final report

Project code: B.AHE.0036

Prepared by: P.D. Kirkland, L. Melville and G. Bellis

NSW Department of Primary Industries; NT Department of Primary Industry and Fisheries; NAQS, Department Agriculture, Australia

Date published: May 2014

ISBN: 9781740361972

PUBLISHED BY
Meat & Livestock Australia Limited
Locked Bag 991
NORTH SYDNEY NSW 2059

Detection of Bluetongue virus and vectors to enhance surveillance

Meat & Livestock Australia acknowledges the matching funds provided by the Australian Government to support the research and development detailed in this publication.

Abstract

Insect borne bluetongue viruses present a disease threat to Australian livestock and a major impediment to trade. With increasing volatility of weather patterns, particularly rising temperatures, southern extension of the distribution of vectors is likely, threatening large sheep populations and rendering large numbers of sheep and cattle ineligible for export. Exotic viruses and vectors continue to enter northern Australia in association with severe weather events. Real time PCR assays have been developed that allow the rapid detection and identification of exotic vectors and the concurrent detection and identification of bluetongue viruses that they are carrying. Results can be obtained for several hundred samples in a day. Current methods may take weeks. These assays are also extremely sensitive and have the potential to detect a single exotic midge in a collection containing more than 20,000 other insects. For virus identification, at present a single test can identify a maximum of 5 serotypes from the potential 10 serotypes present in Australia, or the 26 identified globally. However, a very sensitive prototype assay platform based on magnetic bead technology that will theoretically allow detection of all known serotypes has been developed. Collectively these assays have the potential to revolutionise both insect and virus detection and identification.

Executive summary

Viruses that are spread by insect vectors (arboviruses) present a disease threat to Australian livestock. They are also a major impediment to trade for both live animals and semen and embryos. Viruses belonging to the bluetongue group generally have the greatest impact. Bluetongue viruses (BTV) currently restrict live ruminant exports to many valuable markets. In other countries, these viruses are an important cause of disease in sheep, causing deaths and loss of production. While there are strains of BTV in Australia that have been shown experimentally to cause severe disease and death in sheep, these are currently confined to the far north of Australia. Ultimately the spread of BTV is determined by the movement of their insect vectors which in turn is influenced by climatic conditions, most notably temperature and rainfall. With increasing volatility of weather patterns, particularly rising temperatures, southern extension of the distribution of midges from the genus *Culicoides* is likely, taking with them a range of arboviruses. If pathogenic strains of bluetongue move further south and east, large sheep populations will be at risk of disease outbreaks. Further, some regions currently certified as bluetongue free for export purposes would become zones of possible virus transmission, rendering them ineligible for trade. Recent arbovirus research has also shown that there is occasional entry of exotic bluetongue viruses and vectors into northern Australia in association with severe weather events. Collectively, these changes indicate that the bluetongue situation in Australia is at present unstable and there is potential for these viruses to impact heavily on ruminant health and production.

The key achievements of this project were:

- The elaboration of nucleic acid sequence data for the known *Culicoides* vector species of BTV in Australia as well as the 2 exotic vector species considered as the greatest threat;
- Identification of non-destructive methods for the concurrent purification of DNA from midges and RNA from BTV;
- Development of *Culicoides* species-specific real time PCR assays that allow the rapid detection and identification of midges;
- Application of midge specific assays to evaluate light trap collections of insects from the National Arbovirus Monitoring Program (NAMP);
- Evaluation of the capacity of real time PCR assays to detect exotic *Culicoides* species in NAMP collections;
- Capability to quantify the major vector *C. brevitarsis* in NAMP collections;
- A capacity to detect BTV in insects and to identify insects that are potential vectors of BTV;
- Demonstration of the capacity of these assays to detect BTV in *Culicoides* collections
- Development of novel assays for the detection and identification of BTV

This project has clearly demonstrated the advances that can be made as a result of new nucleic sequencing technologies and the power of real time PCR. While identification of different *Culicoides* species has in the past depended on laborious microscopic examination of specimens by highly trained entomologists, the assays developed during this project make it possible to rapidly screen collections of many thousands of insects and detect a single insect that could be a vector of BTV. As shown during this study, this insect could easily be overlooked by microscopic screening. Without sorting through an entire light trap collection and counting each of many thousands of insects (amongst a large quantity of extraneous ‘by-catch’), it may be possible to quantify the numbers of vector species present to a level of accuracy equivalent to that of microscopic screening. While microscopic examination

could take several hours to examine a single large collection, it is possible in the same time to process up to 100 fold more collections with these assays. Nevertheless, the assays that have been developed thus far are not 'perfect' and from time to time, for reasons that have yet to be elucidated, false "positive" results are encountered. Whether the presumptive detection of a vector species is the result of residual DNA fragments from damaged insects or cross reactivity from closely related hybrids is not clear. However, the use of 'non destructive methods of nucleic acid extraction ensure that the cadavers of the specimens being examined are still available for morphological examination by an entomologist. This technology is not intended to replace the expert *Culicoides* entomologists (few of whom are available nationally) but rather to free them from 'routine examinations and enhance the national entomology capacity. In a similar manner, if a real time PCR assay for an exotic species gives a positive result, it is possible for this result to be confirmed by an entomologist by microscopic examination of the available cadavers. These assays will however potentially challenge the perseverance of the professional entomologist as they have the potential to detect a single exotic midge in a pool of perhaps 20,000 insects.

For BTV detection, several of the assays developed offer major improvements. While BTV detection has depended on laborious cultural methods that can take 5-6 weeks, followed by further time to characterise any virus detected, real time PCR assays can not only directly detect virus in either the blood of sentinel cattle or in *Culicoides* but will also allow the concurrent identification of the serotype of BTV that is present. Results can be obtained for several hundred samples per day. During the course of this project, assays were evaluated to identify several of the more common serotypes of BTV, with some offering optimal results. Further research is required to return optimal results for some serotypes, most notably serotype 1, the most commonly encountered virus in Australia. For virus detection in insects it has been possible not only to detect virus in the body of midges but also to identify insects in which virus replication has occurred and the level of virus, immediately confirming that these are probable vector species. These results can be obtained on the same nucleic acid extract that is used to identify the species of *Culicoides*. While real time PCR assays have great potential, they do have some limitations. At one time, a single test can identify a maximum of 5 serotypes from the potential 10 serotypes present in Australia, or the 26 identified globally. However, a prototype assay platform based on magnetic bead technology that will theoretically allow detection of all known serotypes has been developed and with similar sensitivity to real time PCR. In proof of concept studies it has been possible to detect several serotypes concurrently. Given time, it should be possible to develop a system that can, at the least detect all BTV serotypes present in Australia in a single assay.

This project has shown that real time PCR based assays can be used to rapidly detect both individual *Culicoides* species and BTV in either sentinel cattle or insects. It is expected that in the near future some of the assays developed during this project can be transferred to other laboratories servicing the NAMP to confirm their utility. Preliminary use of these assays in one other laboratory indicates that these assays are very robust and reproducible. If these assays are employed on a routine basis for arbovirus and vector surveillance in NAMP, they offer a range of benefits including the capacity to rapidly detect incursions of exotic vector species, identify serotypes of BTV being transmitted in insects, facilitate studies of vector capacity and offer potential for novel BTV surveillance techniques in remote areas where sampling of sentinel cattle is not possible. As well as more efficient and rapid virus and vector surveillance, it is possible that there may be a reduction in surveillance costs. During the last year of this project, the detection of some serotypes of BTV in Queensland for the first time suggests that the BTV situation in Australia is no longer stable and

the extension of this technology to other serotypes of BTV is warranted. With modest modifications it would also be possible to use this technology for the detection of other arboviruses (such as Epizootic Haemorrhagic Disease Virus (EHDV) and orthobunyaviruses) that have recently attracted global attention.

Table of Contents

1 Background

Biting midges of the genus *Culicoides* are biological vectors of a number of important arbovirus infections of animals. The most well known is BTV which has gained renewed international prominence as several novel serotypes have spread across Europe, resulting in severe disease outbreaks. Furthermore, until recently, strains of BTV that have the potential to cause severe disease in sheep have been confined to the far north of the NT and perhaps the extreme north of WA. Although the reasons for this limited distribution are not understood, the fact that these serotypes have not moved out of this area has provided sheep producers in Australia with a small degree of 'comfort'. However, perhaps 2 or more of the potentially pathogenic serotypes have recently been detected in the Cape York region of north Queensland and there have also been 2 new incursions of previously exotic serotypes detected in the restricted zone of the NT in the last few years. Further, during the course of this research project, serotype 2, one of the serotypes recently introduced to the NT was detected in south western Queensland. It is not known whether the viruses detected in Queensland have moved from the NT or whether they are also new incursions from offshore. Both options may be likely. Regardless, there are signs that the BTV situation in northern Australia is not stable and risks of the southern movement of pathogenic strains towards sheep populations may be increasing. There could also be introductions of other vector borne viruses but this project is limited to the study of BTV and their known vectors.

As the distribution and biology of the insect vector governs the spread of viruses such as BTV, it is important to be able to rapidly identify midges and monitor their geographical distribution. Further, it is essential to understand which *Culicoides* species are the vectors of a serotype of BTV as there are *Culicoides* spp found throughout most of Australia but very few of these are vectors of BTV and these species have a well defined but fluctuating distribution. Their range varies under climatic influences and an expansion of this range, or the incursion of a new species, could rapidly alter the disease status of a country like Australia. An increase in average temperatures, perhaps more likely in the future, would not only allow midges to survive further south but also increase their potential to become infected with BTV and enhance the efficiency of virus transmission.

Current vector monitoring techniques employed under the NAMP involve collection using light traps and microscopic examination of the catch to remove non-target insects then identification and counting of individual specimens of *Culicoides.* The volume of non-target insect by-catch can be considerable and the removal midges from by-catch can be very time consuming. Identification of midges is not only very time consuming but also technically demanding as many midges are similar and identification depends on variations in wing patterns, mouth parts and other features. The ability to differentiate these insects on morphological features is held by only a small number of entomologists. In the last few years, molecular based genotyping techniques have been applied to identify genetic markers for individual species. Initial research in Europe has shown the benefits of this technology to rapidly identify midges, to differentiate difficult species and provide information on potential vectors of BTV. A prerequisite to developing assays for *Culicoides* identification is the availability of nucleic acid sequence data from sections of the midge genome that may allow differentiation between midges and other insects and the identification of individual *Culicoides* species. A collaboration initiated between Dr A. Mitchell (Australian Museum), Dr D. Gopurenko (NSW DPI, Wagga Wagga) and Dr G. Bellis (NAMP reference entomologist) has generated preliminary data for all of the important *Culicoides* that are currently present in Australia. Genetic analyses based

on more than 100 sets of sequence data from Australian *Culicoides* confirms that they can usually be reliably divided into separate species, consistent with the morphological identification. When these data are combined with additional data obtained from Japanese collaborators for almost 300 insects, not only can individual species be recognised but midges from a single species can be identified to their country of origin. While this research has not yet been completed, the existing data set is robust and sufficient to allow its use in other projects, such as the development of species-specific assays based in real time PCR (qPCR) technology.

Preliminary studies undertaken at Elizabeth Macarthur Agriculture Institute (EMAI) leading to this project showed that it is possible to develop species-specific qPCR assays. Additionally, the use of qPCR assays were expected to allow large scale screening to be rapidly undertaken to confirm that the sequences used in these prototype assays are unique, or that minor adjustments to primer and probe sets may be required. In parallel with the development of prototype midge-detection assays, studies were continuing at EMAI to identify a qRT-PCR that would reliably detect all Australian serotypes of BTV. However, it would be preferable, at least for the detection of BTV incursions, to use an assay that is capable of detecting all 26 known serotypes of BTV. Collaborations between EMAI and colleagues in Germany, Switzerland and the UK have the aim of evaluating pan-reactive qRT-PCR assays for BTV. Sequence data for Australasian *Culicoides* species have become available at a time to coincide with the development of these BTV assays. Collectively, this information is sufficient to support research for the development of insect species-specific assays and concurrent virus detection in insect samples. These techniques need to be evaluated in a series of field studies to provide essential tools that are important to improve our understanding of bluetongue disease risks in Australia and enhance future surveillance capacity.

2 Project objectives

The objectives of this project were:

1. To obtain a comprehensive DNA sequence data for major Australian *Culicoides* species from selected locations over their entire geographical range;

2. To obtain additional sequence data for midges that have the potential to present an incursion threat

3. To define essential reagents and develop diagnostic tests for the detection of Australian *Culicoides* species that are considered to be vectors or probable vectors of BTV.

4. To evaluate *Culicoides*-specific assays by testing of NAMP insect collections from a range of locations

5. To undertake studies to identify optimal methods for the detection of BTV in insect collections, (preferably in a manner that will retain the major morphological features of the insects to allow conventional identification if needed) and to allow insect identification assays to be undertaken on the same preparations.

6. To investigate assays for the rapid identification and typing of BTVs based on new real-time PCR and micro-array technology.

7. To apply, ultimately, prototype BTV assays on insect collections from various locations within the BTV endemic zones in Australia, to evaluate the suitability of this technology for bluetongue monitoring in remote locations and for the identification of potential vectors of BTV.

3 Methodology

3.1 Assays for identification of insects

To support the development of diagnostic assays based on real time polymerase chain reaction (qPCR) technology, nucleic acid (DNA) sequence information ("DNA barcoding") was required for each of the midge species that are proven or suspected BTV vectors in Australia, as well as key species of midges that present a threat through arrival in Australia from neighbouring countries. An important consideration in developing these assays was the availability of midges that had been reliably identified to species level. To achieve this, all samples selected for analysis had been individually identified by entomologists who either service the NAMP or who are engaged by the Australian Department of Agriculture to monitor Northern Australia and offshore islands for the arrival of exotic species of *Culicoides* under the Northern Australia Quarantine Strategy (NAQS). Samples from the important Australian species were usually obtained from existing NAMP collections across the known range throughout Australia, although on occasions special collections were made to obtain some of the less common insects. All insects used had been preserved in either 70% or 95% ethanol and stored for no longer than 8 years.

