final report

Project Code: B.STU.0246

Prepared by: Lorna Melville
NT DPI

Date published: July 2015

PUBLISHED BY
Meat and Livestock Australia Limited
Locked Bag 1961
NORTH SYDNEY NSW 2059

Lorna Melville – Mentor for Postdoctoral Fellow

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

Abstract

Through targeted mentorship and by engaging in BEF research activities over the last three years, the postdoctoral fellow enhanced his skills in pathology, immunohistochemistry (IHC), arbovirology, grant writing/management, and leadership. The new IHC protocol he standardised became instrumental in addressing specific research objectives and has potential for use in future BEF research and diagnostics. Overall, the fellow's research efforts elucidated on the tropism of the BEF virus for different bovine tissues, described peripheral nerve lesions in BEF virus-stricken downer cattle, and compared the sensitivity of qRT-PCR and virus isolation in monitoring BEF viraemia. He also studied the kinetics of six cytokines and initiated cellular immunology studies of BEF. Through routine diagnostic work, field post-mortem examinations, and by participating in ongoing arbovirus surveillance, the fellow familiarised himself with animal production systems and vector borne disease issues in the Northern Territory. He presented his research at three national scientific meetings, authored four peer-reviewed papers that are under review, and expects to write two additional papers. The fellow also attained membership of the Australian & New Zealand College of Veterinary Scientists, and was accepted in the MPhil program at the University of Queensland. He was recently appointed Senior Veterinary Pathologist/Virologist at the BVL. In 2016 he took a role in the UAE and resigned.

Table of Contents

Abstract **2**

1 Project objectives **4**

1.1 By 30 June 2015, Dr Lorna Melville will have: 4

1.2 The postdoctoral fellow will: 4

2 Success in achieving milestones **4**

2.1 Project milestone -- By 30 June 2015, Dr Lorna Melville will have: 4

2.2 Project milestone -- By 30 June 2015, the postdoctoral fellow will have: 5

3 Overall progress of the project **10**

3.1 Research plan development and the initial experimental infections 10

3.2 Cytokine studies conducted during the three year project 11

3.3 Research post-mortems, histopathology, and IHC protocol standardisation 11

3.4 Cellular immunology studies 12

3.5 Throughout the 3-year project, Robert Barigye familiarised himself with animal husbandry practices and the status of vector borne diseases in northern Australia through participating in the surveillance activities of the National Arbovirus Monitoring Program (NAMP) 13

4 Appendices **13**

1 Project objectives

1.1 By 30 June 2015, Dr Lorna Melville will have:

1) Mentored the postdoctoral fellow so as to produce a science leader working in animal health of northern beef industry
2) Ensured the postdoctoral fellow has opportunity for development of leadership skills by acting as a mentor and by enabling the postdoctoral fellow to receive formal leadership training on annual basis
3) Develop the research capability of the postdoctoral fellow to a point where he will be successful in obtaining competitive grants

1.2 The postdoctoral fellow will:

1) Develop or enhance skills in arbovirology and understanding of vector borne diseases in northern Australia
2) Develop or enhance skills in histopathology and advanced staining techniques such as in situ hybridisation or immunohistochemistry relevant to bovine ephemeral fever
3) Investigate the relative pathogenicity of different strains of bovine ephemeral fever virus using case material from natural infections or if necessary experimental inoculations
4) Investigate specific immunological and inflammatory response associated with the disease including involvement of particular lymphocyte populations, role of neutrophils, prominent cytokines and other inflammatory mediators involved. This will assist with clinical diagnosis and may help with treatment options
5) Attend and/or present research findings at relevant symposia such as the Arbovirus Research in Australia Symposium
6) Publish three scientific publications in high impact journals with the postdoctoral research fellow as the leading author

2 Success in achieving milestones

The implementation plan for the postdoctoral research project that Robert Barigye developed upon arriving at the Berrimah Veterinary Laboratories (BVL) was approved by the MLA in August 2012 (revised and re-approved in July 2013, and approved yet again by the MLA in July 2014) continued to be implemented during Year 3 of the project. Below is an outline of the successes that were realised in achieving the project objectives per the milestones set by the MLA at the inception of the project:

2.1 Project milestone -- By 30 June 2015, Dr Lorna Melville will have:

1) Mentored the postdoctoral fellow so as to produce a science leader working in animal health of northern beef industry:

During the 3-year-project, Dr Melville mentored the postdoctoral fellow in animal pathology/virology with particular emphasis on how the two disciplines relate to animal health and disease diagnostics and research in general terms and in the context of the beef industry in northern Australia. Specifically, the postdoctoral fellow was provided mentorship in arbovirus and general animal disease monitoring and surveillance in northern Australia. On June 25, 2015 Robert met all the selection criteria set in the position description and was appointed Senior Veterinary Pathologist/Virologist (on permanent basis) at the BVL.

Arbovirus research and diagnostic pathology are his major position responsibilities going forward.

2) Ensured the postdoctoral fellow has opportunity for development of leadership skills by acting as a mentor and by enabling the postdoctoral fellow to receive formal leadership training on annual basis

During the lifetime of the project, the postdoctoral fellow was mentored in leadership through providing ample opportunities that saw him informally lead most of the BEF research project activities. This involved the fellow determining the project implementation timelines and communicating that information to the research team, drafting and obtaining animal ethics committee (AEC) approvals from Charles Darwin University (CDU), planning and coordinating the procurement of consumables, and writing periodic reports that were sent to the MLA and other stakeholders including the CDU-AEC. Besides these activities, whenever required, the postdoctoral fellow provided diagnostic services as lead/duty pathologist and this involved coordinating the testing of submitted samples, assigning diagnostic tests, collating results from the different laboratory sections, and writing reports that were submitted to the clientele of the BVL. In addition, this involvement also provided ample opportunities of chairing weekly meetings at the BVL as well as attending various trainings relevant to enhancing his leadership. For example, Robert attended training on “Respect in the workplace”, two courses on “animal ethics and use of animals in research” among others.

3) Develop the research capability of the postdoctoral fellow to a point where he will be successful in obtaining competitive grants

Throughout the project period, the postdoctoral fellow was mentored in grant writing and project management. Upon arrival at the BVL and during the three years that followed, Robert was mentored in developing research plans that provided the blueprint of project implementation in line with the Year 1, 2 and 3 milestones that the MLA set at the inception of the project. Besides this, the postdoc fellow was mentored in writing and processing AEC approvals for all the experimental work that was done during the 3-year-project. In the final year, Robert developed a pre-proposal titled *“A comparative study of bluetongue virus resistance in Damara, Dorper and Merino sheep breeds”* that he submitted to the MLA. He hopes to further develop this into a three year research proposal that will see him lead research efforts to investigate “the basis of reported differences in BTV resistance in different sheep breeds”.

