manhattan, Kansas. The Congressional Conference report language confirmed Manhattan as the relocation site, with \$1.5M in relocation assistance. The Ag bill was passed by the House on October 7th, and subsequently passed by the Senate on October 8th, 2009. The relocation will become official upon signature by the President of the United States. The move timeline is uncertain, but will be completed by the end of FY10. The ABADRL will be housed with four other ARS research units at the Grain Marketing & Production Research Center (GMPC) in Manhattan and will conduct BSL-2 research in that facility. The ABADRL will conduct BSL-3 laboratory, insect, small animal and large animal research in facilities owned by Kansas State University.

The ABADRL currently has the highest level of funding in its history, thanks to additional funding sources such as Department of Homeland Security, ARS Office of International Research Projects, and the Department of State Biosecurity Engagement Program. Additionally, the lab has the largest number of national and international collaborations in its history, and continues to have a productive research program addressing the needs of our stakeholders.

Evaluation of real time PCR assays for the detection of BTV in bovine semen

Dr. Peter Kirkland, R. Davis and X. Gu

Elizabeth Macarthur Agricultural Institute, Camden, New South Wales, Australia

Although there has been a long term requirement to screen the semen of cattle that have potentially been infected with bluetongue virus (BTV), until recently, there has been little evidence

of the presence of BTV in the semen of naturally infected bulls. Studies conducted in the USA and Australia consistently showed that BTV was not present in the semen of bulls infected with “wild-type” strains of virus. However, if a mature animal was infected with a cell culture adapted strain of BTV, infectious virus could be detected in the semen for a short period soon after the onset of infection. Recently, following the natural infection of cattle in Europe with BTV serotype 8, virus has been readily detected in semen and has also crossed the placenta, causing foetal infections. Although these strains of BTV-8 are naturally circulating, they possess the characteristics attributed to cell-culture adapted or vaccine strains of virus.

During one of the large studies conducted in Australia, mature bulls were experimentally infected with a laboratory adapted strain of BTV serotypes 1. A total of 8 bulls were inoculated and on each occasion that a semen sample was collected, blood samples were also collected to monitor the onset and duration of viraemia. From day 7 after experimental infection, samples were collected twice weekly for 4 weeks then once a week for a further 4 weeks. Methods used to detect infectious virus in blood and semen included the inoculation of embryonated chicken eggs (ECE) followed by passage in insect and mammalian cell cultures, direct passage in both insect and mammalian cell cultures, and inoculation of sheep. For both blood and semen, a large volume of sample was examined to maximise virus detection. Serological methods (AGID, cELISA and VNT) were also employed to monitor infection. The semen samples from these bulls were stored both in liquid nitrogen and also at -80°C. Recently, these samples were tested using a semi-automated method for RNA extraction and both a nested reverse transcriptase polymerase chain reaction (nRT-PCR) assay and a BTV pan-reactive real time reverse transcriptase polymerase chain reaction (qRT-PCR) assay.

BTV was detected intermittently in semen from a number of the mature bulls that had been experimentally infected with this laboratory-adapted strain of BTV-1. These detections occurred during or immediately after the period of detectable viraemia. The duration of viraemia varied from 17 to 31 days. Each of the virus isolation methods had comparable sensitivity if samples were passaged sufficiently. BTV was most readily detected by inoculation of ECE or sheep. When the semen samples were examined by nRT-PCR, similar results were obtained to the virus isolation methods even though a 10 fold lesser sample volume was assayed. Superior results were obtained from the semi-automated magnetic bead based nucleic acid extraction system compared to a manual column extraction method. When the semen extracts were tested in the qRT-PCR, a higher level of sensitivity was achieved than with any of the other virus detection methods. In some bulls, where infectious virus had been detected intermittently, viral RNA was detected consistently. Further, viral RNA was detected for several collections (7-10 days) longer by qRT-PCR than by conventional methods. Comparison of several published qRT-PCR methods and commercially available kits for BTV showed that the “Swiss” method (Hoffmann et al., 2008) had the highest analytical sensitivity.

It was concluded that a combination of a magnetic bead based nucleic extraction method and an appropriate pan-reactive qRT-PCR will readily and reliably detect BTV in the semen of bulls and will permit a large number of samples to be tested in a short time.

