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Rapid detection of bovine respiratory disease pathogens

Queensland Government
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Abstract

Bovine respiratory disease (BRD) is the most important disease of feedlot cattle in Australia with losses estimated at approximately $60 million per year. BRD is a multifactorial disease with multiple viral and bacterial pathogens interacting with environmental conditions all playing roles in increasing the susceptibility to disease. In recent years, vaccines have become available for three of the microbial (two viral and one bacterial) components of BRD. Essential to the adoption of vaccines for the control of a pathogen is the concurrent assessment of the epidemiology of the targeted pathogens. In addition, the capacity to rapidly identify pathogens will enable the development and adoption of techniques to reduce their negative effects in the production environment. The rapid detection methods for BRD pathogens developed in this project will contribute to improved diagnosis of these pathogens and understanding of the roles they play in the development of BRD. This information will be crucial in developing more effective management strategies, such as vaccination, to minimise the negative economic impacts of BRD in the Australian feedlot sector.

Executive Summary

The bovine respiratory disease complex (BRD) has been identified as the most significant infectious disease of feedlot cattle in eastern Australia (DAN.064). Indeed BRD is the most significant disease associated with feedlot cattle worldwide. BRD has a complex aetiology with four viral and three bacterial species along with environmental conditions predisposing cattle to developing the illness. The four viruses commonly associated with BRD are Bovine herpesvirus 1 (IBR), Bovine viral diarrhoea virus (bovine pestivirus), Bovine parainfluenza 3 virus and Bovine respiratory syncytial virus. Serological surveys have shown that all of these viruses infect feedlot cattle in Australia. In addition a number of bacterial species are recognised as important in the BRD complex, for example *Mannheimia haemolytica*, *Pasteurella multocida* and *Histophilus somni*.

The current study was conducted to improve the detection and identification of the pathogens involved in the onset of BRD. While improved detection will have limited impact on the treatment of individual animals it is essential for developing more effective BRD management strategies. The diagnostic tools developed in this project will enable the epidemiology of BRD development in feedlot cattle to be more accurately defined. Once the roles that the various pathogens play in BRD development are determined, this information can then be utilised to develop more effective management practices for disease prevention. It is possible that different pathogens are more important in some feedlots compared to others. Using current serological tests these essential elements of the BRD complex cannot be easily determined.

The tests developed allow for the detection of the BRD pathogens within 24 hours of a sample being received. Previously, confirming the presence of these pathogens was done retrospectively using slow, difficult and expensive techniques. The presence of various viruses was usually determined by detection of antibodies (that develop after the infection is resolved) in paired blood samples collected 21 to 30 days apart. Typically another 7 days or more is required to complete the tests, so by the time the feedlot gets the results, the disease has long passed. Using the test developed in this project it should be possible to identify infections before the infection is resolved. Another improvement is the ability to detect concurrent infections with several viral agents that was not possible using virus isolation procedures, as typically one agent usually outgrows the other(s) which can lead to the under representation of some pathogens in disease investigations. This may result in underestimating the importance of a pathogen. Further a combined assay was developed and successfully applied for the detection of the four major viral agents of BRD. This permits testing for all of these viruses using a single test for a cost of less than $10.00 (excluding labour).

The rapid diagnostic tests developed in this project are ready for testing in field trial situations and we are planning to do this as part of a new activity investigating the epidemiology of BRD over the next three years. A planned outcome of this activity is the development of a best practice manual for the feedlot sector by 2009 that will provide the industry with a set of tools to minimise the impact of BRD. The application of these tests will provide the sector with essential data on the role of various pathogens in BRD development leading to better management practices.

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1 Background

1.1 Bovine respiratory disease

The bovine respiratory disease complex (BRD) has been identified as the most significant infectious disease of feedlot cattle in eastern Australia (DAN.064). Indeed BRD is the most significant disease associated with feedlot cattle worldwide. BRD has a complex aetiology with four viral and three bacterial species along with environmental conditions predisposing cattle to developing the illness.

The four viruses commonly associated with BRD are:

- Bovine herpesvirus 1 (BoHV-1)
- Bovine viral diarrhoea virus (BVDV or bovine pestivirus)
- Bovine parainfluenza 3 virus (BPIV3)
- Bovine respiratory syncytial virus (BRSV)

Serological surveys have shown that all of these viruses infect feedlot cattle in Australia.