Exotic species were collected during overseas visits by NAQS staff or overseas collaborators. As well as species that are exotic to Australia, in some instances, offshore samples were also obtained for species that are already present in Australia. For example, the most common BTV vector in Australia is *C. brevitarsis*. Samples of this species were obtained throughout its known range, including Japan, East Timor, Papua New Guinea and the Solomon Islands. Other species such as *C. actoni* were also obtained from Indonesia. While the focus has been on the vector or potential vector species, other midges have been not been excluded. It was important to compare their DNA profiles with the target species to confirm that they can be distinguished and also to confirm that there is no cross reactivity with irrelevant midges in species-specific assays. The identity of exotic midge species, or of any species where unusual sequence data was obtained, was usually confirmed by the National Reference Entomologist for *Culicoides*, Dr Glenn Bellis.

In addition to accurate identification, an essential requirement for the development of a large DNA library was to extract DNA from insects individually. Owing to the extremely small size (1-2mm) of most *Culicoides* species, identification and subsequent DNA extraction procedures are extremely demanding when insects have to be handled individually and separately from any other midges to minimise any potential for cross contamination. As the genetic data obtained could potentially challenge morphological criteria for midge identification, DNA was extracted from individual midges using non-destructive techniques. Soft tissues from the insects were digested in 120µL of an enzyme solution, leaving the cytoskeleton intact. This was retained and allowed further taxonomic investigation if DNA sequence data revealed conflicting data for samples within a recognised species. DNA was subsequently purified from 50µL of the enzyme digest, using a magnetic bead based purification system (MagMax96 viral RNA, LifeTechnologies, Austin Texas, USA) together with a semi-automated magnetic particle handling system (Kingfisher 96, Thermo, Finland). DNA sequencing was directed at the COI1 gene which is

commonly used for similar studies in other insects. Purified DNA was amplified by PCR and the sequence determined at a commercial sequencing facility. DNA was extracted and purified from approximately 5250 individual midges across more than 70 recognised species. Due to the large number of insects examined, work on this scale, although of immense value to this project, was beyond the scale originally envisaged and was part-funded through collaboration with a related NSW DPI insect taxonomy ("Bar-code of life") project managed by Mr D. Gopurenko and Dr Andrew Mitchell. Purified DNA extracts were prepared through the MLA project and sequencing was undertaken by NSW DPI. Data were shared between the 2 projects. Reliable sequence data were obtained for approximately 1200 midges and used for assay design. The DNA extracts from most of the other midges were used for assay validation.

DNA sequence data were examined and aligned to identify nucleotide sequences that were unique to a species of interest but were conserved within all samples available for that species. PCR primers and a fluorophor labelled probe were then selected using Primer Express software to provide optimal amplification and detection of the target DNA sequence. Real time PCR (qPCR) assays were run using 5µL of purified DNA and AgPath mastermix (LifeTechnologies, Austin Texas, USA) and run on an ABI 7500 Fast Real Time thermocycler. Prototype assays were run under standard cycling conditions for the mastermix to test a representative selection of samples for the species of interest. If the assay did not detect all midge extracts examined, or the assay efficiency appeared to be suboptimal, based on cycle-threshold (Ct) values, cycling conditions were varied until an optimal assay conditions were identified.

As it is not practical to test all insects in NAMP collections on an individual basis, testing was initially limited to the DNA extracts that were used for sequencing. Apart from making efficient use of the extracts, if any anomalous results were obtained, both the sequence data and the cadaver could be examined to confirm the authenticity of the insect identification. Due to the size of the insect collection and sequence database, a subset of more than 1000 DNA extracts, varied by both species and geographical location, were then tested to assess the performance of assays, firstly in homologous tests (i.e. testing of the species for which the assay was designed) as a measure of assay sensitivity and then in heterologous tests (i.e. testing of other species) as a measure of assay specificity. Using this approach, assays were evaluated for the known Australian vector species (*C. actoni, C. brevitarsis, C. fulvus and C. wadai*) and for the exotic species *C. nudipalpis and C. orientalis*.

During the evaluation of assays, it was found that the prototype assay for *C. brevitarsis* did not detect most of the insects from Japan and other parts of SE Asia that were morphologically identified as *C. brevitarsis*. A new "pan-brevitarsis" assay was developed that would detect both Australian and offshore strains of this midge. However, further evaluation of the nucleic acid sequence data by collaborators under the "Bar Code of Life" project has concluded that off-shore collections that have been traditionally recognised as *C. brevitarsis* are indeed a separate species. Consequently, the original *C. brevitarsis* assay was used as the primary assay for this major Australian species.

Ultimately it is expected that the *Culicoides* specific assays will be used to screen entire NAMP collections as one or more large pools rather than as individual insects. Before NAMP collections were tested, several studies were undertaken to investigate the potential to test a large pool of insects as they would be collected in the field. Firstly, the limits of detection of a single insect in a large pool were estimated from

the DNA concentration of a single insect. As qPCR is quantitative, the Ct values could provide a guide to the relative concentration of target DNA and in turn the extent to which the targeted DNA from a single insect might be diluted in a pool of insects. As the Ct values suggested that samples might be diluted significantly and still return a positive result (or be detected in a larger pool), as a proof of principle study, DNA extracted from individual insects was added to an equal volume of individual extracts from large numbers of other insects to provide estimates of the limit of detection. Individual insects from the target species were also added to pools of other 'irrelevant' insects. During these "limit of detection" studies non-destructive methods that had been developed for individual insects were modified to extract total DNA from large numbers of insects in a single mixed pool, as would be encountered in a field collection. Up to 1ml (settled volume) of insects (or a total of approximately 3000 midges) were added to 1mL of digestion buffer, resulting in a concentration of the ratio of midge to digestion buffer of about 8 fold (in contrast individual insects had been placed in 120µL of buffer solution).

To evaluate the sensitivity of assays for exotic midge species, pools of insects were created using known numbers of identified insects from NAMP collections to which varying numbers (from 1 to 5) of the exotic species *C. nudipalpis* and/or *C. orientalis* midges were added. Insects in these 'spiked' midge pools were digested in the optimal manner that had been identified in previous studies. The DNA was then purified using the standard magnetic bead based extraction methods. Each pool was then tested firstly in assays for *C. brevitarsis*, *C. nudipalpis* and/or *C. orientalis* and later in assays for *C. actoni*, *C. fulvus* and *C. wadai.*

For final validation of the *Culicoides* species-specific qPCR assays, a random subset of NSW NAMP collections for 2011-12 that were distributed across both the NSW vector and vector free areas were obtained. Digest solutions from this NAMP collection and the samples spiked with exotic midges were tested both at Berrimah Agricultural Research Centre (BARC), Darwin and at EMAI to assess the robustness and reproducibility of these assays.

The entire NSW NAMP insect collections for 2012-13 and a selection of collections from northern Australia (NT and WA) were also tested at EMAI, using the pooling methods that had been established. All midge extracts were tested in the 4 assays for Australian vector species (*C. actoni, C. brevitarsis, C. fulvus* and *C. wadai*) and assays for the 2 exotic species (*C, nudipalpis* and *C. orientalis*) The 2012-13 collections from NSW and northern Australia were also tested for the presence of BTV.

3.2 Assays for detection and identification of bluetongue viruses

3.2.1 Real time PCR assays

Before the start of this project, several published assays based on real time reverse transcription PCR (qRT-PCR) technology had been evaluated for the detection of BTV (BTV). One assay (Hofmann et al 2008a, 2008b) was shown to be broadly reactive with a capacity to detect all known (26) serotypes of BTV, based on testing of cell culture propagated virus. Studies conducted at EMAI had shown that it had very high analytical sensitivity. However, there had been limited assessment globally of its performance for the direct detection of BTV in animal blood or other specimens such as semen and insects. In the current project there were three main aims:

- to assess the capacity of real time PCR assays to detect BTV directly in blood of animals;

- to establish and evaluate methods to detect BTV in insects
- to establish real time PCR assays that are specific for an individual serotype of BTV and preferably have the capacity to identify individual serotypes in field specimens (either cattle blood or insects) without prior amplification in cell culture.

The goal was to evaluate serotype-specific assays for BTVs 1, 16, 21 and 23. Serotypes 1 and 21 were selected because they are the most widely distributed and most frequently detected serotypes in Australia, and the only serotypes detected in south eastern Australia; serotypes 16 and 23 were suggested as Australian serotypes that had been shown to be highly pathogenic in experimentally infected sheep. However, following the detection of BTV2 in southern Queensland, which was considered to be moderately pathogenic to sheep, priority was given to assays for the detection of BTV2 in preference to BTV16 and 23. There was however still limited testing of prototype assays for BTV23. It was beyond the scope of this project to evaluate a larger number of serotype-specific assays.

Specifications for the prototype serotype-specific assays were provided by the Institute of Animal Health (IAH), Pirbright, England under a collaboration agreement with EMAI. During the design of these assays, to detect an individual serotype reliably, on a global basis it was found that it would be necessary to run 2 separate assays for many of the serotypes. RNA differences between BTVs from the 'eastern' and 'western' hemispheres (with a cross-over in the region of Pakistan) are sufficiently great that it was not possible to design a single assay. Nevertheless, in Australia, in the first instance, it should only be necessary to use one set of assays (Eastern genotypes) for each serotype. The IAH primer and probe specifications were used to assemble individual real time qRT-PCR) assays using 5uL of purified RNA and 20uL of AgPath mastermix (LifeTechnologies, Austin Texas, USA) and run on an ABI 7500 Fast Real Time thermocycler. Prototype assays were run under standard cycling conditions for the mastermix. Prior to running the qRT-PCR, the double stranded RNA in each sample was denatured by heating at 95^{o}C for 5 minutes.

A systematic approach was taken to evaluate these assays. In each instance, samples being used for assay validation were tested in the pan-reactive assay followed by the serotype-specific assay under investigation. Within a serotype the emphasis was on the evaluation of serotype-specific assays for Australian strains of BTV. Firstly, cell culture preparations of reference viruses were tested. In addition to being well characterised reference strains, the quantity of virus is high and the sample has few contaminants that could affect assay performance. In contrast, field collected blood samples are more likely to contain inhibitors of PCR and may also contain RNA from multiple strains. If assays performed in an acceptable manner, their suitability to detect field strains of BTV directly in the blood of both experimentally and naturally infected cattle was investigated. The very sensitive pan-reactive qRT-PCR assay was used as a benchmark for assessment of analytical sensitivity.

Although evaluation of serotype-specific assays was limited to key Australian serotypes, a large reference panel of viruses was used to ensure assay specificity. The reference panel in use consists of 53 viruses and includes viruses for serotypes 1-24, each of the prototype Australian isolates (10 viruses, which in some instances are also the international prototype), 7 South African vaccine strains and a selection of Australian viruses (12 samples) that have novel genotypes, mostly reassortants with SE Asian viruses. The size of panel alone generates a significant volume of work because each sample is tested in each serotype-specific assay, firstly to ensure

that the homologous strains are reliably detected and secondly to check that there is no cross reactivity.

Once an assay had been shown to give acceptable performance on the reference panel, collections of both cell culture adapted field viruses and original cattle blood samples were tested. Samples were tested from sentinel cattle in NSW (2011-2013), NT (2010-2012) and Qld (2010-2012) and, in some instances, from experimentally infected animals. More than 1200 field blood samples and about 170 samples of cell culture adapted virus were tested.

3.2.2 Microsphere based assays

In the original project proposal Objective 6 included a proposal to investigate the suitability of microarray technology for the identification of BTV to serotype level. However, experience with testing for influenza viruses in birds indicated that assays based on Luminex microsphere technology may be more appropriate than those based on micro-array technology and could have a number of advantages. Unlike microarray technology, new assays using Luminex technology can be user-developed for individual targets then combined with existing assays. In contrast, microchips that form the foundation of microarray assays are usually manufactured commercially and cannot be changed unless a new batch of chips is produced. The change of platform in the project was approved and research focussed solely on micro-bead based assays (using MagPix magnetic bead technology) for rapid virus detection and identification was initiated. This assay involves initial amplification of the nucleic acid by RT-PCR with concurrent incorporation of biotin labelled nucleotides. The amplified DNA is then captured on magnetic microspheres and a streptavidin labelled fluorophore is added to enable detection of the final product. The amount of fluorescence detected is related to the amount of amplified nucleic acid. The technology enables detection of multiple targets by the use of microspheres of slightly different colours. The detector both identifies the colour of the bead from which the signal has been detected as well as quantifying the signal from all beads of that colour. Different targets can then be detected simultaneously by the use of a different bead colour for each target. In theory up to 50 different ‘targets’ or individual assay components can be detected concurrently. A high degree of multiplexing would be ideally suited to the detection of different BTV serotypes.

As a “proof of principle”, to investigate the suitability of Luminex technology to detect different bluetongue serotypes, studies were firstly directed at the development of an assay for BTV21. This serotype was selected because it is both widespread in Australia and the corresponding real time PCR assay has a high level of sensitivity, against which comparisons could be made. A prototype BTV21 assay was evaluated using the reference virus panel. An assay for BTV2 was also developed so that the multiplexing capacity of the system can be investigated. A number of reference virus isolates and a limited number of field isolates and blood samples were also tested.

3.2.3 Detection of BTV in insects

To readily evaluate new assays, it is important to have available a large number of BTV infected midges. As none of the Australian species of *Culicoides* have been successfully colonised, all BTV research involving vectors in Australia is dependent on the use of wild caught insects, with obvious logistical constraints. As infection rates in these midges are usually very low and infection in the field can be extremely variable, to reliably complete these studies, midges were experimentally infected with virus. These experimental studies were done in close collaboration with the Biosecurity NSW PhD student Matt van der Saag who was investigating aspects of

the biology of BTV infections in midges. His PhD project has as one objective the development of methods to reliably feed and raise large numbers of *C. brevitarsis*. Due to the related objectives in this MLA project and the PhD project, there has been close collaboration and some of the research efforts have been combined, especially to experimentally infect midges. Consequently, some of the data have also been shared and described in both projects. However, to avoid unduly compromising the project that had primary responsibility for a research area, only an overview of methods and data has been presented in the complimentary project.