Successful development of a recombinant African horse sickness virus vaccine

Dr. James Maclachlan

School of Veterinary Medicine, University of California, Davis

Dr. Maclachlan described collaborative studies with Dr Alan Guthrie and his staff at the Equine Research Centre, Faculty of Veterinary Science, University of Pretoria, Onderstepoort, South Africa and Drs Jules Minke, Jean-Christophe Audonnet and others at Merial Inc. and Sanofi-Pasteur that lead to the successful development of a recombinant African horse sickness virus vaccine [1]. The vaccine is based on the strategy used for successful development of a recombinant bluetongue virus vaccine [2], utilizes the canarypox virus expression vector as well as the genes encoding the outer capsid proteins of African horse sickness virus. This vaccine induces sterilizing immunity in vaccinated horses, and vaccinated animals readily can be distinguished from infected animals using conventional group-specific serological tests (DIVA).

1. Guthrie, A.J., Quan, M., Audonnet, J.C., Minke, J., Yao, J., He, L., Nordgren, R., Gardner, I.A. and Maclachlan, N.J.: Protective immunization of horses with a recombinant canarypox virus

vector co-expressing genes encoding the outer capsid proteins of African horse sickness virus. *Vaccine*, 27: 4434-4438, 2009

2. Boone, J.D., Balasuriya, U.B., Karaca, K., Audonnet, J-C., Yao, J., He L., Nordgren, R., Monaco F., Savini, G., Gardner, I.A. and MacLachlan, N.J.: Recombinant canarypox virus vaccine co-expressing genes encoding the VP2 and VP5 outer capsid proteins of bluetongue virus induces high level protection in sheep. *Vaccine*, 25: 672-678, 2007

Committee Business

George Winegar, USDA, Retired, brought the Import/Export committee resolution requesting that OIE and SPS guidelines be used at the initiation of all international trade for discussions to the BT and related Orbiviruses committee for consideration. The committee endorsed the resolution. No additional resolutions were proposed or other business discussed.

THE MOLECULAR EPIDEMIOLOGY OF BLUETONGUE VIRUS INFECTION IN EUROPE: IMPACT OF VACCINATION

Peter P.C. Mertens¹, Sushila Maan¹, Abraham C. Potgieter², Kyriaki Nomikou¹ and Narender S. Maan¹

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Introduction

Bluetongue virus (BTV) is endemic in warmer regions around the world, between ~45-53°N and 35°S, including North and South America, Africa, the Indian subcontinent, Australasia, and Asia (Mertens et al 2007b). BTV is transmitted primarily by adult female hematophagous midges (genus *Culicoides* - Mellor and Boorman, 1995) and can infect most ruminants or camelids (Chandel et al., 2003; Ruiz-Fons et al., 2008). However, the virus can also be transmitted vertically in cattle and sheep (Menziés et al., 2008), and can infect certain large carnivores via an oral route or injection (Jauniaux et al., 2008). There is also some evidence that BTV can be transmitted vertically in the midge (White et al 2005). The severity of the disease is dependent on host species, breed/strain, and on their serological status for BTV. However, it is also dependent on the virus itself and attenuated strains have long been used as 'live vaccines' (Veronesi et al., 2005).

The nucleotide sequences of BTV isolates reflect their geographic origins (Gould and Pritchard, 1990; Pritchard *et al.*, 1995; 2004), and the majority of the BTV genome segments can clearly be divided into 'eastern' or 'western' groups / topotypes (Maan *et al.*, 2007; 2008; 2009; Mertens *et al.*, 2007a). This indicates that these viruses have evolved, with little genetic exchange between regions, over a very long period of time, allowing them to acquire multiple point mutations and clear regional differences. With the identification of 'Toggenburg orbivirus' (TOV), there are now 25 distinct serotypes of BTV that can be identified by the specificity of reactions between their outer capsid proteins and neutralising antibodies generated during infection of the mammalian host. The BTV genes encoding these outer-capsid proteins show nucleotide sequence variations that correlate with both virus serotype and the geographical origin of the virus isolate (Mertens et al 2007b). Since 1998, nine serotypes of BTV have invaded Europe, which appears to represent a 'cross-roads' between east and west, containing a unique mixture of viruses from both geographic regions.