In addition the following viruses have been associated with BRD in overseas studies:

- Bovine adenovirus (BAV)
- Bovine coronavirus

In a small-scale analysis of paired sera (29 samples) from a feedlot we demonstrated activity of the following viruses: BoHV-1, BVDV, BPIV3, BRSV and BAV.

A number of bacterial species have also been recognised as important in the BRD complex:

For example:

- *Mannheimia haemolytica* (Mh)
- *Pasteurella multocida* (Pm)
- *Histophilus somni* (Hs)

BRD causes economic loss due to morbidity, mortality, loss of feed resources, medication purchases, increased time on feed and associated labour costs.

In North America and in Europe, both live and killed vaccines have been used to control diseases caused by BoHV-1 for over 40 years. QDPI developed an effective BoHV-1 vaccine for Australian conditions. The vaccine is a modified live vaccine (MLV) which has been used in pen and field trials to reduce the effects of BoHV-1 infection in feedlot cattle. These trials were conducted in conjunction with Australian Meat Holdings and with financial assistance of MLA (DAQ.097). The first generation vaccine is currently registered for use in Australian feedlot cattle. The second-generation vaccine, a recombinant BoHV-1 expressing BVDV antigens, is about to undergo pen and field trials as part of the registration process (B.FLT.224). This vaccine is designed to protect feedlot cattle from BoHV-1 and BVDV infection. It is also planned to develop similar vaccines for the other viral components of BRD and also for Mh. For effective application of these vaccines it is essential to be able to specifically identify the pathogens involved in clinical cases.

1.2 Improvement diagnostic capabilities BRD pathogens

Early detection and identification of the pathogens involved in the onset of BRD will have limited impact on the treatment of individual animals. It is however an essential tool in developing more effective BRD management strategies to further reduce the economic impact on the feedlot industry. The diagnostic tools developed in this project will enable the epidemiology of BRD development in feedlot cattle to be accurately defined. Once the roles that the various pathogens play in BRD development are determined, this information can then be utilised to develop more effective management practices for disease prevention. It is possible that different pathogens are more important in some feedlots compared to others. Using current serological tests these essential elements of the BRD complex cannot be easily determined.

Currently, the detection of viral agents is slow, difficult and expensive. In fact, one agent, BRSV, has never been isolated in Australia. The presence of various viruses is usually determined by detection of antibodies (that develop after the infection is resolved) in paired blood samples collected 21 to 30 days apart. Typically another 7 days or more is required to complete the tests, so by the time the feedlot gets the results, the disease has long passed. Detection of bacteria is quicker but these pathogens are thought to only be important when there has been a previous viral infection. Preliminary observations in Canada suggest that concurrent infection with several viral agents may frequently occur but this is difficult to determine using virus isolation procedures as one particular agent usually outgrows the others.

The polymerase chain reaction (PCR) is a technique for amplifying DNA (the genetic material) and is particularly useful for detection of viruses and bacteria in clinical samples. A variation of the PCR is the real-time PCR (RT-PCR), so called because the amplification of DNA in the test tube is measured as it actually happens. This allows for a higher level of sensitivity and also quantification of the amount of starting material. Application of this technology in the feedlot sector will enable an accurate picture of what viruses are playing critical roles in the onset of BRD in feedlots. Potentially this would allow specific disease management strategies to be designed on the basis of pathogen activity. For example, if the incidence of BRD can be associated with a particular virus then a specific vaccination strategy may be required. These assays would also enable the origins of these viral infections to be determined. For example, are cattle being infected before or after arrival at the feedlot? Depending on which is the case, different disease management strategies would be required.

These RT-PCR capabilities will also enable more efficient evaluation of pen trial and field trial samples. Currently traditional virological techniques are used to evaluate how well the vaccine viruses are able to replicate in cattle. This is a long and laborious process; the availability of RT-PCR assays would significantly reduce the time for these analyses. Similar analogies can be drawn for the examination of challenge viruses used in pen trials.

We also expect there will be significant interest from Biosecurity (QDPI&F) in adding these tests to their diagnostic repertoire. While this is not a major focus of our work, the virology section of Biosecurity already have adopted two standard PCR assays developed during FLOT.203 for diagnosis of cattle diseases.

2 Project Objectives

By 31 October 2006:

- To develop real-time PCR assays for the viral and bacterial pathogens implicated in the bovine respiratory disease complex of feedlot cattle.

- Evaluate which pathogens are most important in the development of the bovine respiratory disease complex of Australian feedlot cattle using these assays.