When *Culicoides* midges feed on a BTV infected animal, initially, the only virus present in the insect will be associated with the blood meal and restricted to the gut. Due to the extremely small blood volumes and potentially low virus levels in cattle, if a midge has taken a blood meal containing virus, the virus levels will be very low and may challenge the sensitivity of an assay. Later, if virus replicates in the insect and is present in the salivary glands, virus levels are likely to be much higher and to a titre that should be detected by a sensitive real time PCR assay. As the proportion of BTV infected insects in the field is usually extremely low, and has a distinct seasonal pattern with limited duration, several approaches were taken to experimentally infect insects. These included:

- direct inoculation of insects with virus;
- artificial feeding of insects on various blood-virus mixtures;
- feeding of insects on chicken embryos that had been infected with BTV.

a) Sources of insects

C. brevitarsis has been the main species of interest because these midges are the main vectors of BTV in Australia, and especially of the most commonly encountered serotypes, BTV1 and BTV21. These insects are usually abundant in northern and central coastal NSW during the summer and autumn and are often found in larger numbers than in northern Australia. However, due to a combination of the unusually wet and cool summer in 2011/12, similar to the situation in Darwin, midge populations were much smaller than usual and, when present, their abundance on a daily basis was extremely variable. After difficulties in obtaining sufficient insects by direct collection from the wild, attempts were made to laboratory rear midges. To achieve this, batches of cow dung were collected from northern and central coastal NSW and the midges hatched from the dung pats, collected in cages (paper cups covered with midge proof mesh) and artificially reared.

Initially, midges had limited longevity but with modification of methods and experience, it was possible to raise a high proportion (>90%) of midges for up to 30 days. Such a time span is important for vector infection studies because it is necessary to maintain insects to at least beyond an incubation period for the virus in the midge, thereby providing maximum opportunity for a disseminated infection to occur. These insects have been fed artificially on cotton pads soaked in a 10% sucrose solution.

b) Direct inoculation of midges

For this stage of the project, the plan was for collaborators at BARC Darwin to collect large numbers of midges and to experimentally infect them by direct injection and send the cadavers of the infected midges to EMAI for further study. *Culicoides peregrinus* had been identified as the target species because these are relatively large midges, of sufficient size to allow intra-thoracic inoculation. Once the gut barrier has been by-passed by the use of inoculation, BTV will replicate in a very high proportion of insects. However, as a result of adverse environmental conditions in Northern Australia in 2011, it was not possible to collect any *C. peregrinus* when originally planned and, to avoid delays, other methods were utilised as described

below. In 2012 environmental conditions were more suitable and it was possible to collect *C. marksi* for inoculation. A total of 81 midges were inoculated with BTV1. Full details of the inoculation procedure are described in Appendix 1.

c) Artificial feeding methods

Alternative approaches to direct inoculation of insects include feeding wild caught midges on infected animals or attempting to experimentally infect midges by artificial feeding techniques. As there are considerable advantages in using midges that have been infected by the oral route, thereby simulating a natural route of infection, a decision was made to work with *Culicoides brevitarsis* collected in NSW, recognising the limitations and logistical constraints that this would impose as this is physically the smallest of the midges.

A series of individual experiments were conducted. In each instance, midges were provided access to a sucrose meal on the day of emergence and daily until the time of infection except that they were always starved for 24 hours prior to the inclusion of BTV in the sucrose. Midges were held at approximately 25^{o}C and were allowed access to BTV spiked 10% sucrose overnight. To monitor the uptake of virus, inert, brightly coloured food dye was included in the sucrose to give a relatively intense colour so that after feeding, midges that had fed were easily identifiable (bright green or blue abdomens). Samples of the original virus preparation and virus spiked sucrose were retained on each occasion for comparison with nucleic acid levels extracted from the insects.

On the day after provision of sucrose containing virus, all insects were examined and sorted into fed and unfed. Studies continued separately on the insects that had fed. At this point all sources of virus were removed and insects were placed in new cages to ensure there was no source of virus and to reduce the possibility of ongoing external contamination from the environment in which they were housed. In order to explore the capacity of assays to detect BTV at different stages of infection and circumstances, a number of aspects were studied including:

- Infection of midges of different ages (at 1 and 5 days post emergence);
- Infection of both females and males;
- Comparison of virus level in insects with the virus concentration in the meal immediately after feeding;
- Monitoring of virus levels over time, for up to 25 days post exposure;
- Comparison of destructive and non-destructive nucleic acid extraction methods

d) Embryonated chicken egg model

While it was possible to infect some midges by feeding on virus spiked sucrose/blood mixtures, these methods did not reflect natural feeding behaviour. The use of embryonated chicken eggs was investigated as an alternative to feeding on captive ruminants. Chicken embryos have many advantages in terms of availability in large numbers, cost, no need for restraint and the possibility of prior infection/immunity does not need to be considered. Embryonated chicken eggs are recognised to be one of the most sensitive methods for the culture of wild (non-laboratory adapted) strains of BTV. The embryos were infected at 10-11 days of age by intravenous inoculation in the same manner as is used for isolation of BTV. Virus levels in the chicken embryos were monitored by taking blood samples at regular intervals and testing for BTV RNA by qRT-PCR. When peak levels of viraemia were achieved (bout 48-72 hours after inoculation of the embryos), the shell was removed over the air sac to expose a number of blood vessels and a small ‘cage’ containing midges was placed over the membrane and attached to the shell. The midges were anaesthetised using CO_2 and those that had taken a blood meal were removed from

the cage and reared on sucrose. Samples were taken daily for 14 days to monitor virus levels in midge blood by qRT-PCR.

e) Viral nucleic acid extraction and qRT-PCR

Initially, the non-destructive digestion methods that had been used to extract midge DNA for genetic typing were compared with destructive methods for the extraction of BTV RNA. Whole midges were ground individually in a solution of phosphate buffered gelatine saline. Total nucleic acid was then purified from both insect homogenates and enzyme digests using the magnetic bead nucleic acid purification methods described previously (see section 3.1). The established high throughput pan BTV-reactive qRT-PCR method (See section 3.2.1) was used on all insect samples to detect and quantify BTV RNA. The capacity to quantify viral RNA levels in insects proved to be invaluable. While low virus levels (high Ct values) were likely to be residues from a blood meal, high virus levels (low Ct values) can only be the result of virus replication. By studying insects at different stages after feeding on BTV infected chicken embryos, it was possible to develop profiles for BTV detection in insects that would show whether there had been virus replication in the midge or whether the RNA detected was more likely to be the residue of a blood meal from an infected animal. The methods established for BTV detection in insects were applied to the NAMP collections from NSW in 2012 and 2013 and from a limited collection from northern Australia (NT and WA) in 2013.

4 Results and discussion

4.1 Assays for the identification of Culicoides species

The first objective of this project was, in collaboration with the “Bar-code of Life” project, to obtain nucleic acid (DNA) sequence information (“DNA bar coding”) from each of the midge species that are known to be vectors or are potential vectors of BTVs in Australia, as well as key species of midges that present a threat through arrival in Australia from neighbouring countries. Using non-destructive techniques that leave the cytoskeleton intact, DNA was extracted and purified from approximately 6000 individual midges across more than 70 recognised species. Reliable sequence data was obtained for approximately 3000 midges. DNA profiles are available for all of the major Australian *Culicoides* species known to be vectors of BTV as well as for a number of exotic species of *Culicoides*. Analysis and review of these sequence data will inevitably be on going as nucleic acid sequencing is rapidly becoming an essential element of taxonomic classification. The cytoskeletons have been retained and will allow further taxonomic investigation if required. Examples have already emerged where some midges thought to belong to a single species are most likely to be a complex of several closely related, morphologically similar insects.

Real time PCR assays have been developed for the 4 major Australian species (*C. actoni*, *C. brevitarsis*, *C. fulvus* and *C. wadai*) and for 2 exotic species (*C. nudipalpis, C. orientalis*). Using a ‘perfect’ real time PCR assay with optimal efficiency, if is possible to use ‘absolute’ reactivity (Ct values) without a need to set cut-off values. However, these assays have been rarely applied to detection and identification of DNA from midges and it is not known whether it is possible to achieve the same levels of performance as an assay for detection of a virus. Further, as these insects usually come from mixed collections, the possibility cannot be excluded that there was residual DNA from other damaged insects. This could contribute to the apparent lack of specificity. Finally, on some occasions, the possibility that the midges being tested are actually hybrids of at least 2 species cannot be excluded. This would clearly confound the analysis. Consequently, a Ct cut-off of 33 was chosen and this

resulted in very significant improvement of the relative test performance. To put this Ct value into perspective, it could represent detection of 10-100 copies of the target DNA whereas the reactivity detected in individual insects from the homologous species was usually 1,000 to100,000 times greater. It was decided that this approach was not unreasonable for this application. When testing samples from midges collected in Australia and applying a 'corrected' cut-off value, the assays for 3 of the 4 major Australian species appear to have a good level of sensitivity and specificity, detecting most of the midges from the respective species but with limited cross reactivity with other species (Se=97.6-99.8%; Sp=96.6-99.7%) (Table 1). The assay for *C. fulvus* showed a lower but acceptable level of performance (Se=84.9%, Sp=86.5%). On most but not all circumstances, there were lower levels of test performance when testing offshore collections of midges from the priority species (Table 1).

The 2 assays developed for the exotic species *C, nudipalpis* and *C. orientalis* performed well, although the number of samples examined was limited. Further assessment of the performance of these assays will be considered later in this report during the testing of insect pools.

Although approximately 10,000 assays were completed in sensitivity and specificity analyses, in some instances, there would be benefit in testing of additional midges in both homologous and heterologous assays for locations where there are currently limited numbers of samples, and with any additional exotic species that become available. There is a need to undertake further investiations to determine whether the assay for *C. fulvus* can be refined and an assay for the closely related *C. dumdumi* developed.

As *C. brevitarsis* is the major BTV vector throughout eastern Australia, more extensive testing of the 'brevitarsis' assay has occurred, with almost 4,000 specimens tested. There has been limited cross reactivity with a small proportion of specimens, usually at a very low level that would usually be classified as an inconclusive rather than positive result. As this assay has such high analytical sensitivity, it is possible that some of this reactivity may be arising from residual fragments of cadaver or even free DNA, arising from collections where there have also been large numbers of *C. brevitarsis* in the light trap. Even if all cross reactivity is considered to be 'non-specific', this assay still has a specificity of almost 98%. The level of cross reactivity is low and was almost eliminated by setting a numerical cut-off based on a specific Ct value. When patterns of cross reactivity are examined further (Table 1C), it is apparent that in most instances the cross reactivity is generally limited to particular species and indicates the areas for initial attention. A preliminary morphological examination of some of these insects suggests that they have been correctly identified but further examination will be undertaken. The DNA sequence for these samples will also be examined closely to confirm their speciation.

A notable exception with the *C. brevitarsis* assay is that it did not detect some off-shore (especially Japanese) strains of midges that had been identified as *C. brevitarsis* based on morphological criteria. A more broadly reactive assay that would detect all strains of *C. brevitarsis* (Australian, South-east Asian and Japanese) was developed. Using this new "pan-brevitarsis" assay and the original "Australian" *C. brevitarsis* test, it has become possible to discriminate between Australian and Japanese strains of midges, providing a capacity to detect new incursions of this midge. Unrelated, but during the course of this project, the genetically different midges that were originally thought to be strains of *C. brevitarsis* were examined further by the reference entomologists and, based on genetic and morphological data, have been classified as a new species - *C. asiatica* (Bellis et al., 2014).

The cadavers/cytoskelton for all midges from which DNA was extracted at EMAI have been returned to Glenn Bellis, the national Reference Entomologist for *Culicoides*. When combined with the sequence data, these cadavers represent a substantial national resource for future midge taxonomy studies.

Studies to investigate the analytical 'sensitivity' of these assays have shown that it is possible to identify individual midges of one species in large collections containing many other midge species and also other insects. Collectively, these studies showed that the theoretical limit of detection could range from 1/10,000 to 1/50,000, greatly exceeding the number of midges usually found in a light trap collection.

Additionally, these collections may also contain many species. It would be preferable if nucleic acid extracts could be obtained with little prior sorting. This would need to be achieved in a manner that does not significantly dilute DNA from small numbers of a target species that might be present in a large insect pool. The methods of extraction effectively increased sensitivity of the assay and the limit of detection because insect pools were extracted in 10 times less digestion buffer. The high sensitivity and specificity of the assays for the 4 main Australian vectors has been confirmed during the testing of several of the NAMP collections (See Tables 1A-1C). Interestingly, when the qPCR assays were applied to the 2011-12 NAMP collections from both the NSW vector and vector free areas, some discrepancies were initially encountered. In one instance, a test of a pool from the vector zone returned a negative result, even though the NAMP database indicated that *C. brevitarsis* should be present. Microscopic examination did not detect any *C. brevitarsis* and it was later found that these midges had been removed from the collection for other research. The converse was also encountered on 2 occasions when positive qPCR results were obtained for collections at the margin of the vector zone. Close microscopic examination of these detected single *C. brevitarsis.*

Table 1A - 1C. Assessment of the sensitivity and specificity of assays for the identification of *Culicoides* species

Table 1A. Sensitivity testing
These data show the proportion of insects of an individual species that are detected in the homologous assay.

Species	All samples	Australian Samples
C. brevitarsis	85.44*	99.75*
C. actoni	80.25	98.15
C. fulvus	78.23	84.85
C. wadai	96.89	97.60
C. nudipalpis	100	NA
C. orientalis	86.96	NA

* Percentage of *C. brevitarsis* detected by the *C. brevitarsis* assay. Calculations are based on a positive detection being a Ct value less than 33.

Table 1B. Specifiity testing
These data show the proportion of insects of gave a negative result in the heterologous assay.

Species	All samples		Australian Samples	
	Absolute	With Cut-off	Absolute	With Cut-off
C. brevitarsis	89.77	94.28*	89.20	96.60*
C. actoni	99.16	99.70	99.18	99.65
C. fulvus	69.94	89.64	78.05	86.49
C. wadai	90.62	98.60	90.73	99.49
C. nudipalpis	99.70	99.93	100.00	100.00
C. orientalis	94.18	97.02	94.36	97.24

* Percentage of samples for species other than *C. brevitarsis* that gave a positive result in the *C. brevitarsis* assay. Calculations are based on a positive detection being a Ct value less than 33.

Table 1C. Species giving cross reactivity in heterologous assays

Species	(Australian species only, based on data WITH cut-off)	Non Australian samples
C. brevitarsis	C. nattaiensis*	C. pallidimaculatus**
C. actoni	C. minimus	C. fulvus
C. fulvus	C. dycei, C. marksi, C. cuniculus, C, zentae, C papuensis	C. victoriae((gp), C. brevitarsis
C. wadai	C. oxystoma (1)	C. nudipalpis, C. oxystoma, C. nattaiensis
C. nudipalpis	Nil	C. fulvus (1)
C. orientalis	C. cordiger, C. shivasi	C. vexans

* *C. nattaiensis* was the Australian species that gave cross reactivity in the *C. brevitarsis* assay.