'Molecular epidemiology' studies can be used to compare RNA sequences from novel BTV isolates with those of existing strains from known locations and dates, identifying both virus serotype and topotype. Sequence variations can also identify individual virus lineages within the same area and the presence of 'reassortant' strains containing genome segments from different 'parental' strains. BTV molecular epidemiology studies depend on development of sequence databases for the RNA segments of 'known' virus isolates from defined locations with well documented isolation dates and passage histories. Ideally these viruses should be held in long-term reference-collections (e.g. the BTV-collection at IAH Pirbright - Mertens et al 2007c: http://www.reoviridae.org/dsRNA_virus_proteins/ReolD/BTV-isolates.htm), allowing sequence data to be linked to biological characteristics and epidemiology of specific isolates.

The BTV genome codes for a total of 10 distinct viral proteins, one from each dsRNA segment, seven of which (VP1 to VP7) are structural components of the virus particle. Three distinct non-structural proteins (NS1, NS2 and NS3/NS3a) are also synthesised during replication in infected cells (Mertens *et al.*, 1984; 2005). Cross-hybridisation studies (Huismans *et al.*, 1987; Mertens *et al.*, 1987), and recent nucleotide sequence comparisons (Bonneau *et al.*, 2000; Wilson *et al.*, 2000; Potgieter *et al.*, 2005; Balasuriya *et al.*, 2008; Maan *et al.*, 2007; 2008) have shown different levels of variation in individual BTV genome segments and the proteins they encode. The capsid proteins that are situated on or near to the surface of the virus particle are more variable than components of the virus core, or the non-structural proteins (Mertens 2004; Maan *et al.*, 2008; 2009). Mertens and associates (2007d) describe the individual BTV proteins

and RNAs in detail. VP2 (encoded by Seg-2) is the outermost of the BTV capsid proteins and represents a primary target antigen for neutralising antibodies (Huismans and Van Dijk, 1990; Roy *et al.*, 1990; De Maula *et al.*, 2000). Phylogenetic analyses of Seg-2 show that it represents the least conserved region of the BTV genome, and separates into 25 distinct clades that consistently reflect virus serotype (<33% nt sequence variation within serotype: 29-59% variation between types - Maan *et al.*, 2007; 2009). However, sequence variations in Seg-2 also provides evidence for distinct eastern and western lineages (topotypes) within individual BTV types (<13% Seg-2 nucleotide variation within topotype).

The 'eastern' BTV group includes viruses from India, Indonesia, China or Australia, while the western group includes viruses that are primarily from Africa and North or South America (Maan *et al.*, 2007; 2008; Mertens *et al.*, 2007a). There is also evidence for a far eastern group(s), although few sequences are currently available. Sequence variations in Seg-2 provide a basis for molecular epidemiology studies that can be used to identify/confirm virus serotype, the geographical origins of the virus lineage and distinguish between even closely related virus strains within a single epizootic or region. VP5 (encoded by Seg-6), is the smaller of the BTV outer capsid proteins and Seg-6 is the second most variable region of the BTV genome (<43.9% nucleotide variability overall - Singh *et al.*, 2004; Maan *et al.*, 2008). Although VP5 also influences the specificity of reactions with neutralising antibodies, its contribution is less significant than VP2 (Mertens *et al.*, 1989; DeMaula *et al.*, 2000).

Recent analysis of BTV genes showed that genome Seg-7 (encoding outer core protein VP7) is the third most variable genome segment (<34.7% nt variability), despite VP7 representing the primary target for the majority of BTV serogroup-specific serological assays (Wade-Evans *et al.*, 1990; Afshar *et al.*, 1992). Oligonucleotide primers were also designed targeting Seg-7, for use in BTV virus-species / serogroup-specific RT-PCR assays (Anthony *et al.*, 2007). Genome segments 1, 3, 4 and 9 (encoding structural proteins) and segments 5, 8 and 10 (encoding non-structural proteins) are highly conserved across the entire BTV species/serogroup, although they also show eastern and western grouping (topotypes) with 20 – 31% nt variation. The high levels of conservation in Seg-3 have been used to distinguish and identify members of different *Orbivirus* species (Attoui *et al.*, 2001; Nomikou *et al.* 2009). Sequence comparisons of the polymerase gene have been used to compare even distantly related reoviruses, identifying members of different genera within the family *Reoviridae* (Attoui *et al.*, 2000; Mertens, 2004).