2.2 Project milestone -- By 30 June 2015, the postdoctoral fellow will have:

1) Developed or enhanced skills in arbovirology and understanding of vector borne diseases in northern Australia

Throughout the 3-year project, the fellow familiarised himself with animal husbandry practices and the status of vector borne diseases in northern Australia through participating in the surveillance activities of the National Arbovirus Monitoring Program (NAMP). He directed and took part in the routine collection and analysis of biological specimens from the departmental sentinel cattle and whenever opportunities presented themselves, the fellow conducted field post-mortem examinations and collected biological specimens from cattle naturally infected with the BEF virus. The samples collected in this manner were used in the

BEF pathogenesis studies – in particular, these samples were crucial during the initial evaluation and optimisation of the BEF IHC protocol. In addition, Robert also regularly performed routine diagnostic pathology work as a lead/duty pathologist and thus gained exposure that enabled him enhance his proficiency in the diagnostic pathology of diseases of domestic, wild and aquatic species unique to the NT region including native Australian species.

2) Develop or enhance skills in histopathology and advanced staining techniques such as in situ hybridisation or immunohistochemistry relevant to bovine ephemeral fever

Using experimentally infected BSR cells and biological specimens collected during research post-mortem examinations of several BEF virus stricken cattle, the fellow standardised and validated a new IHC protocol for the detection of BEF virus antigens in bovine tissues. Most importantly, the new IHC protocol was subsequently successfully used alongside qRT-PCR to characterise the tissue tropism of the BEF virus in cattle as had been outlined under the specific research objectives. The latter objectives had been streamlined when the research fellow developed the three year research project plans in Year 1. The highlights of the project that were permitted by the use of the new IHC assay alongside qRT-PCR include the following:

- During the studies on the tropism of BEF virus for different cattle tissues, post-viraemic localisation of the virus within haemal node and spleen (lymphoid tissues) was confirmed by simultaneous detection of viral RNA and BEF virus antigens in a number of animals. Note that the longest period of post-viraemic detection of BEF virus RNA was 120 days after cessation of viraemia
- The BEF virus was cultured from the spleen and characteristic rhabdovirus (bullet-shaped) particles demonstrated by transmission electron microscopy in the haemal node 7 days after cessation of viraemia in one steer.
- Immunohistochemical localisation of BEF virus antigens within areas of brain stem inflammation in a chronically paralysed steer 42 days after the initial BEF diagnosis provided evidence that linked the virus to low-grade brain stem encephalitis that by nature and severity may partially contribute to the neurological symptoms reported in some BEF downer cattle.
- Detailed histopathological studies of peripheral nerve tissues collected from BEF virus-stricken downer cattle demonstrated severe neuropathy interpreted to be secondary to ischaemic and pressure necrosis as a result of prolonged recumbency. Together with brain stem and spinal cord lesions, the peripheral nerve lesions are now believed to be the cause of the chronic paralysis seen in a minority of animals after the acute phase of BEF.

Note that in addition to having been instrumental in addressing an important research objective of this project, the new IHC protocol has great potential as a tool for future BEF research and diagnostics. Besides this, the experiences learnt during the standardisation of the BEF virus IHC will be applied to other arboviruses – eg plans are underway to complete standardisation of the an IHC protocol for detection of bluetongue virus antigens in animal tissues – both for research and BTV diagnosis.

3) Investigate the relative pathogenicity of different strains of bovine ephemeral fever (BEF) virus using case material from natural infections or if necessary experimental inoculations

At the time Robert developed research plans for implementation of the BEF project, a specific study objective was formulated to compare the sensitivity of qRT-PCR and virus isolation (VI) in detecting viraemia during BEF virus infections in cattle. The research objective in question was premised on the fact that in most/all previous published work, the duration of viraemia in BEF has been determined by VI despite anecdotal evidence that qRT-PCR is comparatively a more sensitive assay. To address this research objective, Robert together with the other members of the research team collected data from BEF virus-positive sentinel cattle at Berrimah and Beatrice Hill Research Farms. Preliminary analysis of this data has shown qRT-PCR to be a more sensitive assay than VI as a method of BEF virus detection in viraemic cattle. In addition, the data also suggest some BEF virus isolates may be non-cytopathic in cell culture further rendering VI a less sensitive technique for BEF diagnostics. This data will be subject to a full research paper which will be written and submitted for publication in a peer-reviewed virology journal. Once published, the data will inform the general scientific community as well as veterinary diagnosticians of the superiority of qRT-PCR technology as a more accurate assay for determination of the viraemic period in BEF virus infected cattle. After publication, this knowledge will help improve future diagnostic, surveillance, and research investigations of the cattle disease.

4) Investigate specific immunological and inflammatory response associated with the disease including involvement of particular lymphocyte populations, role of neutrophils, prominent cytokines and other inflammatory mediators involved. This will assist with clinical diagnosis and may help with treatment options

A) Cytokine studies:

At the initiation of the postdoctoral research project in June 2012, the precise role(s) of proinflammatory cytokines (TNF-α, IL-1β, and IL-6) as well as the anti-inflammatory cytokine (IL-10) in the pathogenesis of BEF in cattle had not been thoroughly investigated. This was despite the knowledge that fever and inflammation were hallmark signs of the disease. In addition, no studies had linked Th1 cytokines (IL-2 and IFN-γ) to the initiation of the adaptive immune response in BEF. Against this background, one of the research objectives formulated at the beginning of the project was to describe the precise role of proinflammatory cytokines in the development of fever and related BEF symptoms. The other specific research objective was to define the kinetics of IL-2 and IFN-γ during the early stages of the adaptive immune response during acute BEF virus infections so as to elucidate the possible role(s) played by cell mediated immune mechanisms in augmenting humoral immunity in BEF. During the course of Years 2 and 3, Robert conducted studies on the role of the four cytokines (IL-1β, IL-6, IL-10, and TNF-α) in the development of fever and two Th1 cytokines (IL-2 and IFN-γ) in the early stage immune responses in BEF virus-infected cattle. Blood and other biological specimens were collected from cohorts of sentinel cattle naturally infected with BEF virus and plasma samples tested with specific antigen capture ELISA. Of the six study cytokines, the IL-1β, IL-6, IL-10, and TNF-α assay were concluded by the end of Year 2 and those of IL-2 and IFN-γ completed in Year 3. The data from these studies are subject to a peer-reviewed manuscript that is due for publication in the *Journal of*

Veterinary Immunology and Immunopathology. Prior to submission of the peer-reviewed manuscript, Robert also gave a departmental seminar in which he discussed the significance of the major findings from the cytokine studies. The paper abstract and a video recording of the presentation are currently displayed on the departmental website where the material is accessible to departmental colleagues and the general public. In addition, an oral paper based on the cytokine study data was also presented at the 2014 Annual Conference of the Australian Society of Veterinary Pathology in Adelaide, SA. As the paper was published in the conference proceedings, it is available to the general scientific community in Australia and elsewhere. Besides this, an abstract of the paper based on the same data that Robert posted on his personal "researchgate" page has since been downloaded by several scientists worldwide.