** *C. pallidimaculatus* was the non-Australian species that gave cross reactivity in the *C. brevitarsis* assay.

Examination of the 2012-13 NAMP collections from NSW clearly demonstrates the power of these assays. This consisted of 217 individual light trap collections covering NSW for a period of more than 6 months, with a small number of strategic collections in Victoria, South Australia and Tasmania. The composition of these collections and the frequency and abundance of individual species is shown in Table 2.

Table 2. Composition of NSW NAMP light collections in 2013

Species	Number of Collections	Total
C. austropalpalis	94	6266
C. brevitarsis	95	80666
C. bundyensis	41	385
C. bunrooensis	18	308
C. dycei	43	1037
C. fulbrighti	7	22
C. henryi	15	450
C. marginalis	6	22
C. marksi	76	10640
C. marmoratus	15	329
C. molestus	2	31
C. narrabeenensis	5	13
C. nattaiensis	9	30
C. ornatus group #8	3	104
C. rabauli	3	6
C. sigmoidus	2	4
C. victoriae	93	860
C. wadai	1	1
C. williwilli	4	4
Totals	**217**	**101178**

Generally, there was a very high degree of accuracy with the *C. brevitarsis* assay correctly identifying the presence of this midge in a collection on each occasion it was present. An example of results obtained is included in Appendix 2. The greatest sensitivity that was achieved in an individual collection was the detection of a single *C. brevitarsis* in a pool of more than 2230 other midges at Moree, NSW. The result for this pool (Ct = 22.7) suggests that this sample could have been diluted more than one thousand fold before a negative result would have been obtained. The *C. brevitarsis* assay gave presumptively false positive results for 4 collections from NSW, each of which was on the margin of the known vector zone. On re-examination, 1-3 *C. brevitarsis* were detected in 3 of the 4, including the sample from Moree in which a single *C. brevitarsis* was detected in a large pool of *C. austropalpalis* (600 insects), *C. dycei* (10), *C. marksi* (1590) and *C. victoriae* (30). Single positive results were obtained in the *C. actoni* assay at Lismore and the *C. orientalis* assay yielded 2 reactors (Berry NSW and Orbost Vic), with the Orbost collection also reacting in the *C. brevitarsis* assay. Re-examination of these collections failed to identify the target midges in these collections, confirming that these are false positive results. The assays for *C. fulvus* and *C. wadai* gave similar patterns of reactivity to what had been observed during the initial validation, producing a small number of false positives. However, the *C. wadai* assay did appear to have poor sensitivity against some field collections. As *C. fulvus* is not present in NSW and *C. wadai* is extremely uncommon and confined to the far north coast, true positive results are not expected, though in this collection there was a single *C. wadai* that was detected. Although most of these have been shown to be true positive results, even if they had been false positive, they do not detract from the performance of these assays, especially when 217 collections containing more than 101,000 insects were screened in 6 assays in a very short time frame, leaving a small number for further examination. Taking such an approach would streamline surveillance but also identify areas where assays may potentially require further fine

tuning. Several of the pools that have given unusual results from the 2013 collection are being re-examined by an entomologist.

A selection of NAMP collections from the NT and northern WA were also tested. A total of 31 collections were examined, with one very large collection being divided into 4 sub-lots to facilitate DNA extraction. The composition is summarised in Table 3. There were no positive results obtained in either of the assays for exotic species (*C. nudipalpis* and *C. orientalis*). However, there were 3 collections that gave positive results for *C. brevitarsis*, with 2 of these being relatively strong reactions. Taking into account that these positives were obtained for quite large collections, it would seem from previous experience that these results are likely to be reliable.

Table 3. Composition of light collections from northern Australia in 2013

Species	Number of Collections	Total
C. actoni	11	1576
C. austropalpalis	16	1770
C. brevipalpis	1	168
C. brevitarsis	14	10959
C. bundyensis	3	78
C. bunrooensis	1	4
C. clavipalpis	1	1
C. dycei	5	11
C. flumineus	1	4
C. fulvus	5	1548
C. leanderensis	2	6
C. marksi	15	5857
C. ornatus gp	11	744
C. oxystoma	6	233
C. pallidothorax	1	24
C. peregrinus	10	3445
C. purus	1	1
C. wadai	2	193
C. willi willi gp	1	5
C. zentae	1	19
plain wing species	1	1
Totals	31	26647

The new assays to detect the exotic species, *C. nudipalpis* and *C. orientalis* appear to perform well and have reliably detected these species in spiked insect collections (Appendix 3). These assays were also shown to be robust and reproducible, with the laboratories at EMAI and Darwin correctly classifying all samples spiked with the exotic midges and correctly identifying *C. brevitarsis* in the NSW NAMP collections for 2011. qPCR technology offers considerable power for the detection of nucleic acid for diagnostic purposes as these assays are extremely sensitive, rapid (can be completed in about 2 hours) and can be semi-automated. It is possible to extract and test several hundred samples (which can represent entire trap collections) in one day. This provides considerable capacity for undertaking virus and vector surveillance studies in a contemporary manner, rather than waiting perhaps for several months for results. Additionally, these assays are not subjective and, unlike morphological identification, are not dependent on the skills of a highly trained entomologist. Nevertheless, it is not intended to use them in place of an entomologist but rather to allow more efficient use of the entomologist's time. Highly sensitive assays can be used to rapidly screen collections and if the assay generates what

may be presumptively a false positive result, this can be checked by the entomologist. These assays should prove invaluable for monitoring for incursions of exotic midges and for changes in the distribution of established vectors under the influence of climatic variability.

4.2 Evaluation of assays for the detection of bluetongue viruses

4.2.1 Detection of viruses in sentinel cattle

At present, to reliably identify the serotype of BTV that has infected an animal, it is necessary to culture and identify the virus which usually takes many weeks, or, perhaps, to undertake nucleic acid sequencing on RNA amplified by RT-PCR. The latter is also relatively time consuming and expensive. Through collaboration with overseas colleagues, it has been possible to gain access to specifications for prototype assays for detection of individual serotypes of BTV. However, to cover the global spectrum of BTVs it is necessary to run 2 separate assays for each serotype because there are RNA differences between BTVs from the 'eastern' and 'western' hemispheres (with a cross-over in the region of Pakistan). Nevertheless, for surveillance within Australia only a single assay should be required for each serotype.

Initially the emphasis was to be on BTVs 1, 21 & 23 – the first 2 being the most common serotypes throughout Australia, the latter, potentially one of the most pathogenic and hence a risk of causing a disease outbreak if it moved south. However, following the unexpected detection of BTV serotype 2 in southern Queensland, priorities were changed and BTV23 replaced by BTV2 in the priority list. To measure the performance of the type-specific assays, all samples were tested in the BTV pan reactive qRT-PCR to assess the levels of BTV that might be detected. The Ct value from the pan reactive assay provided a standard against which the serotype-specific assays could be judged. Further, use of the pan-reactive assay confirms that the test specimens do in fact contain BTV. This is especially important when testing field material.

All samples consisted of Australian field strains of BTV that had been isolated at either BARC or EMAI and subsequently amplified in cell culture. After establishing the level of pan reactivity of samples in the 53 virus reference panel, individual assays for BTV serotypes 1, 2, 21 and 23 were conducted on the samples in the panel to establish levels of sensitivity and specificity (Appendix 4). Later, other NSW isolates held at EMAI were also tested. In general, each of the type-specific assays had a very high level of specificity, detecting the homologous virus but showing either no or, on an odd occasion, negligible cross reactivity. This demonstrates one of the merits of using a reference panel of cell culture origin – the virus titres and RNA concentrations are very high and consequently, there is a very wide range of concentrations over which the potential for cross reactivity can be assessed. In general, positive samples could be diluted at least 1/10,000 to 1/100,000 before giving a negative result. Therefore, if cross reactivity is to be a problem, there is great potential to detect it when testing these samples with high RNA concentrations. This research also demonstrates one of the many benefits of real time PCR – the capacity to provide quantitative data that had good reproducibility (both from one test run to another and for multiple replicates within the same test). This then quickly provides a measure of relative analytical sensitivity of each assay.

In regard to assay sensitivity, while each assay did detect the homologous reference strain, there were clear indications of variability. The assays for BTVs 2 and 21 have

a very high level of sensitivity, giving quantitative results similar to those obtained in the pan reactive assay. The difference in Ct values was usually less than 3 units. In some instances, the BTV21 specific assay proved to have higher sensitivity. While the assays for BTVs 1 and 23 did correctly identify cell culture amplified virus stocks in the reference panel, the difference in reactivity compared to the pan reactive assay was quite marked (about 4 $\log_{10}$ less). It was anticipated that such a level of sensitivity would need to be improved to enable routine identification of BTV to serotype level in blood samples, where the virus levels are consistently much lower than encountered in cell cultures.

Testing then commenced on the collection of Australian strains from NSW, NT or Qld that had been progressively archived at EMAI since BTV was first detected. Testing of the panels of field strains provided similar trends but the implications of the lower sensitivity of some assays became apparent. When tests were conducted on blood samples from cattle infected with BTV2, similar, and in some instances slightly higher sensitivity was achieved when compared to the pan reactive assay. Largely comparable results were achieved with the BTV21 and the pan reactive assay on blood samples, correctly identifying BTV21 in all but one sample from which BTV21 was reportedly isolated. Interestingly for this sample, the BTV21 assay also gave negative results with the cell culture fluid. However, when samples were tested in all other assays, even though the BTV1 assay had reduced sensitivity, this sample gave a positive result, suggesting that the virus in this sample has been incorrectly identified. The results for BTVs 1 and 23 were as expected, with a large difference again shown between reactivity of cell culture virus in the pan reactive and type specific assays. Although the NT strains of BTV1 were detected with a modest Ct difference (3-7 or 1-2 $\log_{10}$), the difference in reactivity for most Australian isolates of BTV-1 compared to the pan reactive assay was quite marked (up to 4 $\log_{10}$ less). Very weak reactivity was obtained with most of the BTV1 isolates from NSW and, in some instances, even virus in cell culture was not detected. When the corresponding blood samples were tested, BTV1 was not identified in many of the samples while BTV23 was detected intermittently and with weak positive results. An interesting observation was made with one of the blood samples from an animal in Queensland where BTV1 was readily detected and with the results similar to the pan reactive assay.

As one of the final goals of this phase of the project was to apply the BTV type-specific assays to NAMP collections, priority was given to improvement of the BTV1 assay as this virus is much more frequently transmitted than any other serotype. Additional RNA sequencing data were obtained from collaborators at IAH Pirbright and the BTV1 assay was redesigned. In general, this improved the results for the BTV1 qRT-PCR (difference in Ct values of 3-6) but the high level of sensitivity that had been achieved with the BTV2 & 21 assays was not achieved (see Appendix 5). Evidence of genetic variability between BTV1 isolates in different parts of Australia, and at times in the same region, is still apparent. However, it is now usually possible to routinely identify of BTV1 in cattle blood samples. Similar trends were observed for the assay for BTV23 although this assay did not show quite the same decline in sensitivity as the BTV1 assay. Overall, the results for BTVs 1 and 23 suggest that the assays need to be refined to give optimal detection of field strains but with some care as there are some field strains that are detected well.

It is important to achieve maximal sensitivity with these assays not only because of the degree of genetic variability observed with field strains of BTV but also because the levels of virus in cattle blood are usually quite low. There is also the potential for infections with multiple serotypes. The presence of potent inhibitors of PCR in whole blood is well recognised and this is also an important consideration when testing

animal samples. The higher the sensitivity of the assay, the less likely genetic variability or inhibitors of PCR will completely suppress detection of the viral RNA.

Blood samples and virus isolates from cell culture were also tested from sheep and cattle that had been experimentally infected with different serotypes of BTV. For 2 sheep infected in the NT with BTV2, the BTV2-specific assay readily and consistently gave strong positive results on each of the 19 occasions that these animals were sampled over a period of 5 weeks. Samples from 2 sheep infected with BTV20 were also tested. While these gave strong positive results in the BTV pan-reactive assay, as expected they were consistently negative in the assay for the serotypes for which testing took place (BTV-1, BTV2, BTV21 and BTV23).

Blood samples from cattle infected with BTV23 in the NT were also tested, together with the corresponding isolates in cell culture. Although all samples were consistently detected by the BTV23 specific assay and did not react in assays for BTV1, BTV2 and BTV21, there was a moderate (5-10 Ct) difference between the results for the pan reactive assay and the BTV23 specific assay. These results would suggest that the BTV 23 assay could probably be improved.

For testing of NAMP samples, the same approach was taken as was used during the assay development phase. Each sample was first screened in the pan-reactive BTV group assay to identify any samples that contain BTV RNA and to provide an indication of the quantity of BTV RNA that was present, providing a benchmark against which the performance of the type-specific assay could be assessed. The reactivity detected in the pan-reactive assay was also useful when interpreting results. For example, if there was a large quantitative difference between the pan reactive assay and a type-specific assay that should have high sensitivity, these results could indicate that the virus was a variant, or that there was a dual infection, with high levels of a second virus contributing to the reactivity of the pan BTV assay.

Initially, archived samples from NSW sentinel cattle were tested, generally with good results. Whole blood samples collected from Qld sentinel cattle in 2010 were also tested, resulting in the direct detection of both BTVs 2 & 21. The type-specific qRT-PCR assays detected BTV RNA directly in cattle blood and, in many instances, provided identification of an infecting virus where virus isolation had failed. These assays were then used to test NAMP sentinel cattle samples for 2011-2013 for NSW, 2012 for Qld and selected samples from Darwin in 2011 and 2012. The assays for BTV2 & 21 continued to give good results, detecting BTV RNA over a prolonged period and often when virus isolation was unsuccessful (see Appendix 6). In NSW in 2013, different patterns of reactivity were detected on the far north-coast compared to the lower north coast (See Appendix 7). In most instances, the presence of BTV1 could be confirmed but not with the confidence for other serotypes. Inevitably this would probably reduce the time over which infection with this serotype might be detected in an animal.