Real-time RT-PCR assays targeting BTV Seg-1 (Shaw *et al.*, 2007) and Seg-9 (Maan *et al.* – in preparation) can be used to identify RNA from either eastern or western BTV topotypes, or combined in a pan-BTV-specific assay (available from Qiagen Germany).

BTV incursions into Europe: BTV has been recorded on the fringes of Europe for many years with several serotypes present in Turkey, Cyprus, Israel and Africa (Taylor and Mellor, 1994). Before 1998, BTV had caused only periodic and relatively short-lived epizootics within southern Europe, involving a single serotype on each occasion (Mellor & Wittmann, 2002). However, since 1998 Europe has experienced multiple BT outbreaks, involving at least 13 incursions / strains, belonging to a total of nine different BTV serotypes (BTV-1, 2, 4, 6, 8, 9, 11, 16 and 25) (reviewed by Maan *et al.* 2009; Mellor *et al.* 2009). BTV-15 and 24 were also identified in Israel during 2006 and 2008 respectively (the first time in the Mediterranean region) suggesting that they could represent future threats to Europe.

The majority of the European viruses in were identified by conventional (gel based) RT-PCR using serotype specific primers targeting Seg-2 and sequence analysis (Mertens *et al.*, 2007a; e). However real-time RT-PCR assays are now also available for all of the European BTV serotypes (from Laboratoire Service International – France).

BTV-1: Sequencing and phylogenetic analyses of Seg-2 have identified three separate introductions of BTV-1 into the Mediterranean region, in 2001, 2006 and 2007 (Mertens *et al.* 2007g). These include an eastern strain (in Greece), related to viruses from India, and two incursions of a western strain, one of which spread to south-west France during November 2007. This provided the first overlap between the northern European outbreak of BTV-8 (see below) and any other BTV strain/serotype and opportunities for genome segment exchange / reassortment.

BTV-2: BTV-2 was first identified in Tunisia during February 2000, then on the Italian island of Sardinia during August 2000 and by October it was confirmed in Sicily, Calabria

(southern mainland Italy), Corsica and the Spanish islands of Menorca and Mallorca (Anon 2000a, b, c and d). Seg-2 of the Tunisian BTV-2 is almost identical (99.8%) to isolates from Corsica and Sardinia, indicating that like BTV-1, BTV-2 spread northwards from Africa into Italy and the western Mediterranean islands. The initial European BTV-2 outbreaks were not caused by the live, attenuated vaccine, however, subsequent BTV-2 isolates from mainland Italy were as much more closely related to the vaccine strain used in the region, indicating that it had been transmitted in the field (Ferrari *et al.*, 2005, Batten *et al.*, 2008).

BTV-4: A western strain of BTV-4 was first detected in Greece during 1999, although it had previously been recorded to the south and east of Europe (e.g. Cyprus, Syria, Jordan, Israel, and Turkey - Taylor and Mellor, 1994). BTV-4 was also isolated in Israel during 2001, 2006 and 2008. Phylogenetic analyses demonstrate that the European and Israeli strains are closely related to earlier isolates from Cyprus (CYP1969/01) and Turkey (TUR1978/01), and to the IAH reference strain of BTV-4 (RSAr4/04) which also originated from Cyprus (ASOT-1) (Sellers *et al.*, 1979). This suggests the 1999-2000 Greek strain of BTV-4 has been circulating in the Mediterranean region for at least 40 years (Mertens *et al.* 2007k). In 2003 a distinct strain of BTV-4 was detected in the western Mediterranean islands (Menorca - SPA2003/02 and Corsica - FRA2003/01) (Breard *et al.*, 2007). The virus is believed to have a sub-Saharan origin, entering Europe from North Africa, possibly from Tunisia or Algeria. It was concluded that there had been introductions of BTV-4 into Europe, from North Africa in both 2003 and 2004.