- Develop multiplex assay systems to ensure rapid and economical application of these assays to clinical samples.

3 Methodology

3.1 Real-time PCR Assays for Viral Components

3.1.1 Design and Optimisation of Real-time PCR Assays

The individual real-time PCR assays were designed and optimised with the ultimate objective of grouping the assays to form multiplex reactions. The primers were designed with a T_m of approximately 60°C and the probes were specifically designed with a T_m of approximately 70°C. The primers and probes were designed within a narrow annealing temperature range to facilitate the optimisation of the multiplex reactions. All subsequent real-time PCR primers and probes were designed to conform to these specifications. Where possible, the primer and probe sets were sourced from the literature. However, this was frequently not possible because the published methods were not compatible with the relevant multiplex reactions.

Three different mastermixes were evaluated throughout the study. The TaqMan® PCR and RT-PCR Mastermixes (Applied Biosystems) were chosen for the individual reactions and the QuantiTect® Multiplex PCR and RT-PCR Mastermixes (Qiagen) were chosen for the multiplex reactions.

Primer and probe sets were designed manually, with the aid of the computer program Primer Express (Applied Biosystems). Regions of high conservation in the species of interest were identified through literature searches and nucleotide alignments using the computer programs Sequencher (GeneCodes) and GeneDoc (Nicholas and Nicholas, 1997). Throughout the project, primers and probes were synthesised by various biotech companies including, GeneWorks, Proligo, Operon and Applied Biosystems. The primer and probe concentrations were optimised for all of the real-time PCR assays by titration. The concentrations of primers and probes were then re-optimised for the multiplex reactions.

3.1.2 Testing of Bovine Clinical Samples

Clinical samples (n=55) were tested for BRD pathogens using the real-time PCR methods described below. Samples from animals exhibiting clinical signs of BRD were provided by Dr. Tony Batterham (Quirindi Veterinary Laboratory), Dr. Peter Young (Q-Vax Pty Ltd), Dr. Ibrahim Diallo and Dr. Bruce Corney (Department of Primary Industries and Fisheries, Queensland). The clinical samples tested included nasal and eye swabs, and trachea, lung, heart and bronchial lymph node samples.

Extraction of nucleic acids from clinical samples and cell culture material was achieved using the Roche High Pure Viral Nucleic Acid Extraction Kit. This kit was selected after comparison with several extraction kits and boiling methods. The added advantage of the Roche kit was that the extraction of total nucleic acid allowed the eluate to be utilised for PCR and RT-PCR methods. This kit was also found to be effective for the extraction of bacterial DNA.

Positive controls for the real-time PCR assays were extracted from virus-infected cell culture supernatants, and bacterial cultures for the three bacterial species included in this study. Viral and bacterial positive control material was sourced from the QABC collection and also from Dr. Janice Smith (James Cook University) and Dr. Pat Blackall (Department of Primary Industries and Fisheries, Queensland).

3.1.3 Real-time PCR Assay for Bovine Pestivirus (Bpest)

Novel real-time PCR (RT-PCR) primers and probe were designed to detect all known isolates of Bpest of the type 1 genotype. A nucleotide alignment of the 5' untranslated region (5' UTR) of

87 Bpest isolates was produced using the computer program Sequencher. The 5' UTR region is the most highly conserved region in the Bpest genome, therefore specific oligonucleotides were designed from this region using the computer program Primer Express. The Bpest real-time PCR assay was evaluated and optimised using cell culture and clinical material.

The final, optimised Bpest real-time PCR assay was as follows. The PCR was conducted in a 20 µl reaction mix containing 1 µl of the nucleic acid sample, 600 nM of each primer (BVDVfwd and BVDVrev), 100 nM of the probe (BVDVmbg), 1x TaqMan® One-Step RT-PCR Master Mix (Applied Biosystems), 1x MultiScribe® RT (Applied Biosystems) and sterile deionised water. The cycling parameters were as follows: 48°C for 30 minutes and 95°C for 10 minutes, followed by 40 cycles of 95°C for 15 seconds and 60°C for 60 seconds. The primer and probe sequences for the Bpest real-time PCR are presented in Table 1.

3.1.4 Real-time PCR Assay for Bovine Herpesvirus (BoHV-1)

BoHV-1 primers and probe were sourced from Lovato *et al*. (2003). The primers and probe from this assay were targeted for the gC region of the BoHV-1 genome. The BoHV-1 real-time PCR assay was evaluated and optimised using cell culture and clinical material.