When compared with the NAMP virus isolation results, the value of the qRT-PCR assays is apparent. BTV isolates were not obtained from a number of animals, while in others dual infections were not identified by virus isolation. Generally, the qRT-PCR correctly detected over many weeks the serotype with which an animal had been infected whereas virus isolation was mostly successful during the first few weeks of infection. Virus was isolated from about 35% of samples that were shown to contain BTV RNA. This is also related to the biological characteristics of the infection whereby virus remains infective for a limited period whereas RNA may be detected for many months. Further, about 10 days after the onset of infection, the production of type-specific antibodies can also impede the efficiency of virus isolation. These

results clearly have implications for BTV surveillance where animals are not sampled at short intervals. In most sentinel herds in Australia, sampling and hence virus isolation is only conducted monthly (in contrast to the weekly samples in a key herd in the NT, from which these samples shown in Appendix 6 were obtained). The longer period over which RNA can be detected means that it is more likely that by qRT-PCR will confirm the activity of a particular serotype than virus isolation. To put this into perspective, in the NT sentinel herd that was sampled weekly, viruses were isolated from the sample panel on 38 occasions whereas the serotype specific qRT-PCR assays identified the serotype with which the cattle had been infected on 107 occasions. Nevertheless, this should not be taken to suggest that virus isolation is not important because there are many additional situations where it is critical to have the virus isolate available for further study. There are some samples in which an individual serotype of BTV was not identified with the type-specific assays in use. The results of virus isolation and serology showed that this was not due to assay failure but, rather, the infecting virus was either BTV7 or BTV20, a relatively uncommon serotype for which a type specific assay has not been evaluated.

The prototype assays evaluated in this project have already been utilised as a means of rapidly identifying virus strains being transmitted in new locations in eastern Australia. An example was the spread of BTV into north western NSW in the 2011-12 arbovirus transmission season, close to significant sheep populations. Blood samples from infected sentinel cattle were tested in the type specific PCR assays and BTV21 transmission was identified in this region for the first time in more than 20 years. The possibility of ongoing southward movement of BTV2 from Queensland was also excluded with confidence by application of the BTV2 specific assay. Collectively these events already provide practical evidence of the benefits of these technologies, with results being available in about 24 hours.

In NSW, the pan-reactive and type-specific assays have also been used to guide virus isolation attempts. Whenever a sentinel animal seroconverted, all available blood samples around the time of seroconversion were screened in the pan-reactive assay. Virus isolation was then only attempted on samples in which BTV RNA was detected.

4.2.2 Detection of bluetongue viruses in insects

The aim of this stage of the project was to establish whether BTV could be detected in *Culicoides* midges with the use of techniques that would not destroy the morphology of the insect. This would then allow detection of BTVs in insects and, if necessary, re-examine the insects from a taxonomic perspective to confirm the identity of the insect. As infection rates in these midges are usually very low and infection in the field can be extremely variable, to reliably complete these studies, midges were experimentally infected with virus. Three different methods were eventually utilised – direct inoculation of insects, feeding of virus-spiked sucrose and feeding on BTV infected chicken embryos.

4.2.2.1 Direct inoculation of midges

Of the 81 midges that were inoculated intra-thoracically with BTV, 75 were killed and nucleic acid extracted after 1-4 days to assess background BTV RNA levels arising from the inoculum. Low virus levels were detected (mean Ct value = 31.56). Two midges were sacrificed at Day 8 and 4 at Day 10. There was clear evidence of virus replication in 5 of the 6 insects (Ct range 19.59 - 21.36 (mean 21.42) for the 5 insects; Ct=35.00 for the remaining midge in which virus had not replicated). These

results would suggest that the samples in which there was evidence of virus replication could be diluted more than 1/1800 before background levels of BTV RNA would be reached.

4.2.2.2 Feeding on virus-spiked sucrose solutions

Although the abundance of field populations of *C. brevitarsis* was extremely variable and often low due to the unusually wet and cool summer, large numbers of midges were successfully hatched from cow dung and reared in the laboratory to support a range of studies. The success of feeding of midges with virus spiked sucrose or with sucrose blood mixtures was readily determined by the staining of abdomens with the brightly coloured food dye that was included in the sucrose mixture. A very high proportion (74-100%) of midges took up BTV RNA from the sucrose meal. The higher proportions of midges containing BTV RNA were observed, not unexpectedly with high titred preparations of BTV23 which had been used to maximise chances of infection of the midges. This result was not necessarily related to a specific serotype as similar trends were observed with a limited study of midges fed sucrose containing high titred BTV-1.

Viral RNA was successfully extracted from experimentally infected *C. brevitarsis* using the same non-destructive nucleic acid extraction methods that were used to obtain DNA to identify the insects. There was no significant difference in the levels of nucleic acid recovered whether the midges had been destroyed and fully homogenised by grinding in a buffer solution or had been subjected to the enzymatic digestion protocol. Using estimates of the volume of a blood meal taken by *C. brevitarsis* and comparing the BTV RNA levels in the virus spiked sucrose with those detected in the insects confirmed that the recovery of viral nucleic acid from the midges was extremely efficient. Because of the need to be able to detect any serotype of BTV, and also because of its proven high analytical sensitivity, the pan reactive BTV qRT-PCR assay was initially used in all studies to detect BTV in insect preparations. The viral RNA extracted from the insects was readily detected but virus levels soon after feeding were extremely low, a direct reflection of the minute volume that had been taken up by the midges. During the time of these studies, more than 1000 midges were successfully reared and examined individually. The results showed that:

- The level of BTV RNA detected on Day 1 post feeding closely follows the predicted volume of meal taken up by the midges;
- There were no appreciable differences in levels of infection when midges were fed on Day 1 or Day 5 after emergence. Once good survival rates were achieved, midges were usually fed at 5 days;
- The proportion of midges containing BTV RNA, presumptively residual RNA from the virus spiked sucrose, progressively declined until about Day 10 and then reached a plateau, with the proportion of insects in which BTV RNA was detected remaining at about 15-20% after about 3 weeks post exposure. RNA levels were extremely low (Ct values >35).
- There appeared to be no difference in results between males and females;
- These techniques provide a capacity to examine large numbers of individual insects in a relatively short period of time;
- Viral RNA levels can be readily quantified in individual midges;
- Although based on limited numbers of insects, there do not appear to be any differences in infection rates between different serotypes. However further research is needed using standardised but high levels of virus to maximise the potential for each midge to ingest an infective dose of BTV.

- The low virus levels detected in these experimentally infected midges demonstrate the high sensitivity of these techniques.
- As these methods are at least semi-quantitative and reproducible, it should be possible to discriminate between residual RNA from a blood meal and RNA arising from virus replication in a midge that is acting as a vector of BTV. It was expected that an infected vector would have virus levels 100-1000 times higher than found in these experimentally infected midges. Such differences in virus levels were confirmed in later studies.

Collectively these results show that the methods employed readily allow both the detection and quantification of BTV RNA levels in individual midges after extraction of the nucleic acid using non-destructive methods. Nevertheless, although BTV RNA was easily detected in midges that had ingested sucrose containing BTV, there was usually no evidence of BTV replication because the RNA levels became progressively lower (higher Ct values) from a few days after the ingestion of BTV spiked sucrose. There was a single exception where an extract from one midge sampled on Day 17 after feeding returned a Ct value of 21.4, conclusively confirming virus replication in that insect. A summary of these results is provided in Appendix 8.

As these data were generated from an unscheduled experiment undertaken at short notice due to the shortage of midges for infection by direct inoculation, there were many variables, a number of which were not controlled to a high level (e.g. varying virus doses, times after emergence, feeding with sucrose etc). Therefore, data from these sucrose feeding studies were only interpreted in a general manner but they did provide valuable samples for the evaluation of assays for the detection of BTV. As one of the major limitations was considered to be uptake of virus in a blood meal, further research was undertaken in the PhD program to develop methods that would allow insects to be experimentally exposed to virus in blood.

4.2.2.3 Embryonated chicken egg model

In the UK, colonised *Culicoides variipennis* are routinely infected orally by placement on an artificial membrane under which virus infected warm blood is circulated. This equipment was also trialled in NSW without success, probably as a result of the very small size of *C. brevitarsis* and perhaps the thickness of membranes that were available. This led to the development and application of the embryonated chicken egg model. An average of about 10-20% of insects that were given access to blood vessels of chicken embryos did take a blood meal. Although on occasions, up to 50% of midges fed. While the feeding rate is much lower than attained with virus spiked sucrose, this method had a number of advantages. Firstly, it more closely resembled natural infection methods, it was possible to work directly with field ('wild' type) strains that have not been adapted to cell culture and secondly it was possible to consistently produce blood with similar and high virus loads. Further, midges were exposed to virus exclusively by feeding on an infected source of blood.

Studies were undertaken at both Darwin and EMAI. At Darwin, wild caught *Culicoides* were artificially fed using the embryonated chicken egg model and held for at least 10 days. While low virus levels consistent with residues from a blood meal were detected in most insects soon after feeding and in some for up to 10 days, in a small proportion of insects, high virus levels consistent with virus replication were detected. The BTV RNA levels (Ct=32) in midges soon after feeding were directly linked the virus level in the embryo on which they had fed (using the average volume of blood taken by midges as 0.1µL). In midges in which virus replication had occurred, the Ct values were markedly lower, ranging from 19.2 to 26.94 (Appendix 9). At Darwin *C. actoni*, *C. marksi* and *C. peregrinus* were studied. An infection rate

of 2.5% was detected in *C. actoni* that were fed on embryos inoculated with BTV1. No evidence of virus replication was observed for *C. marksi* and *C. peregrinus.* These results show that the methods that have been developed to detect BTV in insects are effective in practical situations as extracts from several thousand insects have already been successfully tested in this project. These studies also confirmed that the model system can be used to investigate the replication of BTV in wild caught Australian *Culicoides* species.

4.2.2.4 NAMP insect collections

The methods that have been used in the vector competence studies clearly show that BTV can be detected in nucleic acid acid extracts from individual insects using non-destructive methods. Further, it was possible to differentiate between RNA that is the residue from a blood meal or from active virus replication. The RNA levels associated with virus replication were sufficiently high that virus might still be detected after a high degree of dilution, perhaps as much as 1/10,000. Evaluation of these assays for the detection of BTV in routine NAMP collections was therefore appropriate. However, these studies had involved RNA extraction from individual insects whereas a NAMP light trap collection could contain a few midges or perhaps several thousands of midges. For the application of the *Culicoides*-specific assays, methods had been developed to extract DNA from large insect collections. As a total nucleic acid purification method had been used, it was expected that if BTV RNA was present, it should also be possible to detect it in a large collection. Consequently, the purified RNA from the same insect pools that had been used for detection of individual species of midges were tested without further preparation. All sample pools were first screened in the pan-reactive BTV assay and then if positive, the type specific assays for BTV1, BTV2 and BTV21 were employed. From NSW, a total of 383 insect pools were tested (166 in 2012, 217 in 2013). From northern Australia, 31 collections were tested. One very large collection was subdivided into 5 smaller collections for processing. BTV serotype 1 was detected in a single pool of midges collected at Lismore, NSW on 09/04/2013. This pool consisted of mostly *C. brevitarsis* (51/53). Even though the pool of midges was relatively small and the ratio of *C. brevitarsis* high, the reactivity of this sample (Ct = 26.2) was sufficiently strong that it could still probably be diluted more than 1/1,000 before it would no longer be detected in the pan reactive assay. Additionally, the level of BTV RNA is perhaps 10-50 fold lower than has been usually observed in experimentally infected insects and would be consistent with a single infected midge in the pool. As the incidence of infection in competent midges is very low, given the relatively long (monthly) sampling interval, it is not surprising that there was only one positive pool detected. Nevertheless, these studies have shown that the real time PCR assays perform extremely well for the testing of large essentially “unsorted” mixed pools of insects.

4.2.2 Development of new assays for virus detection.

As a “proof of principle”, a microbead-based assay for BTV21 was first developed to investigate the suitability of Luminex technology on a MagPix platform for the detection of different bluetongue serotypes. This serotype was selected because it is both widespread in Australia and the corresponding real time PCR assay has a high level of sensitivity, against which comparisons could be made. This prototype assay was first evaluated by testing RNA from a range of serotypes in the reference virus panel (Appendix 10A). The results were good with the assay having a high level of sensitivity and specificity, similar to or perhaps even sometimes higher than real time PCR. On a limited number of field isolates, one strain was correctly identified with a

much higher level of sensitivity than the corresponding qRT-PCR (Appendix 10B). Following on from this success, an assay for BTV2 was also developed so that the multiplexing capacity of the system could be investigated. This assay was shown to have very similar performance characteristics to the BTV21 assay, again generally with sensitivity comparable to the BTV2 real-time PCR assay. To expand this 'proof of concept' study, preliminary studies were undertaken to determine whether the 2 assays (BTV2 and BTV21) could be multiplexed – i.e. whether all reagents could be mixed and run in a single assay. This was also achieved, and evaluated by concurrently running the individual ('singleplex') assays at the same time as the duplex assay to test the same sample panel. The results obtained were almost identical for each serotype, confirming that there is potential to develop multiplex assays using this technology. Once high quality purified RNA is available, assuming that the correct PCR primers are available, there should be little or no difference between the capacity to test reference or field samples for an individual serotype. Nevertheless, a selection of virus isolates and blood and insect samples were tested, with results again comparable to those obtained by qRT-PCR.

While real time PCR assays can be run in a shorter time than the MagPix based assay, this new magnetic bead based platform may provide a capacity for large scale multiplexing. It provides several advantages. In theory up to 50 different 'targets' or individual assay components can be detected concurrently whereas most real time PCR systems are limited at present to about 5 assays. If there is a need to expand the range of MagPix assays, magnetic beads of a different colour signature (specific to the new test component) can be added to the mix. A high degree of multiplexing would be ideally suited to the detection of different BTV serotypes. In theory, it should also be possible to multiplex assays for both the identification of insects and simultaneous detection of BTV. When a large number of assays are multiplexed, both costs and total 'turn around time' are significantly reduced. The only limitation of this detection technology is that it is a qualitative system and would only be suitable for screening of collections. It would not support the differentiation of RNA levels in an insect as a residue from a blood meal or virus replication. However, further studies using this technology are warranted.