BTV-6: In October 2008, 4 animals on different farms in eastern Holland tested positive for BTV by RT-PCR. Full genome sequence confirmed the introduction of a new western strain of BTV-6 into northern Europe, showing that it was derived from the live South African vaccine (with 99.7 – 100% nucleotide identity in most genome segments). However, it was also identified as a reassortant virus, with Seg-10 derived from another (unknown) parental strain (most closely related to the BTV-2 vaccine strain RSAvvv2/02 – 98.4%). There was also evidence of reassortment with BTV-8 (Seg-7), indicating that it had been circulating in the region from some time. Like BTV-8, it is still unclear how BTV-6 first arrived in Europe (although illegal use of live vaccines has been suggested) (ProMED 31-OCT-2008).

BTV-8: In August 2006, BT was recognised for the first time in northern Europe (OIE, 2006; Toussaint *et al.*, 2006). The outbreak was initially mild and subsided during the winter of 2006/7 but reappeared in the same regions during May-June 2007 with greatly increased severity killing thousands of animals (mainly sheep). Although these BTV-8 outbreaks stopped during the winter of 2007/8, export of infected animals into northern Spain and Italy led to further outbreaks in both regions (EFSA report, 2007). Sequence analyses of Seg-2 and 6 from the first virus isolate from the outbreak demonstrated that it belongs to a western lineage from sub-Saharan Africa but is distinct from the South African BTV-8 vaccine strain (Maan *et al.*, 2008; Mertens *et al.* 2007m).

During January 2008, a group of cows, some of which had previously been infected with BTV-8, were imported from the Netherlands into Northern Ireland. Although all of these animals were negative by RT-PCR assays of blood samples, three of their calves were born RT-PCR +ve for BTV-8 RNA, providing clear evidence of vertical transmission *in utero*. Two in-contact adult cows also became infected with the same virus, providing evidence of horizontal transmission despite the absence of adult *Culicoides* (Menzies *et al.* 2008).

In May 2009 there were BT outbreaks in Israel due to type 8. The Israeli strain showed 99.7% similarity in Seg-2 to the Netherlands isolate, and is therefore thought to be derived from the European BTV-8 outbreak. BTV-8 was also isolated in Oman during 2009 but showed only 95.2% similarity in Seg-2 to the Netherlands BTV-8, indicating that although it is a western/African strain from a similar lineage, it was not derived from the European BTV-8 outbreak.

BTV-9: The first outbreaks of BT in Europe since the 1980s, occurred on four Greek islands (Rhodes, Leros, Kos and Samos) close to the Anatolian coast of Turkey during October 1998, caused by BTV-9 (Mertens *et al.* 2007n; Anon. 1998a, b). Sequence analysis of Seg-2 identified an eastern strain of BTV-9, distinguishing it from the South African BTV-9 vaccine strain which belongs to a western group (Mertens *et al.* 2007o). BTV-9 had previously been reported in Anatolian Turkey, Syria, Jordan and Israel (Taylor and Mellor, 1994).

The virus spread to mainland Greece, south-eastern Bulgaria and European Turkey during 1999, (Anon. 1999a and b), then in 2001 to Serbia, Montenegro, Kosovo, Macedonia,

Bulgaria, Croatia (Anon. 2001a; Anon. 2001b - f), mainland Italy and Sicily. In 2002 BTV-9 was identified again in Bosnia, Bulgaria, Montenegro, Yugoslavia and Albania and there was an unconfirmed report of BT in Kosovo (Calistri *et al.*, 2004). Although BTV-9 was also isolated in Sicily in 2003, this was from an animal that died within week after vaccination with the live BTV-9 vaccine and the virus that was recovered proved to be identical (Seg-2) to the South African vaccine strain which may reflect the use and persistence of the live vaccine strain in the region (Savini *et al.*, 2008). BTV-9 also caused further outbreaks in Italy, during 2004.

BTV-11: On 20 December 2008, blood from an animal from East Flanders in Belgium was tested positive for BTV-11 using conventional RT-PCR targeting genome segment 2 (Mertens *et al* 2007a, e). Seg-2 sequence data from this sample showed 99.74% nucleotide identity with South African reference strain of BTV-11 (RSAr11/11) and 100% identity with that of the modified 'live' vaccine strain of BTV-11 from South Africa. Only around 84% similarity was found to BTV-11 strains from the USA. A Zimbabwe strain of BTV-11 gave an intermediate level of similarity in Seg-2 (De Clercq *et al* 2009).