In summary, data collected from the cytokine studies undertaken during the three-year postdoctoral BEF research project have elucidated on the role and kinetics of proinflammatory and early stage adaptive immune response-specific cytokines during acute BEF virus infections. As anticipated at the beginning of the project, these data have bridged decades-long knowledge gaps in the understanding of the pathogenesis of BEF in cattle. Most importantly, the new knowledge will lay the foundation for future research towards development of improved diagnostic and research tools as well as informing novel BEF treatment protocols and the design of improved BEF vaccines. As the data have been widely disseminated in peer-reviewed media and other fora, the new knowledge is now available to various stakeholders including the greater scientific community. This way, the outcomes of this research will have a broader impact on future BEF research, improved BEF diagnostics as well as on treatment strategies and management of the disease.

B) Cellular immunology studies:

Experiments on immunological aspects of BEF were initiated in Year 3 but remain partially implemented at the time of writing the final report (sample collection and completion of the CD4+ and CD8+ cell kinetics studies will be concluded in September, 2015). During the course of Year 3, peripheral blood mononuclear cells (PBMCs) as well as plasma and serum samples were collected from a total of 12 cattle during a 2 weeks study period that also involved inoculating live BEF virus into three BEF virus-immune cattle (the other three groups of three animals each were the different controls). The PBMCs were separated from peripheral blood of the experimental animals and cryopreserved in liquid nitrogen. Flow cytometry analysis needed to determine the kinetics of CD4+ and CD8+ lymphocytes will be done as soon as additional PBMC collections are made from 8 more animals in September, 2015. The plasma and serum samples collected at the time of the initial PBMC collections were also cryopreserved at -80^{o}C and cytokine assays and the virus neutralising antibody test will be done at a later date respectively. Note that majority of the necessary reagents needed for the flow cytometry studies have already been procured and the relevant collaborations established with personnel at the Immunology Laboratory at the Menzies School of Health Research (MSHR) in Darwin. The flow cytometry will be done at the MSHR as soon as the final collection of PBMCs is done in September 2015.

5) Attend and/or present research findings at relevant symposia such as the Arbovirus Research in Australia Symposium

Throughout the 3-year-project, Robert attended conferences and/or symposia relevant to veterinary pathology and arbovirology in Australia. The papers he presented at the national scientific meetings include the following:

- **Barigye, R.,** Melville, L. *(Joint Oral Presentation).* Neutralizing Antibodies and Pathology of Bovine Ephemeral Fever; 11th Arbovirus Research in Australia Symposium, Gold Coast, Queensland, Australia, September 9th—14th 2012.
- **Barigye, R.,** Davis S, Walsh S, Banurp C, Auman S and Melville L*. (Oral Presentation)*. Immunohistochemical detection of bovine ephemeral fever virus antigen in bovine tissues; Annual Conference of the Australian Society of Veterinary Pathology, Adelaide, SA 10-13th, October, 2013. *(Best Oral Presentation)*
- **Barigye R**, Burnup C, Davis S, Aumann S, Day C, Susan Walsh, Melville L.F . (2014). Kinetics of proinflammatory cytokines and persistence of viral RNA and antigens during natural bovine ephemeral fever infection; the paper was submitted to the XXVIII World Buiatrics Congress (Cairns, QLD, 27th July to 1st August 2014) where it was accepted for an oral presentation. *However, Robert's attendance was not possible due to budgetary constraints.*
- **Barigye, R.,** Davis S, Walsh S, Burnurp C, Auman S and Melville L. (Oral presentation). Kinetics of plasma cytokines, viraemia, and onset of virus neutralizing antibody responses during natural bovine ephemeral fever virus infection; Annual Conference of the Australian Society of Veterinary Pathology, Adelaide, South Australia 9th-11th, October, 2014.
- **Barigye, R.,** Burnup, C., Davis, S., Aumann, S., Day, C., Walsh, S. and Melville, L.F. (Orap presentation). Immunohistochemical detection of bovine ephemeral fever virus antigens in bovine tissues. Besides conference papers, Robert presented the seminar as part of the departmental "knowledge seminar" series (Ref to abstract under Appendix 4).

6) Publish three scientific publications in high impact journals with the postdoctoral research fellow as the leading author

At the time of compiling the final report, Robert Barigye has so far written four manuscripts based on research data collected during the 3-year-project. These papers have been submitted for peer review and publication to the Australian Veterinary Journal (3 papers) and the Journal of Veterinary Immunology & Immunopathology (1 paper). He expects to write two additional peer reviewed papers -- one based on the CD4+/CD8+ kinetics studies and the other based on the data that compared the sensitivity of qRT-PCR and virus isolation for detection of viraemia in BEF virus infected cattle.