The final optimised BoHV-1 real-time PCR assay was as follows. The PCR was conducted in a 20 µl reaction mix containing 1 µl of the nucleic acid sample, 600 nM of each primer (BHVfwd and BHVrev), 200 nM of the probe (BHVprobe), 1x TaqMan® PCR Master Mix (Applied Biosystems), and sterile deionised water. The cycling parameters were as follows: 50°C for 2 minutes and 95°C for 10 minutes, followed by 40 cycles of 95°C for 15 seconds and 60°C for 60 seconds. The primer and probe sequences for the BoHV-1 real-time PCR are presented in Table 1.

3.1.5 Real-time PCR Assay for Bovine Control Gene (BCG).

The inclusion of a bovine control gene in the multiplex real-time PCR tests has a two-fold advantage. Primarily, detection of the BCG is utilised to ensure that the sample contains sufficient bovine cells for the detection of cell-associated viruses. In addition, the BCG assay can be used to confirm that the PCR amplification was successful and that the absence of any detectable pathogens is a true result and not a function of inhibition of the assay. The BCG selected was 18S rRNA, because it is a multi-copy gene found only in eukaryotic cells which has been shown to be an effective control gene for many real-time PCR applications. The 18S rRNA primers and probe were supplied by Applied Biosystems as a single mastermix solution. The BCG real-time PCR assay was evaluated and optimised using cell culture and clinical material.

The final optimised BCG real-time PCR assay was as follows. The PCR was conducted in a 20 µl reaction mix containing 1 µl of the nucleic acid sample, 1x pre-developed TaqMan® 18S rRNA assay (Applied Biosystems), 1x TaqMan PCR Master Mix (Applied Biosystems), and sterile deionised water. The cycling parameters were as follows: 50°C for 2 minutes and 95°C for 10 minutes, followed by 40 cycles of 95°C for 15 seconds and 60°C for 60 seconds.

3.1.6 Real-time PCR Assay for Bovine Respiratory Syncytial Virus (BRSV)

Real-time PCR primers and probe were designed from a nucleotide alignment of 24 BRSV sequences from the fusion-protein region of the viral genome. A large number of clinical samples were tested with this PCR in conjunction with a previously published BRSV real-time PCR method (Achenbach *et al*., 2004). Fluorescent antibody analysis of CRIB-1 cell culture was also used in an attempt to identify BRSV-infected clinical samples. The full evaluation of the real-time PCR and fluorescent antibody methods were hindered by the unavailability of a BRSV strain for use as a positive control. Although a high level of BRSV activity was detected by serology in

feedlot cattle after induction during the DAN.064 study, this virus has never been isolated in cell culture or detected by RT-PCR from Australian cattle.

3.1.7 Real-time PCR Assay for Bovine Adenovirus (BAV).

Real-time PCR primers and probe were designed to target the E1A region of BAV-3. However, full evaluation of this method was hindered by the unavailability of a BAV-3 strain for use as a positive control. A large number of BRD clinical samples were tested using the BAV real-time PCR. Fluorescent antibody analysis of CRIB-1 cell culture was also used in an attempt to identify BAV-infected clinical samples. Although BAV-3 activity was detected by serology in feedlot cattle after induction during the FLOT.203 study, this virus has never been isolated in cell culture or detected by PCR from Australian cattle.

3.1.8 Real-time PCR Assay for Bovine Parainfluenza Virus 3 (BPIV3).

Initially, attempts to design a real-time PCR assay for BPIV3 were hindered by the inability of the test to detect all of our BPIV3 isolates. Subsequent sequence analysis revealed that a previously unidentified genotype of BPIV3 was circulating in Australian feedlot cattle (see Section 3.4). The sequence data generated in this study allowed us to design specific primers and probe to detect all of the known genotypes of BPIV3. The matrix (M) protein region was selected for oligonucleotide design due to the high level of conservation between the different strains of PIV3. The BPIV3 real-time PCR assay was evaluated and optimised using cell culture and clinical material.

The final optimised BPIV3 real-time PCR assay was as follows. The PCR was conducted in a 20 µl reaction mix containing 1 µl of the nucleic acid sample, 600 nM of each primer (BPI3fwd and BPI3rev), 200 nM of the probe (BPI3mbg), 1x TaqMan® One-Step RT-PCR Master Mix (Applied Biosystems), 1x MultiScribe® RT (Applied Biosystems) and sterile deionised water. The cycling parameters were as follows: 48°C for 30 minutes and 95°C for 10 minutes, followed by 40 cycles of 95°C for 15 seconds and 60°C for 60 seconds. The primer and probe sequences for the BPIV3 real-time PCR are presented in Table 1.