BTV-16: Outbreaks caused by BTV-16 occurred in Greece during 1999-2000, and in the Turkish province of Izmir during 2000 (Mellor *et al* 2009). During 2002 and 2004 further outbreaks occurred on Sicily, mainland Italy, Sardinia, and Corsica, and on Cyprus during 2006 (Mertens *et al* 2007p). Sequence analysis of Seg-2, indicates that all of the Mediterranean / European isolates of BTV-16 belong to an eastern group of viruses and are closely related to the South African BTV-16 vaccine (<0.7% variation), suggesting a recent common ancestry (Mertens *et al* 2007q). The BTV-16 vaccine strain can cause severe disease in some European breeds of sheep, with viraemia levels ($>10^6$ TCID₅₀/ml) that are sufficient to allow infection of feeding *Culicoides* and therefore onward transmission of the virus (Veronesi *et al.*, 2005). The vaccine strain was originally derived from the reference strain that was originally isolated from Hazara in West Pakistan during 1960 (Howell *et al.*, 1970). This close relationship suggests that the live, attenuated vaccine may be involved in the origins of all of the European incursions of BTV-16. The live vaccine strain of BTV-16 was used as part of an annual vaccination campaign in Israel (Shimshony, 2004) and could represent a source for the European viruses. However, the Italian field strain of BTV-16 appears to have an even closer relationship to the vaccine strain than the viruses from either Greece or Turkey (~99.9% in Seg-2), suggesting that it was not derived from the earlier Greek or Turkish outbreaks (Batten *et al.*, 2008; Savini *et al.*, 2008). The Italian BTV-16 strain from 2002 is a reassortant, containing Seg-5 derived from the BTV-2 vaccine strain that was also used as part of the multivalent vaccine in Israel (Batten *et al* 2008). The outbreak in Sardinia during 2004 was caused by the BTV-16 vaccine used in Italy during 2004 (Savini *et al.*, 2008) and was not caused by the strain from Greece and Turkey. In November 2008, there were reports of further outbreaks due to BTV-16 in Greek island of Lesvos returning after ~7 years (ProMED 20-JAN-2009), and in both Israel and Oman.

BTV-25: During late 2008, a novel BT-like virus (Toggenburg orbivirus (TOV)) was detected in goats from Switzerland (Hoffman *et al* 2008). Initial molecular epidemiology studies indicate that the maxim sequence identity to any BTV ranged from 63% (segment 2) to 79% (segments 7 and 10). Therefore this virus may represent a previously unknown 25th serotype of BTV and appears to belong to a distinct topotypic group within the *Bluetongue virus* species. Initial attempts to isolate this virus proved unsuccessful, hindering serotyping, although antisera from infected animals failed to neutralise any of the existing 24 BTV types, again indicating that it represents type 25.

Identification of reassortants: Phylogenetic analyses of BTV core / non-structural proteins

In attempts to minimise BTV circulation, live attenuated monovalent vaccines for BTV-2, 4, 8, 9 (western topotype) and BTV-16 (eastern topotype) were used in the Mediterranean region. The South African 'Group B' multivalent live attenuated vaccine (containing types 3, 8, 9, 10 and 11) was also used briefly in Bulgaria during 2000 (Panagiotatos, 2004; Savini *et al.*, 2007). These activities have generated an unprecedented mix of BTV field and vaccine strains within Europe and have provided multiple and widespread opportunities for the exchange / reassortment of genome segments (Monaco *et al.*, 2005; Batten *et al.*, 2008; Nomikou *et al* – in preparation). Reassortment has been detected in genome Seg-3 between western BTV-2 from Corsica and Italy with the western BTV-4 strains from Morocco and Spain (Maan *et al.*, 2008). Similar

comparisons of Seg-5 from the eastern strains of BTV-16 from Greece and BTV-9 from Bulgaria showed 99.9% identity indicating that they have also been involved in reassortment (Maan *et al.*, 2008).