The list of the papers so far submitted for peer review and publication is indicated below:

- **Barigye R**, Davis S, Walsh S, Elliott N, Burnup C, Aumann S, Day C, Dryting K, Weir R, Melville LF. (2015). Post-viraemic detection of bovine ephemeral fever virus by use of autogenous lymphoid tissue-derived bovine primary cell lines, qRT-PCR and transmission electron microscopy. *Manuscript under review (Australian Veterinary Journal)*
- **Barigye R**, Burnup C, Davis S, Aumann S, Hunt R, Hunt N, Day C, Walsh S, Weir R, Dyrting K, Elliott N, Melville FL. (2015). Validation of an immunoperoxidase assay for the detection of bovine ephemeral fever virus antigens in cattle tissues. *Manuscript under review (Australian Veterinary Journal)*

- **Barigye R,** Burnup C, Davis S, Aumann S, Hunt R, Hunt N, Day C, Walsh S, Elliott N, Melville FL. (2015). Viral neurotropism, peripheral neuropathy and other morphological abnormalities in bovine ephemeral fever virus-infected downer cattle. Australian Veterinary Journal *(reviews were recently received and the minor revisions recommended by reviewers are being addressed ahead of resubmission and publication)*
- **Barigye, R**., Melville, L, Davis, S., Hunt, R., Hunt, N., Walsh, S. Elliott, N. (2015). Kinetics of proinflammatory cytokines and virus neutralising antibodies during acute bovine ephemeral fever virus infections in Brahman cattle. Accepted -J. Veterinary Immunol. & Immunopathol. *(reviews were recently received and the minor revisions recommended by reviewers are being addressed ahead of resubmission and publication)*

7) Other achievements made by the postdoctoral fellow:

- After intense preparations lasting about 3 months in mid-2014, Robert, the postdoctoral fellow passed the written and practical anatomic veterinary pathology examination of the Australian & New Zealand College of Veterinary Scientists (ANZCVS). He is now a member of the professional organisation and recognised as a veterinary pathologist in Australia and New Zealand. The Veterinary Pathology qualification is also recognised by the National Association of Testing Authorities, Australia (NATA) and hence Robert now meets the NATA requirements to work as a diagnostic veterinary pathologist at the BVL and elsewhere within Australian and New Zealand.
- In February 2015, Robert enrolled in an MPhil degree program at the School of Veterinary Science, University of Queensland. His thesis titled *"A study of viral tissue tropism, cytokine expression, and T-cell responses in bovine ephemeral fever"* is based on the research data he has collected during implementation of postdoc project since arriving at the BVL in June 2012.
- On June 25, 2015 Robert was appointed Senior Veterinary Pathologist/Virologist on permanent basis at the BVL. Arbovirus research and diagnostic pathology are the major position responsibilities going forward.

3 Overall progress of the project

3.1 Research plan development and the initial experimental infections

Robert Barigye started working at the BVL in June, 2012. Upon his arrival at the laboratory, he embarked on writing a detailed research plan that was later approved by the MLA in October 2012. Midway Year 1, a group of 10 cattle and an assortment of chemical reagents and other laboratory supplies were sourced in preparation for the experimental inoculation of cattle. An application submitted to the CDU-AEC was approved for the use of live cattle in this research. To evaluate the infectivity of BEF virus-positive bovine blood before attempting the experimental infections, seven BEF virus-naïve cattle were at different time points inoculated with 6 ml of BEF virus-positive bovine blood. Quite surprisingly and to the disappointment of the BEF research team, none of these animals went down with experimental BEF – the animals later on confirmed to have seroconverted following the experimental inoculations. Around that time, data from ephemerogroup virus ELISA studies obtained by a collaborator working at the Australian Animal Health Laboratory suggested that a substantial number of cattle in the NT have prior exposure to various ephemerogroup viruses like Adelaide River, Fukuoka, Obodhiang, Puchong and Kimberly viruses. At the

time, we suspected that exposure to these viruses might render these animals immune to experimental BEF virus infection. This hypothesis was, in part, later substantiated after batches of the BEF virus-infected blood from our collection availed to Zoetis collaborators successfully produced experimental BEF infections in VIC-based cattle (contrary to having failed to induce clinical BEF in NT cattle at BRF).

3.2 Cytokine studies conducted during the three year project

In the course of implementing the Year 1 plan, it became inevitable that most of the Year 1 objectives had to be carried forward to Year 2. Further re-approved in 2013 (Year 2), the plan described in great details the implementation strategies for the Year 1/2 project objectives. Subsequently, the plasma samples collected from study cattle during Years 1 and 2 were tested for IL-2, IL-1β, Il-6, IL-10. The assays for TNF-α, and IFN-γ were completed in the course of Years 2 and 3. Subsequently, analysis of the cytokine data has shown statistically significant differences between BEF virus-infected and BEF-naïve cattle. In particular, a temporal correlation was shown between the kinetics of IL-1β expression and fever in acutely infected cattle, and an increase in expression of IL-2 and IFN-γ was also showed in cattle at the onset of the virus-specific adaptive immune response. Overall, the cytokine data have generated new knowledge on the pathogenesis of the disease and elucidated on the likely involvement of cellular mechanisms alongside humoral mechanisms during the early adaptive immune response of BEF. The manuscript based on the cytokine data has received good reviewer comments at the *Journal of Veterinary Immunology and Immunopathology* where its publication is pending minor revisions recommended by reviewers. The cytokine data has also been presented at a national conference in Australia and at a departmental seminar within the Department of Primary Industry & Fisheries in Darwin, NT. Besides this, a paper summarising the cytokine data that Robert recently posted on his personal “researchgate” page has already been downloaded by a number of scientists worldwide (16 downloads and 30 views at the time of writing the final report). In summary, the cytokine studies have characterised the kinetics of proinflammatory and early stage adaptive immune response-specific cytokines during acute BEF virus infections and findings clarified decades-long knowledge gaps in the understanding of the pathogenesis of BEF in cattle. As the data have been widely disseminated in peer-reviewed media and other fora, and the research findings are available to various stakeholders including the greater scientific community. This way, the outcomes of this research will have a broader impact on future BEF research and diagnostics as well as on treatment strategies and management of the disease.

3.3 Research post-mortems, histopathology, and IHC protocol standardisation

Not only did studies implemented during Years 1, 2 and 3 permit the standardisation and validation of a new IHC protocol for detection of BEF virus antigens in bovine tissues, they helped in elucidating on the tissue tropism of the BEF virus. In addition, these studies helped in providing additional evidence on the histopathological basis of the chronic paralysis seen in some BEF virus-stricken downer cattle. The data on standardisation of the new IHC protocol are subject to a research paper that is in review and consideration for publication in the Australian Veterinary Journal *(Title: Barigye R et al. (2015). Validation of an immunoperoxidase assay for the detection of bovine ephemeral fever virus antigens in cattle tissues).*

To realise these achievements, research post-mortem examinations were performed and tissue and other biological specimens collected from 10 BEF virus-stricken cattle at different time points following cessation of viraemia. The qRT-PCR assay was used to test the various tissue and body fluid samples for viral RNA and the new IHC assay systematically used to screen formalin-fixed tissues for BEF virus antigens. The data obtained from these studies were then analysed and the tissue tropism of the virus inferred from the distribution of BEF virus RNA and/or antigens in the tissues/organs determined. In a time dependent manner, qRT-PCR demonstrated viral RNA in different tissues several weeks after cessation of viraemia with the longest detection consistently demonstrated in the spleen and haemal node (lymphoid tissues). In particular, the longest detection of virus RNA was in the haemal node 4 months after the initial BEF diagnosis. Besides these findings, the BEF virus was cultured from the spleen and characteristic rhabdovirus particles demonstrated by transmission electron microscopy in the haemal node of a Brahman steer 7 days after cessation of viraemia. The novel findings made during the virus tropism characterisation studies are subject to a research paper that was submitted for review and publication at the Australian Veterinary Journal *(Title: Barigye R et al (2015). Post-viraemic detection of bovine ephemeral fever virus by use of autogenous lymphoid tissue-derived bovine primary cell lines, qRT-PCR and transmission electron microscopy)*.