5 Success in achievingoObjectives

5.1 Assays for the identification of *Culicoides* species

The overall objectives for this project were to develop a large database of nucleic acid sequence data for species of *Culicoides* found in Australia (both vector and non-vector species) and exotic vector species that present a threat to Australia. These data would in turn be used to develop and evaluate rapid diagnostic assays for the detection and identification for *Culicoides* species that are vectors of BTV. Finally, these assays were to be evaluated for routine use by testing of NAMP insect collections. Each of these objectives has been achieved in full. Although some improvements are possible for one of the assays, each is considered to be "fit for purpose". These assays have been shown to be a valuable alternative to morphological identification of light trap collections by entomologists and have very high analytical sensitivity, allowing the detection of single midges of interest in very large insect collections without any prior sorting or other screening. Indeed, in some instances, it would appear that the species-specific assays have the potential for greater sensitivity and accuracy in detection than an entomologist. The availability of non-destructive techniques to extract total nucleic acid from unsorted collections both facilitates testing and also allows retrospective morphological examination of samples that have given unexpected or problematic results. Finally, without further

processing, the same preparation that was used for the identification of insects can be used for the detection and quantification of BTV in potential or known vector species.

The scale of some aspects of the work and extent of investigations undertaken in this project were much greater than originally planned. The capacity to experimentally infect wild caught insects introduced a number of opportunities that had not been anticipated.The productive collaboration with our Biosecurity NSW funded PhD student lifted the outcomes of this project to a level higher than expected. The seven *Culicoides* specific assays that are now available provide a valuable resource to underpin future vector research and the refinement of methods for vector monitoring.

5.2 Assays for the detection and identification of bluetongue virus

The second stage of this project consisted of a series of studies with the objective to develop rapid assays for the rapid detection and identification of BTV in either insects or the blood of sentinel animals. As well as using pan-reactive assays that would detect any serotype of BTV, there was an objective to investigate assays for the rapid identification and typing of BTVs based on real-time PCR and micro-bead based technology. It was expected that these assays would identify BTV to serotype in either NAMP insect collections or in sentinel cattle blood. The project has met the defined objectives in all respects. The results of this research demonstrate that the serotype specific qRT-PCR assays have a wider spectrum for detection of BTV than other methods as well as having the potential to provide results in less than 1 day compared to many weeks. The application of prototype BTV assays to test insect collections from various locations within the BTV endemic zones in Australia and to test experimentally infected insects have demonstrated that this technology should be suitable for bluetongue monitoring in remote locations and for the identification of potential vectors of BTV.

6 Impact on meat and livestock industry – Now and in five years time

While this project has been highly successful, it has in practical terms delivered a suite of tools that now need to be taken to an implementation stage for either practical application through NAMP with on-going refinement or to provide the principles upon which additional assays are developed. In five years time, it would be expected that these and similar assays would be in routine use in the NAMP, providing a greater capacity for surveillance and increased efficiencies by reduction in the time that professional entomologists need to commit to relatively routine insect sorting and identification.

Although these tools may not be expected to bring benefits in the immediate future, the potential for short term benefits should not be underestimated. At times circumstances can change rapidly for vector-borne diseases such as bluetongue. There could be situations where these assays could be deployed as part of an emergency response and result in almost immediate returns. The extension of BTV transmission into north western NSW in 2012 provides a good example. In this instance it was possible to screen blood samples from sentinel cattle and within 24 hours there were results that indicated that the virus being transmitted was BTV21 and there was rapid exclusion of the possibility of BTV2 transmission in NSW.

However, at this stage the range of serotype-specific assays is limited and needs to be expanded to meet the national needs for comprehensive BTV surveillance.

This project will provide animal health authorities and the Australian livestock industries with a range of tools to use in NAMP and NAQS to more accurately and efficiently monitor the distribution of existing *Culicoides* and BTVs in Australia, particularly in remote locations where conventional sampling methods are not possible. Further, it will provide tools for rapid monitoring for incursions of insects and BTVs and in a time frame that is much shorter than using current techniques. These assays should also provide indicative information on the probable vectors of individual serotypes of BTV, as well as exploring potential overwintering mechanisms for BTV in the vector population. Finally, these tools will be useful for monitoring for the possible spread of BTVs, especially the pathogenic northern serotypes, beyond their current limits and into areas where there may be a threat of disease. While sampling of sentinel cattle provides a sensitive system for this purpose, in a number of critical locations there have been significant logistical problems in locating and sampling cattle. Using this alternative approach to test insect samples, it should be possible to develop strategies to make regular insect collections with infrequent visits by field personnel. If collected in sufficient numbers, BTV detection could be undertaken on insects with identification of viruses to the serotype level. While novel, such an approach would be very efficient and is only possible with the tools that have been produced in this project.

7 Conclusions and recommendations

This project has delivered a suite of rapid diagnostic assays for the detection of both insect vectors and BTV. These should be used as a foundation upon which to build a complete set of assays for the detection of all serotypes of BTV that are either known to be present in Australia or which present a threat. This would allow the detection and identification of an individual serotype of BTV either in animal blood or insects within a few hours of receipt. This would facilitate early warning of emerging disease threats in a realistic time frame and overcome many of the shortcomings of existing technologies.

As well as further refining some of the assays, in particular those for BTV1 and for *Culicoides fulvus*, additional assays would be invaluable for some potential vector species such as *C. dum dumi* which is closely related to *C. fulvus*. Further applications for some of the midge assays should be explored – for example, the capacity to estimate midge numbers in light trap collections based on the results of a real time PCR result on a pooled sample.

For these assays to achieve their potential for enhanced arbovirus surveillance in remote areas, there is at present one limitation. In many remote areas there are limitations or even prohibitions on the supply of ethanol which is currently the preferred preservative for insects. Without such a preservative, insects deteriorate before they can reach an entomologist. Alternative preservatives that will allow both midge identification and virus detection must be identified as a priority.

Although its development was supplementary to this project, the embryonated chicken egg model has proven to be invaluable to study virus-vector biology. Further studies with BTV should be undertaken and could include researching the vector capacity of other midge species and other serotypes of BTV. This model could be readily used to explore scenarios associated with environmental variability, such as

investigating the impact of higher temperatures on midge infection rates and subsequent transmission. The application of the model to provide convincing biological data that southern midge species that are abundant (eg *C. victoriae*) cannot become competent under altered climatic conditions would be extremely valuable for preparedness planning. There are several other groups of arboviruses of livestock that are important to animal health, in particular Akabane and other related viruses belonging to the Simbu serogroup. Studies of infection rates and changes in vector capacity under the influence of climatic variability could also be readily investigated using this model system. This would provide an insight into the degree of disease risk that these viruses could present for livestock producers in southern Australia as there is a likelihood that these viruses would move southwards as seasonal temperatures rise. Should this occur, devastating disease outbreaks would be almost inevitable.

Finally, new diagnostic assays based on microbead technology should continue to be explored as they show considerable potential for broad-based multiplexed assays. The possibility that these assays could be utilised to concurrently identify the midge species present in a light trap collection and to establish whether these insects are carrying bluetongue or perhaps other important arboviruses would be extremely useful for future surveillance. Surveillance for exotic midge species provides a sensitive early warning because it is highly improbable that the arrival of a new species will immediately result in virus transmission. Rather, new arrivals need to survive and multiply to generate a population of sufficient size to support virus transmission. Being able to detect new insect arrivals, or alternatively the introduction of new viruses would significantly enhance preparedness and allow refinement of ongoing surveillance to assess future risks. In future, these assays would also be invaluable to facilitate serotype-specific BTV surveillance in overseas locations where efficient monitoring has not been possible.

8 Bibliography

Bellis, G., Dyce, A., Gopurenko, D., Yanase, T., Garros, C., Labuschange, K. and Mitchell, A.. (2014) Revision of the *Culicoides* (Avaritia) Imicola complex Khamala & Kettle (Diptera: Ceratopogonidae) from the Australasian region. Zootaxa 3768 (4): 401–427

Hofmann M, Griot C, Chaignat V, Perler L, Thür B. Bluetongue disease reaches Switzerland [in German]. Schweiz Arch Tierheilkd. (2008a)150:49–56. DOI: 10.1024/0036-7281.150.2.49

Hofmann MA, Renzullo S, Mader M, Chaignat V, Worwa G and Thuer B (2008b) Genetic characterization of Toggenburg Orbivirus, a new BTV, from goats, Switzerland. Emerg. Infect. Dis., 14: 1855–1861

Acknowledgements

The authors are indebted to NAMP and NAQS entomologists for the collection of *Culicoides*, to Mr David Gopurenko and Dr Andrew Mitchell for the sharing of midge DNA sequence data, to Dr Xingnian Gu for the design and refinement of real time PCR assays for the detection of *Culicoides* and to staff of the virology laboratories at BARC and EMAI for the testing of midge and BTV samples. We are indebted to Matthew van der Saag for his close collaboration and exchange of data throughout this project.

9 Appendices

9.1 Appendix 1

Method for the direct inoculation of *Culicoides spp*

In order to develop assays for the detection of BTV in insects, samples from experimentally infected midges were required. Previous development work used *C.peregrinus* as the test species as the larger size makes intra- thoracic inoculation easier and post-inoculation survival is greater than for smaller species. This species is readily infected by inoculation, is abundant at Beatrice Hill Farm (BHF) and is reported to support bluetongue replication by natural means. All *Culicoides* spp. so far investigated, with the exception of those inoculated with low quantities of virus, have been susceptible to infection with BTV following inoculation.

All insects used in the trial were sourced from BHF located approximately 50km South East of Darwin on the Adelaide River flood plain. (12' 39" S 131' 20 E).

Initial attempts at insect collections were conducted during 2011 but were unsuccessful due to low environmental numbers. During February and March 2012 numbers of insects were sufficiently high to justify collecting for inoculation.

Only insects clearly identifiable as nulliparous and unfed were used in the trials. Collections of insects were made by aspiration from penned cattle using an air broom / leaf blower as a vacuum. A bag was fitted between the intake and the power source to retain the insects. A screen at the intake excluded larger insects which could cause physical damage to the *Culicoides*.

Aspirated collections were made for periods of 5 minutes commencing approximately 1/2 hour before sunset and ceasing no more than 15 minutes after sunset. At the completion of each 5 minute aspiration period the bag was tied off with an elastic band and placed in a modified anaerobic jar. The jar was flushed with CO_2 and then flooded to approximately 1 Kpa with CO_2 and held for about 2 minutes. The anaesthetised insects were then transferred via a glass funnel to a paper cup receptacle approximately 100mm high and 80mm diameter. The insects were confined by a light cloth screen secured by two elastic bands. Access to insects in the container was by a 30mm diameter port approximately midway up the vertical surface. The port was screened with two sheets of Dental Dam taped in place with slits at 90^0 to each other. The paper cups were placed in an insulated container and transferred to the laboratory in most cases within two hours of collection. As a food source a tissue pledget of approximately 25mm square was moistened with 10% sucrose and placed on the screen covering the pot. All insects were held at 28^0C and 85% relative humidity in a small incubator.

Preliminary examination of collected insects was done from 12-24 hours after being placed in pots. This was undertaken on a refrigerated surface at approximately -20^0C. The insects were anaesthetised and placed on a blotting paper surface attached to an aluminium tray of 2 mm thickness. Insects were examined at 10 x magnification and all apparently strong, healthy, viable nulliparous females were removed by aspiration using a small vacuum pump fitted with a glass receptacle to retain the insects. At completion the insects were returned to a container for a period > 1 hour to permit recovery prior to inoculation.

Only insects which were strong and mobile were used for inoculation. Insects resting on the screen or on the wall were removed from the holding pot by aspiration via a modified 10ml glass pipette attached to a vacuum pump. Selected insects were aspirated <20 per time and anaesthetised with CO_2 while in the pipette. Once comatose, they were transferred to a plate on a cold table as previously described. The used for inoculation procedures was 6mm aluminium with blotting paper fixed by adhesive. The inoculum was BTV serotype 1 (BTV1), titre $10^{4.25}$ TCID/50µl.

Inoculations were made using needles made from 1mm outside diameter glass capillary tubing which was pulled in a Narishige PB7 needle puller. Tips were formed by snapping the end of each tube. This was done using entomological forceps under 8X magnification. For optimum suitability tip diameter was approximately 0.05mm. Tip shape was not a critical issue in achieving satisfactory penetration and inoculation of the insect.

The needle was mounted in a holed silicone stopper and attached to the same vacuum pump used in aspirating insects from the cold table. Inoculum was loaded in the needle by closing a port on the handpiece in which the needle was mounted. Inoculation was effected in a similar manner, with the needle being attached to the outlet line rather than the inlet.

Following anaesthesia the insect was rolled on to it's back using forceps. The forceps were then placed against the body of the insect on the opposite side to which the needle was being inserted.

Post inoculation the insects were returned to pots at 26^{o}C and 85% RH. Insects were given access to 10% sucrose and monitored daily. Insects which died or failed to fly or crawl strongly were harvested at 24 hour intervals up to 10 days post inoculation, when all surviving insects were taken. Harvested insects were stored at 4^{o}C in 70% ethanol and the interval post inoculation recorded.

9.2 Appendix 2

Examples of C. brevitarsis assays conducted on NSW NAMP collections, 2013.