Impact of vaccination

The spread of BTV can be controlled by vaccination of susceptible ruminant populations. Currently two different forms of BTV vaccine are used for this purpose: inactivated, mostly monovalent vaccine formulations; or modified live virus vaccines (MLVs), many of which are available as multivalent preparations from South Africa. There concerns over the virulence of BTV-MLV in naïve animal populations and their transmission / persistence in the field (Veronesi *et al.* 2005). MLVs can also exchange genome segments / reassort with other BTV strains, generating novel progeny viruses, which may have novel biological characteristics (Batten *et al.*, 2008). BTV MLVs have not been licensed for use in northern Europe and authorities in affected countries decided to wait for production of inactivated BTV-8 vaccines. Although inactivated BTV-2 and BTV-4 vaccines had already been used in southern Europe (Savini, *et al.* 2008), almost two years elapsed between the initial BTV-8 incursion in the Netherlands / Belgium in 2006 and the first field vaccinations in the UK during 2008. However, the efficacy of inactivated vaccines has clearly demonstrated by the voluntary vaccination campaign in England and Wales, which prevented re-emergence of BTV-8 in the UK during 2008, after >1000 cases in 2007 (Carpenter, *et al.* 2009).

Polyvalent, cross-serotype, inactivated BTV vaccines are not widely available although a bivalent inactivated BTV-1 and 8 vaccine is now available in Spain (www.fortdodge.eu). The incursion of different BTV types into new European locations is unpredictable and vaccine production is largely 'reactive', potentially resulting in significant periods between incursion and the availability of a vaccine against the homologous type. Some local authorities may therefore consider the use of MLVs, and two distinct MLV's (BTV-6 and 11) were detected in animals in Belgium, the Netherlands and Germany during 2008. It was suggested that they may have been used illegally and were transmitted by northern European *Culicoides* (ProMED 05-FEB-2009; ProMED 18-OCT-2008) (Maan *et al.* in preparation; de Clercq *et al.* 2008).

Conclusions / Discussion

Sequence analyses of European BTV isolates and comparisons to other strains from around the world have shown variations in segments 1, 3, 4, 5, 8, 9 and 10 that correlate primarily with the geographical origins of the virus lineage from which they were derived. These variations divide the viruses into eastern and western topotypes and even into 'local' topotypes within each group (Maan *et al.*, 2008). This indicates that the viruses in these different regions have been separated and have acquired point mutations over a long period of time (Pritchard *et al.*, 1995; Wilson *et al.*, 2000; Balasuriya *et al.*, 2008; Maan *et al.*, 2008).

The involvement of VP7 in BTV infection of insect cells and of NS3 in the release of virus particles from insect cells, suggests that variations in Seg-7/VP7 and Seg-10/NS3 could relate to different groups/populations of insect vectors in different geographic regions from which these viruses were derived (Balasuriya *et al.*, 2008; Maan *et al.* 2008). Further analyses of additional BTV isolates from around the world will help to define the nature and distribution of different topotypes for each of the BTV genome segments.

There is compelling evidence linking local climate change to changes in the distribution of *Culicoides imicola* in southern Europe (Purse *et al.*, 2005). Higher ambient temperatures appear likely to have increased the vector competence of certain northern Palearctic *Culicoides* species in the region. These changes have coincided with outbreaks of BT in Europe, caused by eleven distinct BTV strains from nine different serotypes. Several of these virus lineages (with the exception of the eastern strain of BTV-1 from Greece 2001) have persisted and spread both westwards and northwards across much of southern and central Europe. With the arrival of BTV-8 in northern Europe during 2006, there have been incursions of BTV into Europe in ten of the last 11 years (1998 – 2009). There have also been disease outbreaks caused by persistence and transmission of BTV-vaccine strains in the field (Ferrari *et al.*, 2005; Monaco *et al.*, 2006; Savini *et al.*, 2008). Molecular epidemiology studies have not only helped to identify the origins and

serotypes of the European viruses, they have also confirmed that they arrived via four distinct routes (Maan et al 2009).

Once a *Culicoides*-borne disease (such as BT) becomes established in an area containing competent vector species (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) (Takamatsu *et al.*, 2003, White *et al.*, 2005). The vertical and horizontal transmission of BTV-8 that was observed in Northern Ireland and several other countries in northern Europe, appears likely to provide at least one such overwintering mechanism for BTV (Menzies et al 2008). Continuing changes in global climate may also increase the distribution and competence of vector-insect populations in the region allowing the virus to spread still further north. BTV therefore represents a significant continuing threat to animal health across the whole of Europe. Consequently, effective control / vaccination programmes are required that will reduce disease in affected countries and help to create barrier regions to prevent further spread of these viruses.