The other important findings of these studies were the spatial localisation of virus antigens in areas of brain stem encephalitis in a chronically paralysed steer 42 days after the initial BEF diagnosis, and demonstration of severe neuropathy within major peripheral nerves of the brachial plexus and those of the hind limbs including the gluteal and fibular nerves. Rather than a direct effect of the BEF virus however, the peripheral nerve lesion was believed to be secondary to ischaemic/compression and pressure necrosis resulting from prolonged recumbency. Nevertheless, together with brain stem and spinal cord lesions, the peripheral nerve lesions were believed to be a contributory factor in the pathogenesis of the chronic paralysis seen in a minority of BEF virus-stricken cattle following the acute phase of BEF. These data are subject to a research paper that was submitted for review and publication at the Australian Veterinary Journal *(Title: "Barigye R et al (2015) Viral neurotropism, peripheral neuropathy and other morphological abnormalities in bovine ephemeral fever virus-infected downer cattle").*

3.4 Cellular immunology studies

During Year 2, Robert wrote a research plan in which he proposed to use sentinel cattle to evaluate immunological aspects of BEF with particular emphasis of CD4+/CD8+ cellular responses and how the latter correlate with initiation of the virus neutralisation antibody response that is crucial for generation of protective immunity in BEF. With the research protocol already approved by the CDU-AEC, partial implementation of this research objective was done when samples were collected from 12 cattle earmarked for the study. During the course of Year 3, PBMCs as well as plasma and serum samples were collected from a total of 12 cattle during a 2 weeks study period that involved inoculating live BEF virus into three BEF virus-immune cattle (the other three groups of three animals each were the different controls). The PBMCs were separated from peripheral blood of study animals and cryopreserved in liquid nitrogen and flow cytometry analysis for the kinetics of CD4+ and CD8+ lymphocytes will be done as soon as additional PBMC collections are made from 8 more animals in September, 2015. The plasma and serum samples collected at the time of

the initial PBMC collections were cryopreserved at -80^{o}C and cytokine assays and the virus neutralising antibody test will be done at a later date respectively. Note that majority of the necessary reagents needed for the flow cytometry have already been procured and the relevant collaborations established with personnel at the immunology laboratory at the Menzies School of Health Research (MSHR) in Darwin. The flow cytometry will be done that the MSHR as soon as the final collection of PBMCs is done in September 2015.

3.5 Throughout the 3-year project, Robert Barigye familiarised himself with animal husbandry practices and the status of vector borne diseases in northern Australia through participating in the surveillance activities of the National Arbovirus Monitoring Program (NAMP)

From the time of his arrival at the BVL to the end of the project, Robert visited several cattle stations including government and private properties around Darwin. The purpose of these visits were to conduct post-mortem examinations on BEF virus stricken cattle and to familiarise himself with the animal production systems, and current situation of vector borne diseases in the NT. In total, 10 downer cattle with history of clinical BEF were necropsied and biological samples taken for the BEF research project. Throughout the 3 years project, Robert also continued to participate in the routine monitoring of sentinel cattle for BEF and other arboviruses including blue tongue and Akabane viruses among others. From the time of his arrival in June 2012 to the end of the project, Robert contributed to the routine diagnostic veterinary pathology work at the BVL. During the three year period, he was Duty (lead) Pathologist on hundreds if not thousands of diagnostic submissions encompassing all domestic livestock and avian species as well as a number of aquatic and wildlife species majority if not all originating from the NT.

4 Appendices

1) Abstract of the paper presented at the ASVP Conference, Adelaide SA, October, 2014
2) Abstract of the paper accepted at the XXVIII World Buiatrics Congress, Cairns, QLD, July, 2014
3) Abstract of the paper presented at the ASVP Conference in Adelaide SA, October, 2013.
4) Abstract of the paper presented at Dept of Primary Industry & Fisheries, November, 2013
5) Synopsis of Year 3 Research Plan
6) Progress on Year 3 Research Plan Implementation

Appendix 1

Kinetics of plasma cytokines, viraemia, and onset of virus neutralisation antibody responses during natural bovine ephemeral fever virus infections (2014)

[1]Barigye R, [1]Burnup C, [1]Davis S, [1]Aumann S, [1]Day C, [1]Susan Walsh, [1]Melville L.F

[1]Berrimah Veterinary Laboratories, Department of Primary Industry & Fisheries, GPO Box 3000, Darwin, NT 0801, Australia

While fever and inflammation are hallmark features of acute bovine ephemeral fever (BEF), conclusive studies have not been done to characterise the proinflammatory and other cytokine responses that underlie the febrile and related symptoms of acute BEF. The present study was therefore done to define the important cytokine networks that characterise the viraemic and post-viraemic phases of BEF virus infection in adult Brahman cattle. Plasma samples from virus-infected (n=3) and negative control (n=3) animals were tested by a quantitative competitive inhibition ELISA for IL-1β, TNF-α, IL-6, IL-10 over a 6-day-period while the IL-2 and IFN-γ assays done by antigen capture ELISA, viraemia monitored by qRT-PCR, and virus neutralisation antibody titres determined using a standard protocol. In comparison with the negative controls, plasma concentrations of IL-1β, TNF-α, IL-6, and IL-10 were consistently elevated in viraemic cattle and upregulated cytokine expression was already in progress by the time viraemia was detected. Not only was IL-1β the most strongly expressed proinflammatory cytokine, it also correlated with fever in two virus-infected heifers. Elevations in plasma TNF-α showed two peaks 3 and 5 days after fever had subsided and IL-6 and IL-10 manifested intermediate plasma elevations that seemed diametrically opposed to each other. The OD indices for plasma IL-2 and IFN-γ were consistently higher in the BEF virus infected cattle (n=4) compared to the negative controls (n=4) and the increase in cytokine production preceded seroconversion. In most cases, viraemia resolved a day after seroconversion except for one heifer in which prolonged viraemia was observed despite elevated virus neutralisation antibodies. This animal produced the least amount of both IL-2 and IFN-γ. The present results underscore the relative roles of IL-1β, IL-6 and TNF-α in the pathogenesis of BEF and further elucidate on the cytokine events (IL-2 and IFN-γ) that underlie the early stage adaptive immune response. These findings further justify the rationale of using anti-inflammatory agents to treat symptomatic BEF. On the other hand elevation of plasma IL-10 suggests a modulatory role played by the anti-inflammatory cytokine in the transient nature of BEF clinical signs. In addition to virus neutralisation antibodies, it is apparent that cellular mechanisms are likely to play a role in the resolution of viraemia during BEF infections. Specific studies will be done to define the role of CD4+ and CD8+ lymphocytes in IL-2/IFN-γ production and hence protective antiviral immunity in BEF.