Location	Date collected	Species	Number	C. Brev Ct value
Wentworth	16/01/2013	C. austropalpalis	150	Neg
		C. marksi	880	
Kempsey	13/02/2013	C. brevitarsis	1400	13.50
		C. austropalpalis	5	
		C. bundyensis	5	
		C. victoriae	5	
		C. narrabeenensis	1	
Mudgee	14/11/2012	C. austropalpalis	6	Neg
		C. bundyensis	20	
		C. bunrooensis	3	
		C. dycei	1	
		C. marksi	2	
		C. victoriae	30	
Bellingen	15/05/2013	C. brevitarsis	21	17.92
		C. austropalpalis	1	
		C. sigmoidus	3	
		C. victoriae	1	
		C. fulbrighti	2	
		C. narrabeenensis	1	
		C. wadai	1	
Wauchope	15/03/2013	C. brevitarsis	220	15.25
		C. austropalpalis	4	
		C. bundyensis	20	
		C. victoriae	20	
		C. fulbrighti	2	
		C. narrabeenensis	6	
Scone	16/01/2013	C. brevitarsis	2	22.34
		C. austropalpalis	16	
		C. bundyensis	4	
		C. bunrooensis	2	
		C. dycei	4	
		C. marksi	32	
		C. victoriae	2	
		C. williwilli group	1	
Scone	12/02/2013	C. brevitarsis	6	22.38
		C. austropalpalis	22	
		C. bundyensis	26	
		C. bunrooensis	16	
		C. dycei	170	
		C. marksi	54	
		C. victoriae	24	
		C. marginalis	1	
Narrabri	16/01/2013	C. austropalpalis	50	Neg
		C. dycei	90	
		C. henryi	25	
		C. marksi	25	
		C. victoriae	5	
Denman	14/01/2013	C. austropalpalis	44	Neg
		C. bundyensis	12	
		C. bunrooensis	35	
		C. dycei	48	
		C. marksi	11	
		C. nattaiensis	9	
		C. victoriae	38	
Denman	15/02/2013	C. brevitarsis	3	19.75
		C. austropalpalis	10	
		C. bundyensis	80	
		C. bunrooensis	30	
		C. dycei	35	
		C. marksi	8	
		C. nattaiensis	7	
		C. victoriae	28	
		C. marginalis	4	

9.3 Appendix 3

Examples of pools of insects spiked with exotic midges (C. nudipalpis and C. orientalis)

Sample ID	Pool Composition		qRT-PCR assay					
	Species	Number	C. nudipalpis	C. orientalis	C. actoni	C. brevitarsis	C. fulvus	C. wadai
1	C. nudipalpis	1	23.37	–	–	–	–	–
2	C. orientalis	1	–	24.27	–	–	–	–
3	C. brevitarsis	1	–	–	–	21.25	31.78	–
4	C. nudipalpis	1	20.00	–	–	–	–	–
15	C. orientalis	1	–	18.98	–	–	–	–
16	C. austropalpalis	35	–	–	–	21.24	28.54	–
	C. brevitarsis	1						
	C. marksi	9						
17	C. austropalpalis	80	–	–	–	21.38	18.75	–
	C. brevitarsis	1						
	C. fulvus	19						
18	C. actoni	9	21.96	–	18.77	–	31.3	–
	C. austropalpalis	40						
	C. nudipalpis	1						
19	C. actoni	9	–	–	19.35	22.96	28.51	–
	C. austropalpalis	90						
	C. brevitarsis	1						
23	C. austropalpalis	49	–	22.00	–	27.39	29.61	–
	C. orientalis	1						
24	C. austropalpalis	90	–	–	–	22.79	29.73	–
	C. brevitarsis	1						
	C. wadai	10						
34	C. austropalpalis	47	21.04	19.89	–	18.87	29.67	–
	C. brevitarsis	1						
	C. nudipalpis	1						
	C. orientalis	1						
35	C. austropalpalis	30	–	–	–	20.28	22.70	–
	C. brevitarsis	1						
	C. fulvus	17						
36	C. austropalpalis	39	21.63	–	–	19.81	21.79	–
	C. brevitarsis	1						
	C. fulvus	20						
	C. marksi	30						
	C. nudipalpis	1						
	C. wadai	9						
37	C. actoni	13	–	22.66	22.02	21.15	20.64	–
	C. austropalpalis	80						
	C. brevitarsis	1						
	C. fulvus	5						
	C. orientalis	1						
38	C. actoni	15	22.72	23.30	20.63	–	28.50	29.00
	C. austropalpalis	80						
	C. nudipalpis	1						
	C. orientalis	1						
	C. wadai	27						
40	C. actoni	21	23.35	24.22	21.59	19.65	20.69	–
	C. austropalpalis	20						
	C. brevitarsis	1						
	C. fulvus	6						
	C. nudipalpis	1						
	C. orientalis	1						
45	C. actoni	18	32.97	23.82	21.41	21.96	21.23	32.42
	C. austropalpalis	61						
	C. brevitarsis	1						
	C. fulvus	15						
	C. orientalis	1						
	C. wadai	3						

Sample ID	Pool Composition		qRT-PCR assay					
	Species	Number	C. nudipalpis	C. orientalis	C. actoni	C. brevitarsis	C. fulvus	C. wadai
46	C. actoni	7	21.89	20.91	20.88	–	22.39	30.12
	C. austropalpalis	23						
	C. marksi	50						
	C. nudipalpis	1						
	C. orientalis	1						
	C. peregrinus	10						
	C. wadai	7						
67	C. actoni	14	–	24.40	27.86	21.47	27.92	–
	C. brevipalpis	5						
	C. brevitarsis	1						
	C. fulvus	9						
	C. orientalis	1						
	C. oxystoma	1						
	C. peregrinus	54						
	C. wadai	3						
	Lasohelia spp	11						
68	C. fulvus	13	23.08	20.93	–	–	23.72	–
	C. nudipalpis	1						
	C. orientalis	1						
	C. peregrinus	56						
	Lasohelia spp	30						
70	C. actoni	7	–	20.40	26.4	19.61	23.57	–
	C. austropalpalis	47						
	C. brevitarsis	1						
	C. fulvus	8						
	C. orientalis	1						
	C. oxystoma	15						
	C. peregrinus	20						
71	C. austropalpalis	47	20.12	21.12	–	–	–	–
	C. nudipalpis	1						
	C. orientalis	1						
	C. oxystoma	11						
	C. peregrinus	40						
73	C. austropalpalis	47	–	22.83	–	19.19	29.72	–
	C. brevitarsis	1						
	C. orientalis	1						
	Lasohelia spp	50						
90	C. actoni	20	21.18	–	20.12	21.62	28.91	–
	C. austropalpalis	47		–				
	C. brevitarsis	3						
	C. marksi	8						
	C. nudipalpis	2						
	Lasohelia spp	24						
91	C. actoni	30	20.82	–	21.90	22.51	26.90	–
	C. austropalpalis	47						
	C. brevitarsis	2						
	C. marksi	20						
	C. nudipalpis	4						

9.4 Appendix 4

Reactivity of pan-reactive and selected serotype specific assays with BTV reference panel

ID	S'type	Origin	Pan	1w	2w	7w	16w	21w	23w
R3338-1	1	ref	24.00	**23.2**	N	N	N	N	N
R3338-2	2	ref	24.32	N	N	N	N	N	N
R3338-3	3	ref	24.80	N	N	N	N	N	N
R3338-4	4	ref	25.84	N	N	N	N	N	N
R3338-5	5	ref	24.74	N	N	N	N	N	N
R3338-6	6	ref	25.18	N	N	N	N	N	N
R3338-7	7	ref	24.61	N	N	N	N	N	N
R3338-8	8	ref	24.43	N	N	N	N	N	N
R3338-9	9	ref	24.67	N	N	N	N	N	N
R3338-10	10	ref	24.21	N	N	N	N	N	N
R3338-11	11	ref	24.82	N	N	N	N	N	N
R3338-12	12	ref	24.16	N	N	N	N	N	N
R3338-13	13	ref	22.21	N	N	N	N	N	N
R3338-14	14	ref	24.19	N	N	N	N	N	N
R3338-15	15	ref	23.39	N	N	N	N	N	N
R3338-16	16	ref	24.68	N	N	N	N	N	N
R3338-17	17	ref	23.85	N	N	N	N	N	N
R3338-18	18	ref	24.55	N	N	N	N	N	N
R3338-19	19	ref	24.59	N	N	N	N	N	N
R3338-20	20	ref	24.85	N	N	N	N	N	N
R3338-21	21	ref	26.00	N	N	N	N	**24.1**	N
R3338-22	22	ref	25.12	N	N	N	N	N	N
R3338-23	23	ref	23.74	N	N	N	N	N	**27.9**
R3338-24	24	ref	23.72	N	N	N	N	N	N
R3338-25	3	sa vaccine	23.84	N	N	N	N	N	N
R3338-26	5	sa vaccine	22.66	N	N	**31.5**	N	N	N
R3338-27	7	sa vaccine	24.35	N	N	**23.5**	N	N	N
R3338-28	8	sa vaccine	26.22	N	N	N	N	N	N
R3338-29	10	sa vaccine	24.55	N	N	N	**36.9**	N	N
R3338-30	12	sa vaccine	24.05	N	N	N	N	N	N
R3338-31	14	sa vaccine	25.01	N	N	N	N	N	N
B323	1	EMAIWS	20.74	**36.2**	N	N	N	N	N
Y919	3	EMAIWS	24.03	N	N	N	N	N	N
D941	9	EMAIWS	23.08	N	N	N	N	N	N
B326	15	EMAIWS	24.25	N	N	N	N	N	N
B327	16	EMAIWS	23.97	N	N	N	**29.9**	N	N
B328	20	EMAIWS	24.85	N	N	N	N	N	N
B329	21	EMAIWS	21.72	N	N	N	N	**28.8**	N
A918	23	EMAIWS	29.69	N	N	N	N	N	N
D554	16	AUSTA	19.77	N	N	N	**30.1**	N	N
D555	1	AUSTA	22.62	**37.8**	N	N	N	N	N
D556	20	MALAYSIA	23.74	N	N	N	N	N	N
D557	20	MALAYSIA	25.71	N	N	N	**31.4**	N	N
D558	9	AUSTA	17.31	N	N	N	N	N	N
D559	21	JAVAA	18.85	N	N	N	N	**23.7**	N
D560	21	AUSTA	18.74	N	N	N	N	**24.6**	N
D561	20	JAVAA	18.21	N	N	N	N	N	N
D562	20	JAVAC	18.54	N	N	N	N	N	N
D563	1	JAVAC	25.28	**38.2**	N	N	N	N	N
D564	7	AUSTA	20.14	N	N	N	N	N	N
D565	2	MALAYSIA	22.62	N	**23.4**	N	N	N	N
D566	9	V7634	18.52	N	N	N	N	N	N

9.5 Appendix 5

Differential reactivity of BTV1 (standard and modified) & BTV21 serotype-specific assays compared to BTV pan reactive assay

VIRUS No	Serotype	DATE	Location	Pan BTV	BTV1	BTV1 Diff	BTV1 Mod	BTV1 Mod Diff	BTV21	BTV21 Diff
X245	1	6/04/1988	TOCAL	30.79	Neg	9.21	38.22	7.43	Neg	-
X250	1	18/04/1988	SINGLETON	25.19	34.42	9.23	31.66	6.47	Neg	-
X329	1	16/01/1989	GLOUCESTER	12.96	21.11	8.15	16.69	3.73	Neg	-
X347	1	8/02/1989	GLOUCESTER	13.18	21.37	8.19	17.09	3.91	Neg	-
X326	1	6/04/1989	MAITLAND	13.66	21.96	8.30	16.52	2.86	Neg	-
Y355	1	16/04/1993	CASINO	15.02	22.54	7.52	18.70	3.68	Neg	-
Y999	1	3/04/1996	CASINO	35.15	Neg	4.85	Neg	-	Neg	-
Y985	1	17/09/1996	LISMORE	13.56	20.33	6.77	17.39	3.83	Neg	-
Z66	1	2/12/1996	CASINO	13.34	20.89	7.55	17.05	3.71	Neg	-
Z177	1	10/01/1997	CASINO	13.31	21.37	8.06	16.72	3.41	Neg	-
Z159	1	10/01/1997	CASINO	12.86	20.72	7.86	17.15	4.29	Neg	-
Z169	1	4/03/1997	WINGHAM	13.68	21.57	7.89	17.86	4.18	Neg	-
Z108	1	1/04/1997	RALEIGH	12.01	19.07	7.06	15.66	3.65	Neg	-
A246	1	3/05/2000	BELLINGEN	11.20	19.50	8.30	16.42	5.22	Neg	-
B483	1	3/06/2002	BELLINGEN	10.58	18.57	7.99	14.88	4.30	33.00	-
A903	1	31/03/2003	BELLINGEN	13.47	21.3	7.83	16.46	2.99	Neg	-
A913	1	9/05/2003	TAREE	13.20	20.73	7.53	16.31	3.11	Neg	-
A949	1	9/05/2003	TAREE	12.61	20.38	7.77	16.37	3.76	Neg	-
A905	1	6/08/2003	KEMPSEY	13.54	21.87	8.33	17.65	4.11	30.70	-
B443	1	11/02/2005	CASINO	13.21	18.77	5.56	16.62	3.41	Neg	-
B478	1	7/04/2005	TAREE	12.72	18.57	5.85	16.34	3.62	Neg	-
B465	1	7/04/2005	WOLLONGBAR	12.46	18.42	5.96	15.77	3.31	Neg	-
C274	1	4/04/2006	BELLINGEN	13.31	20.70	7.39	16.77	3.46	36.01	-
D432	1	3/02/2009	CASINO	12.25	24.72	12.47	16.47	4.22	Neg	-
D439	1	4/02/2009	BEX HILL	12.99	22.04	9.05	16.54	3.55	Neg	-
E395	1	7/04/2010	MOTO	18.28	26.29	8.01	22.14	3.86	Neg	-
E416	1	12/05/2010	KEMPSEY	14.39	21.89	7.50	18.47	4.08	Neg	-
W511	21	7/05/1981	WOLLONGBAR	14.75	Neg	-	37.82	23.07	14.31	0.44
Z448	21	4/02/1998	CASINO	13.66	Neg	-	Neg	-	13.92	0.26
Z452	21	7/04/1998	KEMPSEY	12.74	Neg	-	Neg	-	12.91	0.17
Z453	21	7/05/1998	WINGHAM	14.80	Neg	-	Neg	-	14.98	0.18
Z472	21	14/05/1998	WOLLONGBAR	10.97	Neg	-	Neg	-	11.24	0.27
Z485	21	14/05/1998	WOLLONGBAR	11.51	Neg	-	Neg	-	11.73	0.22
A232	21	18/05/2000	SINGLETON	11.84	Neg	-	Neg	-	11.67	-0.17
E404	21	4/03/2010	BOWRAVILLE	14.15	Neg	-	Neg	-	14.49	0.34
E424	21	15/12/2010	WOLLOMOMBI	30.62	Neg	-	38.37	7.75	30.32	-0.30

9.6 Appendix 6

BTV serotype specific and virus isolation results on blood from sentinel cattle in the NT