It is clear that the use of live BTV vaccines increases the genetic diversity in the virus population and poses a risk of genome segment reassortment between vaccine and field viruses potentially generating virus strains with novel biological properties. The use of live vaccines has clearly not eradicated BTV from Southern Europe. However, inactivated vaccines based on European BTV strains, developed by Merial, Fort Dodge, Intervet and other companies, do not depend on virus replication in the vaccinated host, and are incapable of causing 'vaccine outbreaks' or of becoming involved in genome segment reassortment. In addition, the inactivated vaccine campaign organised by Defra during 2008, appears to have eradicated BTV-8 from the UK, and represents a major success for veterinary science.

Recent studies of orbivirus isolates from Alabama, Missouri and Florida since 1999, using Seg-2 specific RT-PCR assays have identified eight BTV serotypes that were previously exotic to the USA (BTV types 1, 3, 5, 6, 14, 19, 22 and 24) (Johnson *et al.*, 2007; Mertens *et al.*, 2007b; Maan et al – in preparation). These observations indicate that the effects of climate change are not restricted to Europe. They are also unlikely to be restricted to *Culicoides* species or the viruses that they transmit. These changes may therefore affect other vector groups (e.g mosquitoes), potentially changing the distribution and incidence of other arthropod transmitted diseases, both in Europe and elsewhere.

Sources of Information

- Mertens et al 2007b: www.reoviridae.org/dsrna_virus_proteins/btv-serotype-distribution.htm
Mertens et al 2007c: www.reoviridae.org/dsRNA_virus_proteins/ReoID/BTV-isolates.htm
Mertens et al 2007d: www.reoviridae.org/dsrna_virus_proteins/BTV.htm .
Mertens et al 2007e: www.reoviridae.org/dsrna_virus_proteins/ReoID/BTV-S2-Primers-Eurotypes.htm
Mertens et al 2007f: www.reoviridae.org/dsrna_virus_proteins/outbreaks.htm#top
Mertens et al 2007g: www.reoviridae.org/dsRNA_virus_proteins/ReoID/btv-1.htm.
Mertens et al 2007h: www.reoviridae.org/dsRNA_virus_proteins/ReoID/btv-2.htm.
Mertens et al 2007i: www.iah.bbsrc.ac.uk/dsRNA_virus_proteins/BTV2-segment-2-tree.htm.
Mertens et al 2007j: www.reoviridae.org/dsRNA_virus_proteins/ReoID/btv-4.htm.
Mertens et al 2007k: www.iah.bbsrc.ac.uk/dsRNA_virus_proteins/btv4-segment-2-tree.htm.
Mertens et al 2007l: www.reoviridae.org/dsRNA_virus_proteins/ReoID/btv-8.htm.
Mertens et al 2007m: www.reoviridae.org/dsRNA_virus_proteins/BTV-8-Seg-2-tree.htm.
Mertens et al 2007n: www.iah.bbsrc.ac.uk/dsRNA_virus_proteins/ReoID/btv-9.htm.
Mertens et al 2007o: www.iah.bbsrc.ac.uk/dsRNA_virus_proteins/btv9-segment-2-tree.htm.
Mertens et al 2007p: www.reoviridae.org/dsrna_virus_proteins/ReoID/btv-16.htm.
Mertens et al 2007q: www.iah.bbsrc.ac.uk/dsRNA_virus_proteins/btv16-segment2-tree.htm.
BTV isolates by country: www.reoviridae.org/dsRNA_virus_proteins/ReoID/virus-nos-by-country.htm
BTV isolates by serotype: www.reoviridae.org/dsRNA_virus_proteins/ReoID/BTV-isolates.htm

BTV accession numbers: www.reoviridae.org/dsRNA_virus_proteins/orbivirus-accession-numbers.htm
European outbreaks: www.reoviridae.org/dsRNA_virus_proteins/ReoID/BTV-mol-epidem.htm
BTV RNA and proteins: www.reoviridae.org/dsRNA_virus_proteins/BTV.htm
BTV serotype distribution: www.reoviridae.org/dsRNA_virus_proteins/btv-serotype-distribution.htm

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