APPENDIX 2

Title: Kinetics of proinflammatory cytokines and persistence of viral RNA and antigens during natural bovine ephemeral fever infection (2014)

Abstract No. 0145

Title KINETICS OF PROINFLAMMATORY CYTOKINES AND PERSISTENCE OF VIRAL RNA AND ANTIGENS DURING NATURAL BOVINE EPHEMERAL FEVER INFECTION

[1]**Barigye R**, [1]Burnup C, [1]Davis S, [1]Aumann S, [1]Day C, [1]Susan Walsh, [1]Melville L.F[1]Berrimah Veterinary Laboratories, Department of Primary Industry & Fisheries, GPO Box 3000, Darwin, NT 0801, Australia

Objectives While the inflammatory events seen during acute bovine ephemeral fever (BEF) are believed to be mediated by proinflammatory cytokines, immune complexes have been suggested as the initiator mechanism underlying the vasculoinflammatory lesions typically seen in non-resolving cases of the disease. Besides this, the likelihood of viral persistence and potential replication tissue sites of the virus has not been established. The present study was therefore done to define the important cytokine networks that characterise the viraemic phase of the BEF infection in cattle and to investigate tissue tropism and the likelihood of viral persistence in peripheral sites.

Method Briefly, a novel polymer-based immunohistochemistry (IHC) protocol and a standard reverse transcriptase PCR (rtPCR) assay were used to investigate viral persistence and potential virus replication sites in five BEF-virus-infected adult Brahman cattle. Plasma samples from viraemic and negative control animals were also tested by a quantitative competitive inhibition ELISA and the kinetics of interleukin-1β (IL-1β), tumour necrosis factor-α (TNF-α), IL-6, IL-10 over a 6-day-period determined. Testing of plasma samples for IL-2 and interferon-γ (IFN-γ) is in progress.

Results Viral antigens were detected in the spleen, haemonode, lung, liver, and brain of all the four cattle with most of the immunoreactivity appearing intracellulary within histiocytic and dendritic-like cells and pericytes. The spatial localisation of viral antigens correlated with mild lymphoplasmacytic inflammatory lesions in the brain stem and viral RNA was demonstrated in the spleen and haemonodes of three animals from 0 to 4 months after viraemia was first detected. In comparison with the negative controls, plasma IL-1β, TNF-α, IL-6 and IL-10 quantities were elevated in the viraemic cattle (results for IL-2 and IFN-γ are pending).

Conclusions For the first time ever, the present data documents the persistence of BEF viral RNA in the bovine spleen and haemonode and of viral antigen in several bovine tissues several months after detectable viraemia. The results also underscore the role of proinflammatory cytokines in the pathogenesis of the disease and further elucidate on the immunological events that underlie the early phase anti-BEF adaptive immune response. The persistence of viral antigen in lymphoid tissues might, in part, provide the immunostimulation needed to sustain the typically long-lived post-infection virus neutralisation antibody titres. While of possible diagnostic significance, persistent viral antigen may also contribute to immune-mediated pathology as was postulated by previous researchers.

APPENDIX 3

Immunohistochemical detection of bovine ephemeral fever virus antigens in bovine tissues (2013)

[1]**Barigye R**, [1]Burnup C, [1]Davis S, [1]Aumann S, [1]Day C, [1]Susan Walsh, [1]Melville L.F
[1]Berrimah Veterinary Laboratories, Department of Primary Industry & Fisheries, GPO Box 3000, Darwin, NT 0801, Australia

Abstract

Bovine ephemeral fever (BEF) is an arthropod-borne viral disease of cattle caused by a single stranded RNA virus that belongs to the rhabdovirus group. In cattle, the disease is characterised by short-lived viraemia, fever, inappetance, lameness, and paresis. Due to the brief viraemia, BEF diagnosis may be difficult particularly when acute and convalescent sera are not available to support recent viral exposure. While the inflammatory events seen during acute BEF are believed to be mediated by proinflammatory cytokines, immune complexes have been suggested as the initiator mechanism underlying the vasculoinflammatory lesions typically seen in non-resolving cases of the disease. Besides that point, whether the BEF virus persists beyond the acute phase of the disease is not known and the potential viral replication sites in peripheral tissues have not been established. Briefly, immunohistochemistry (IHC) and reverse transcriptase PCR (rtPCR) were used to investigate viral persistence in five adult Brahman cattle. Viral antigen was detected in the spleen, lung, liver, and brain of all the four cattle with most of the antigen appearing within histiocytic cells and pericytes. The spatial localisation of viral antigens correlated with the distribution of lymphoplasmacytic inflammatory lesions in the brain stem and viral RNA was detected in the spleen of three animals several weeks after the clinical disease. While preliminary, these findings suggest the BEF virus or viral RNA may persist in cattle tissues beyond the acute phase of the disease. The persistence of viral antigen, in particular, might underlie the immunostimulation that possibly sustains the long-lived post-infection virus neutralisation titres. While of possible diagnostic significance, persistent viral antigen may arguably contribute to immune-mediated pathology as has been postulated by previous researchers.

Acknowledgement: *This research was funded by the Meat & Livestock Australia through Grant No. B.STV.0246*

APPENDIX 4

Bovine Ephemeral Fever

When: Monday 4 November at 10am.

Where: Ground Floor Conference Room, John England Building, Berrimah Farm, Makagon Road.

Speaker: Robert Barigye, Research Fellow, Berrimah Veterinary Laboratories.

Immunohistochemical detection of bovine ephemeral fever virus antigens in bovine tissues

Barigye, R., Burnup, C., Davis, S., Aumann, S., Day, C., Walsh, S. and Melville, L.F.