Animal No.	Lab No.	Date	Pan BTV	BTV1	BTV2	BTV21	Virus Isolation	Serotype isolated
17	11/0008	6/01/2011	29.37	30.83	-	-	+	1
17	11/0028	13/01/2011	27.49	31.1	-	-	+	1
17	11/0044	20/01/2011	28.41	33.3	-	-	+	1
17	11/0055	27/01/2011	30.41	35.61	-	-	-	
17	11/0069	3/02/2011	31.30	34.51	-	-	-	
17	11/0098	10/02/2011	31.58	35.99	-	-	-	
17	11/0113	17/02/2011	31.04	36.3	-	-	-	
17	11/0131	24/02/2011	31.24	35.57	-	-	-	
17	11/0192	10/03/2011	32.72	37.63	-	-	-	
17	11/0215	17/03/2011	32.46	37.32	-	-	-	
17	11/0239	24/03/2011	33.20	39.17	-	-	-	
17	11/0278	31/03/2011	29.67	37.17	29.98	-	+	2
17	11/0302	7/04/2011	30.29	37.7	31.22	-	-	
17	11/0343	14/04/2011	32.70	38.18	32.42	-	+	20
17	11/0381	21/04/2011	32.61	39.07	33.89	-	+	20
17	11/0389	28/04/2011	33.98	39.98	34.97	-	-	
19	11/0008	6/01/2011	34.32	-	-	-	-	
19	11/0028	13/01/2011	25.42	31.01	-	-	+	1
19	11/0044	20/01/2011	27.22	33.28	-	-	+	1
19	11/0055	27/01/2011	28.82	34.8	-	-	-	
19	11/0069	3/02/2011	29.87	34.64	-	-	-	
19	11/0098	10/02/2011	30.40	35.38	-	-	-	
19	11/0113	17/02/2011	29.61	34.93	-	-	-	
19	11/0131	24/02/2011	29.90	35.33	-	-	-	
19	11/0156	3/03/2011	30.43	35.25	-	-	-	
19	11/0192	10/03/2011	30.72	34.77	-	-	-	
19	11/0215	17/03/2011	29.76	35.32	-	-	-	
19	11/0239	24/03/2011	31.27	37.44	-	-	-	
19	11/0278	31/03/2011	30.34	36.37	-	-	-	
19	11/0302	7/04/2011	30.86	37.46	-	-	-	
19	11/0343	14/04/2011	29.67	36.82	-	-	+	20
19	11/0381	21/04/2011	29.95	37.37	-	-	+	20
19	11/0389	28/04/2011	30.83	38.23	-	-	-	
33	12/0012	5/01/2012	28.26	32.67	-	-	+	1
33	12/0026	12/01/2012	25.50	30.51	-	-	+	1
33	12/0040	19/01/2012	27.11	31.39	-	-	+	1
33	12/0058	25/01/2012	27.88	31.59	-	-	+	1
33	12/0077	2/02/2012	28.44	31.77	-	-	+	1
33	12/0106	9/02/2012	29.09	32.55	-	-	+	1
33	12/0138	16/02/2012	28.37	33.12	-	-	+	1
33	12/0161	23/02/2012	27.94	33.37	-	-	+	1
33	12/0177	1/03/2012	28.64	33.53	-	-	-	
33	12/0195	8/03/2012	28.17	33.82	-	-	-	
33	12/0219	15/03/2012	28.63	33.34	-	-	-	
33	12/0235	22/03/2012	30.54	32.94	-	-	-	
33	12/0255	29/03/2012	29.68	33.11	-	-	-	
33	12/0278	4/04/2012	30.34	34.54	-	-	-	
33	12/0301	12/04/2012	31.58	35.02	-	-	-	
33	12/0314	19/04/2012	31.37	34.87	-	-	-	
33	12/0330	26/04/2012	31.87	35.62	-	-	-	
33	12/0342	3/05/2012	31.56	35.88	-	-	-	
33	12/0362	10/05/2012	32.24	35.39	-	-	-	
33	12/0394	17/05/2012	32.35	36.65	-	-	-	

9.7 Appendix 7

Serotype-specific real time PCR results for sentinel cattle in NSW, 2013

Location	Date	Animal ID	BTV PCR*	Type Sp. PCR BTV 1e	Ct Diff BTV1-Pan	Mean Ct Diff	Type Sp. PCR BTV 21
Lismore	5/06/2013	1780	34.15	-	10.85	9.24	-
		1782	32.56	-	12.44		-
		1783	31.91	37.61	5.70		-
		1784	30.79	40.03	9.24		-
		1785	29.97	-	15.03		-
		1787	30.49	40.74	10.25		-
		1789	35.90	-	9.10		-
		1790	30.50	37.63	7.13		-
		1792	31.89	38.58	6.69		-
		1793	29.72	35.64	5.92		-
Casino	2/04/2013	233	27.00	33.27	6.27	9.68	-
		235	32.94	37.94	5.00		-
		236	29.97	-	15.03		-
		237	27.59	37.10	9.51		-
		239	35.25	-	9.75		-
		240	33.59	-	11.41		-
		241	29.26	34.24	4.98		-
		242	29.55	-	15.45		-
Kempsey	14/05/2013	1448	28.10	33.63	5.53	4.55	-
		1449	30.87	35.57	4.70		-
		1451	29.54	33.81	4.27		-
		1452	29.64	34.53	4.89		-
		1453	31.11	34.29	3.18		-
		1454	30.73	35.03	4.30		-
		1942	31.64	36.04	4.40		-
		1944	28.18	33.31	5.13		-

9.8 Appendix 8

BTV RNA levels in *C. brevitarsis* fed on BTV-spiked sucrose

Virus	Days post feeding	Number positive/ number tested (%)	Av Ct value*	Range of Ct values
BTV-1	1	74/97 (76)	36.16	29.08-45.00
	2	17/26 (65)	38.48	32.8-45.00
	3	28/33 (85)	36.19	32.29-45.00
	4	19/41 (46)	40.26	33.63-45.00
	5	14/34 (41)	40.70	32.48-45.00
	6	9/16 (56)	38.80	29.88-45.00
	7	8/14 (57)	38.63	30.57-45.00
	8	3/12 (25)	42.83	35.67-45.00
	10	17/48 (35)	41.82	32.3-45.00
	11	4/19 (21)	41.47	35.87-45.00
	12	15/28 (54)	40.08	33.76-45.00
	17	12/94 (13)	43.83	33.89-45.00, **21.43****
	18	3/28 (11)	44.06	35.4-45.00
	20	3/32 (10)	42.19	31.47-45.00
	22	14/68 (21)	42.99	31.03-45.00
BTV-2	1	8/11 (73)	36.34	31.85-45.00
	14	9/78 (12)	44.02	35.66-45.00
	21	3/26 (12)	44.03	34.4-45.00
	24	6/26 (23)	40.79	32.8-45.00
BTV-23	1	17/17 (100)	30.65	28.53-45.00
	10	56/82 (68)	38.00	32.79-45.00
	14	4/12 (33)	41.55	33.79-45.00
	16	16/38 (42)	40.86	30.1-45.00
	17	10/29 (35)	40.21	29.54-45.00

* For insects in which RNA was not detected, a Ct value of 45 was used

** Indicative of virus replication

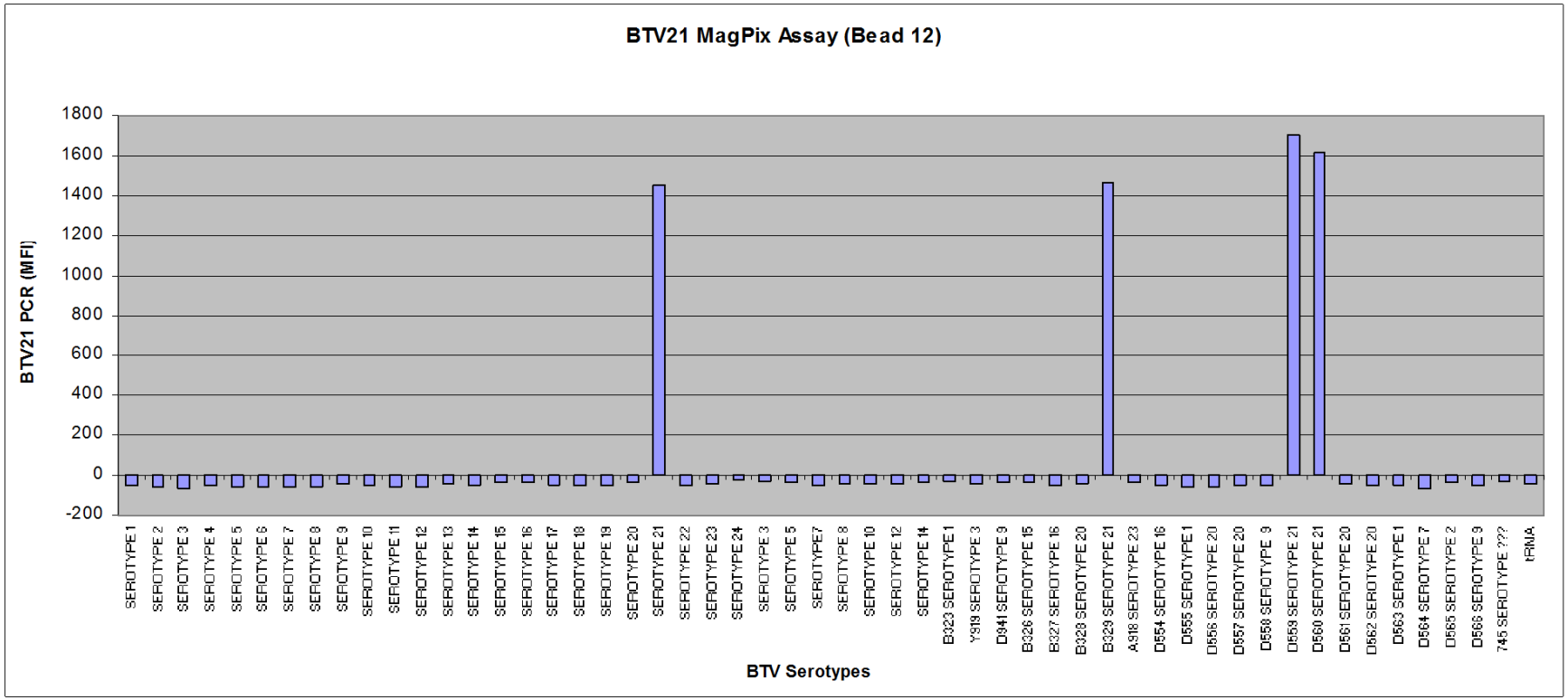
9.9 Appendix 9

Example of Ct values of *C. brevitarsis* feeding on BTV1 infected chicken embryo. Values in bold are indicative of virus replication in the insects.

Midge number	Species	Fed on	Days post infection	Ct value
	ECE		48hr pi	30.30
1	C. brevitarsis	Egg 11	0	32.83
2	C. brevitarsis	Egg 11	0	Neg
3	C. brevitarsis	Egg 11	0	Neg
4	C. brevitarsis	Egg 11	11	Neg
5	C. brevitarsis	Egg 11	11	Neg
6	C. brevitarsis	Egg 11	11	Neg
7	C. brevitarsis	Egg 11	11	**26.94**
8	C. brevitarsis	Egg 11	11	Neg
9	C. brevitarsis	Egg 11	11	Neg
10	C. brevitarsis	Egg 11	11	Neg
11	C. brevitarsis	Egg 11	11	36.56
12	C. brevitarsis	Egg 11	11	36.23
13	C. brevitarsis	Egg 11	11	36.11
14	C. brevitarsis	Egg 11	11	**23.58**
15	C. brevitarsis	Egg 11	11	**24.24**
16	C. brevitarsis	Egg 11	11	Neg
17	C. brevitarsis	Egg 11	11	Neg
18	C. brevitarsis	Egg 11	11	36.35
19	C. brevitarsis	Egg 11	11	Neg
20	C. brevitarsis	Egg 11	11	**23.63**
21	C. brevitarsis	Egg 11	11	**22.20**
22	C. brevitarsis	Egg 11	11	Neg
23	C. brevitarsis	Egg 11	11	Neg
24	C. brevitarsis	Egg 11	11	36.41
25	C. brevitarsis	Egg 11	11	Neg
26	C. brevitarsis	Egg 11	11	35.10
27	C. brevitarsis	Egg 11	11	Neg
28	C. brevitarsis	Egg 11	11	36.17
29	C. brevitarsis	Egg 11	11	Neg
30	C. brevitarsis	Egg 11	11	Neg
31	C. brevitarsis	Egg 11	11	Neg
32	C. brevitarsis	Egg 11	11	Neg
33	C. brevitarsis	Egg 11	11	Neg
34	C. brevitarsis	Egg 11	11	Neg
35	C. brevitarsis	Egg 11	11	**27.47**
36	C. brevitarsis	Egg 11	11	Neg
37	C. brevitarsis	Egg 11	11	36.28
38	C. brevitarsis	Egg 11	11	Neg
39	C. brevitarsis	Egg 11	11	Neg
40	C. brevitarsis	Egg 11	11	Neg
41	C. brevitarsis	Egg 11	11	Neg
42	C. brevitarsis	Egg 11	11	36.59
43	C. brevitarsis	Egg 11	11	Neg
44	C. brevitarsis	Egg 11	11	33.56
45	C. brevitarsis	Egg 11	11	29.24
46	C. brevitarsis	Egg 11	11	**16.80**
47	C. brevitarsis	Egg 11	11	Neg
48	C. brevitarsis	Egg 11	11	36.53

9.10 Appendix 10

A. Testing of BTV Reference panel in MagPix microsphere assay. (Positive = MFI >100)



B. Comparison of BTV21 MagPix assay with BTV21 qRT-PCR

Sample	Identity	BTV (pan) qRT-PCR (Ct)	BTV21 qRT-PCR (Ct)	BTV21 MAGPIX (AvNetMFI)*
W16	EHDV	-	-	-
W17	EHDV	-	-	-
W214	EHDV	-	-	-
W272	EHDV	-	-	-
W525	EHDV	-	-	-
X359	EHDV	-	-	-
X245	BTV1	26.24	-	-
x250	BTV1	26.33	-	-
X326	BTV1	13.56	-	-
X355	BTV1	14.57	-	-
X1004	BTV1	13.27	-	-
Z108	BTV1	12.09	-	-
Z159	BTV1	13.36	-	-
A246	BTV1	7.97	-	-
A949	BTV1	10.67	-	-
C274	BTV1	12.83	-	-
E395	BTV1	18.07	-	-
E416	BTV1	14.47	-	-
E420	BTV1	35.43	-	-
Y999	BTV21	31.09	37.00	1017
Z448	BTV21	13.64	17.80	905
Z452	BTV21	13.22	17.00	1101
Z453	BTV21	15.01	18.70	1297
Z472	BTV21	10.44	15.80	1202
Z485	BTV21	8.78	15.10	1256
A232	BTV21	10.16	15.10	1726
E424	BTV21	29.97	34.50	1380
E404	BTV21	14.27	18.20	970
A905	BTV21	13.90	35.80	1005
B465	BTV21	13.61	37.00	1171
B483	BTV21	11.02	39.00	1401
W225	BTV21	35.00	38.00	1204
BTV21 Control	BTV21	28.10	29.50	254

*Values >100 positive