Bovine ephemeral fever (BEF) is an arthropod-borne viral disease of cattle caused by a single stranded RNA virus that belongs to the rhabdovirus group. In cattle, the disease is characterised by short-lived viraemia, fever, inappetance, lameness, and paresis. Due to the brief viraemia, BEF diagnosis may be difficult particularly when acute and convalescent sera are not available to support recent viral exposure. While the inflammatory events seen during acute BEF are believed to be mediated by proinflammatory cytokines, immune complexes have been suggested as the initiator mechanism underlying the vasculoinflammatory lesions typically seen in non-resolving cases of the disease. Besides that point, whether the BEF virus persists beyond the acute phase of the disease is not known and the potential viral replication sites in peripheral tissues have not been established. Briefly, immunohistochemistry (IHC) and reverse transcriptase PCR (rtPCR) were used to investigate viral persistence in four adult Brahman cattle. Viral antigen was detected in the spleen, lung, liver, and brain of all the four cattle with most of the viral proteins appearing within histiocytic cells and pericytes. The spatial localisation of viral antigens correlated with the distribution of inflammatory lesions in the brain stem and viral RNA was detected in the spleen of two animals several weeks after the clinical disease. While preliminary, these findings suggest the BEF virus may persist in cattle tissues beyond the acute phase of the disease. The persistence of viral antigen, in particular, might underlie the immunostimulation that possibly sustains the long-lived post-infection virus neutralisation titres. While of possible diagnostic significance, persistent viral antigen may arguably contribute to immune-mediated pathology as has been postulated by previous researchers.

APPENDIX 5

Year 3 Research Plan

1. Background:

During the wet season that occurred in Year 2 of the research project, an outbreak of bovine ephemeral fever (BEF) at Berrimah and Beatrice Hill Research Farms in the NT presented an exceptional opportunity and biological specimens needed to address a number of research objectives for Year 2 of the BEF project were collected. In particular, plasma samples were collected and tested for cytokines crucial in the induction of acute inflammation and the early adaptive immune response (IL-1β, IL6, IL-10 and TNF-α) during BEF virus acute infection. However due to shortages in the test kits at the time, some of the plasma samples had been cryopreserved which were then tested for IL-2 and IFN-γ during Year 3. During the same BEF outbreak, 3 animals that were positively diagnosed with BEF were selected and post-mortem examinations later performed a month apart to determine the duration of viral tissue tropism and persistence of viral RNA in different tissues and body fluids. Durign Year 3, formalin-fixed tissue specimens that had been collected from BEFV-infected animals were tested by BEF immunohistochemistry (IHC) and relevant data collected. In addition, 6 animals that survived the BEF infection during the outbreak were earmarked for the final study objective titled "*A study of CD4+ and CD8+ lymphocyte responses in bovine ephemeral fever virus immune cattle"*. During Year 3, peripheral blood mononuclear cells were separated from the 6 animals and cryopreserved in liquid nitrogen.

2. The objectives/aims for the Year 3 research program therefore include the following:

- **Objective 1**: Finalise antigen capture ELISA tests for IL-2 and IFN-γ on plasma samples collected during the BEF outbreak that occurred during the wet season in Year 2.
- **Objective 2**: Perform BEF virus-IHC studies on formalin fixed tissue specimens collected during Year 2
- **Objective 3**: Initiate and finalise studies on CD4+ and CD8+ lymphocyte responses in BEF virus immune cattle
- **Objective 4**: Finish data analysis and write 3 manuscripts for publication in peer-reviewed journals

3. Methodology

The following strategy/methodology will be used to address Year 3 objectives cited above:

- **Strategy to address objective 1:** Antigen capture ELISA tests will be done using a commercial kit already procured from CUSABIO. These are quantitative antigen capture ELISA kits that are used to determine the concentration of cytokine molecules in plasma in the magnitude of picogram quantities. Robert Barigye has already successfully used the kits to determine the kinetics of the other four study cytokines (IL-1β, IL6, IL-10 and TNF-α).
- **Strategy to address objective 2:** Immunohistochemistry studies on formalin fixed tissue specimens collected during Year 2 will be performed using the BEF virus IHC protocol

that Robert Barigye developed and standardised during Years 1/2 of the project. For the primary antibody, this assay is based on the monoclonal DB5 developed against the envelope glycoprotein of the BEF virus by Dr Daisy Cybinski at CSIRO, Long Pocket Laboratories, Brisbane, QLD, Australia in the early 1990s. The rest of the assay was standardised by Robert using the DAKO Envision + System-HRP kit.

- **Strategy to address objective 3:** As the main research objective for Year 3, flow cytometry studies will be performed on peripheral blood mononuclear cells (PBMCs) from cattle that had prior exposure to the BEF virus and the cellular immune responses inferred from that data compared with those of age-matched heifers that have never been exposed to the virus. The proportions of CD4+ and CD8+ lymphocytes in the PBMCs will be determined by flow cytometry using fluorescent dye-tagged monoclonal antibody probes that target specific cell markers located on the surface of cells -- in this case CD4+ and CD8+ molecules located on the surface of two lymphocyte subpopulations. The CD4+ and CD8+ cell indices inferred from the flow cytometry data will be compared in two age-matched groups of experimental cattle intravenously challenged with cell-culture derived BEF virus to stimulate secondary immune responses (Group 1 = immune cattle with prior BEF virus exposure; Group 2 = naïve cattle that have never been exposed to the virus). This work will be done in conjunction with a collaborator based on the Menzies Institute of Health Research in Darwin NT.

4. Timelines of key steps in the methodology

	2014						2015				
Activity	July	Aug	Sept	Oct	Nov	Dec	Jan	Feb	Mar	Apr	May
Cytokine ELISA tests (IL-2 & IFN-γ)	x										
BEF-virus immunohistochemistry		x	x	x							
CD4+/CD8+ studies					x	x					
Data analysis						x	x	x	x		
Writing manuscripts						x	x	x	x	x	x
Final report to the MLA											x

5. Results/outputs expected to be delivered by the end of Year 3.

- **Objective 1**: Data on kinetics of IL-2 & IFN-γ once generated and analysed, along with the data already obtained for IL-1β, IL6, IL-10 and TNF-α, will elucidate the important inflammatory and immunological events that take place during the first 5 days of a BEF infection. This will provide insights into the pathogenesis of the disease.

- **Objective 2**: After completion of the IHC assays, inferences from the data analysis will provide insights on spatial tissue tropism of the BEF virus as well as the temporal persistence of viral antigen (and viral RNA) in bovine tissues following a BEF infection.
- **Objective 3**: The data generated from CD4+ and CD8+ lymphocyte responses will provide insights into the basis of the protective immunity that typically occurs following BEF infections. By relating the CD4+ and CD8+ cell activation indices with the virus neutralisation antibody titres (VNT), we shall generate basic information on the relative roles of the two lymphocyte subpopulations in mediating and/or sustaining immunity against the virus during the post-infection period. This knowledge will greatly inform efforts to design better vaccines against BEF.
- **Objective 4**: By writing and publishing 3 manuscripts in peer-reviewed journals, the information generated during this research will be disseminated for the benefit of the broader scientific community.

APPENDIX 6

Progress on implementation of Year 3 research plan

Summary:

During Year 3, pending IL-2 and IFN-γ assays were completed by testing plasma samples collected during the 2013-wet-season-BEF outbreak at Beatrice Hill Farm. The serum samples collected during the same period were also tested for virus neutralising antibody titres and immunohistochemistry for the detection of BEFV antigen performed on the formalin-fixed tissues that had been collected during research post-mortem examinations that were pending by the time milestone #4 was written. Also, peripheral blood mononuclear cells (PBMCs) as well as serum and plasma samples were collected from 12 cattle including 9 with prior BEFV exposure. The PBMCs were cryopreserved in liquid nitrogen (and after additional PBMC collections in September, 2015) and will be tested by flow cytometry in line with the final study objective ("*A study of CD4+ and CD8+ lymphocyte responses in bovine ephemeral fever virus immune cattle")*. The serum samples collected during the 2-week study objective were also tested for virus neutralising antibodies and testing for selected cytokines will be done on the plasma samples that remain in cryopreservation. The results collected from serology and the pending cytokine assays will be collated with those of the CD4+/CD8+ lymphocyte kinetics by the end of 2015.

1. Progress made in addressing Year 3 study objectives

The following strategies/methodologies were used to address Year 3 objectives as outlined above:

- **Progress in addressing Objective 1 (Finalise antigen capture ELISA tests for IL-2 and IFN-γ on plasma samples collected during the BEF outbreak that occurred during the wet season in Year 2).**

Using commercial kits procured from CUSABIO, antigen capture ELISA tests for IL-2 and IFN- γ were done on plasma samples collected in 2013 from BEFV-infected cattle. The data collected from those studies were added to those from IL-1β, IL6, IL-10 and TNF-α testing done in Year 2, analysed, and a peer reviewed manuscript written and submitted for peer review and publication in the Journal of Veterinary Immunology and Immunopathology. Fantastic reviews were recently received and corrections are underway ahead of publication of the paper in the esteemed journal. An oral paper based on the same data was also presented and well received at the 2014 annual conference of the Australian Society of Veterinary Pathology in Adelaide, SA.

- **Progress in addressing Objective 2 (Perform BEF virus-IHC studies on formalin fixed tissue specimens collected during Year 2):**

Immunohistochemistry studies on all the formalin fixed tissue specimens collected during Year 2 were performed using the BEF virus IHC protocol that was developed and standardised during Years 1/2 of the project. The data collected from the IHC testing of all formalin-fixed tissues collected during research post-mortem examinations done during the course of the BEF project were analysed and used to write a peer reviewed paper that was

submitted to the Australian Veterinary Journal. The landmark paper which describes the new IHC tropical and how the latter has been used alongside qRT-PCR to characterise the tissue tropism of the BEFV in cattle remains in review at the AVJ.

- **Progress in addressing objective 3 (Initiate and finalise studies on CD4+ and CD8+ lymphocyte responses in BEF virus immune cattle):**

Peripheral blood mononuclear cells (PBMCs) as well as plasma and serum samples were collected from a total of 12 cattle over a 2 weeks study period. The PBMCs were cryopreserved in liquid nitrogen and flow cytometry will be done as soon as additional PBMC collections from 8 more animals are concluded in September, 2015. The plasma and serum samples collected at the time of PBMC collections were cryopreserved at -80^{o}C and cytokine assays and virus neutralising antibody testing will be tested at a later date. The 12 animals from which PBMCs and other specimens were collected included 9 cattle with prior BEFV exposure along with 3 naïve animals. Once samples are collected from the additional 8 cattle in September, 2015, flow cytometry will be done on the PBMCs to determine the kinetics of CD4+ and CD8+ during mounting of secondary anti-BEFV immune responses. Note that the necessary reagents have already been procured and collaborations established with the immunology laboratory at the Menzies School of Health Research (MSHR) in Darwin. The flow cytometry will be done that the MSHR as soon as the final collection of PBMCs is done in September 2015.

- **Objective 4 (Finish data analysis and write 3 manuscripts for publication in peer-reviewed journals):**

Based on data collected during Years 1, 2 and 3 of the BEF Research Project, four manuscripts were written and submitted for peer review at the *Australian Veterinary Journal* (3 papers) and *Journal of Veterinary Immunology & Immunopathology* (1 paper). These include:

1. **Barigye R**, Davis S, Walsh S, Elliott N, Burnup C, Aumann S, Day C, Dryting K, Weir R, Melville LF. **(2015**). Post-viraemic detection of bovine ephemeral fever virus by use of autogenous lymphoid tissue-derived bovine primary cell lines, qRT-PCR and transmission electron microscopy. *Manuscript under review (Australian Veterinary Journal)*
2. **Barigye R,** Burnup C, Davis S, Aumann S, Hunt R, Hunt N, Day C, Walsh S, Weir R, Dyrting K, Elliott N, Melville FL. (**2015**). Validation of an immunoperoxidase assay for the detection of bovine ephemeral fever virus antigens in cattle tissues. *Manuscript under review (Australian Veterinary Journal)*
3. **Barigye R**, Burnup C, Davis S, Aumann S, Hunt R, Hunt N, Day C, Walsh S, Elliott N, Melville FL. (2015). Viral neurotropism, peripheral neuropathy and other morphological abnormalities in bovine ephemeral fever virus-infected downer cattle. ***Manuscript under review Australian Veterinary Journal***
4. **Barigye, R**., Melville, L, Davis, S., Hunt, R., Hunt, N., Walsh, S. Elliott, N. (**2015**). Kinetics of proinflammatory cytokines and virus neutralising antibodies during acute bovine ephemeral fever virus infections in Brahman cattle. *Manuscript under review-J. Veterinary Immunol. & Immunopathol.*