3.2 Real-time PCR assays for bacterial components

3.2.1 Real-time PCR Assay for *Mannheimia haemolytica* (Mh).

A real-time PCR assay was developed for *Mannheimia haemolytica* (Mh) that targeted the leukotoxin gene, *lktA*, of the genome. The Mh real-time PCR assay was evaluated and optimised using bacterial culture and clinical material.

The final optimised Mh real-time PCR assay was as follows. The PCR was conducted in a 20 µl reaction mix containing 1 µl of the nucleic acid sample, 600 nM of each primer (Mhfwd and Mhrev), 200 nM of the probe (Mhprobe), 1x TaqMan® PCR Master Mix (Applied Biosystems), and sterile deionised water. The cycling parameters were as follows: 50°C for 2 minutes and 95°C for 10 minutes, followed by 40 cycles of 95°C for 15 seconds and 60°C for 60 seconds. The primer and probe sequences for the Mh real-time PCR are presented in Table 1.

3.2.2 Real-time PCR Assay for *Pasteurella multocida* (Pm)

Novel real-time PCR primers and probes were designed to detect pathogenic strains of Pm by targeting a virulence gene called DAM. This assay was evaluated and compared to another Pm real-time PCR assay (targeting the 16S rRNA region) developed by Dr Pat Blackall's group at Yeerongpilly. The performance of both assays was comparable when run in parallel using the same samples. However, the Yeerongpilly assay was adopted for this study as it had already been rigorously validated and optimised using a reference collection of Pm isolates and also

tested for cross reactivity against a range of bacteria. The Pm real-time PCR assay was evaluated and optimised in our laboratory using bacterial culture and clinical material.

The final optimised Pm real-time PCR assay was as follows. The PCR was conducted in a 20 µl reaction mix containing 1 µl of the nucleic acid sample, 600 nM of each primer (Pmfwd and Pmrev), 200 nM of the probe (Pmprobe), 1x TaqMan® PCR Master Mix (Applied Biosystems), and sterile deionised water. The cycling parameters were as follows: 50°C for 2 minutes and 95°C for 10 minutes, followed by 40 cycles of 95°C for 15 seconds and 60°C for 60 seconds. The primer and probe sequences for the Pm real-time PCR developed in this study are presented in Table 1. Dr Blackall has requested we not disclose the primer/probe combination details of his assay at the present time.

3.2.3 Real-time PCR Assay for *Histophilus somni* (Hs)

A novel real-time PCR assay for Hs was developed by targeting the 16S rRNA region of the bacterial genome. The Hs real-time PCR assay was evaluated and optimised using bacterial culture and clinical material.

The final optimised Hs real-time PCR assay was as follows. The PCR was conducted in a 20 µl reaction mix containing 1 µl of the nucleic acid sample, 600 nM of each primer (Hsfwd and Hsrev), 200 nM of the probe (Hsprobe), 1x TaqMan® PCR Master Mix (Applied Biosystems), and sterile deionised water. The cycling parameters were as follows: 50°C for 2 minutes and 95°C for 10 minutes, followed by 40 cycles of 95°C for 15 seconds and 60°C for 60 seconds. The primer and probe sequences for the Hs real-time PCR are presented in Table 1.

3.3 Development of multiplex assays

3.3.1 DNA Viral Multiplex Assay (BCG, BoHV-1, & BAV).

The real-time PCR assays for BoHV-1 and BCG were combined to form a multiplex real-time PCR assay. The primer and probe concentrations for both assays were re-optimised. In addition, two reagent master-mix kits were evaluated to optimise assay sensitivity. The BAV primers and probe were not included in the multiplex because the lack of a positive control meant that the assay could not be validated.

The DNA viral multiplex real-time PCR was carried out and optimised using the Corbett Rotor-Gene 3000. The PCRs were conducted in a 20 µl reaction mix containing 1 µl of the nucleic acid sample, 400 nM of BHVfwd, 400 nM of BHVrev, 200 nM of BHVprobe, 1x pre-developed TaqMan® 18S rRNA assay (Applied Biosystems), 1x QuantiTect® Multiplex PCR Mastermix (Qiagen) and sterile deionised water. The cycling parameters were as follows: 95°C for 15 minutes, followed by 40 cycles of 94°C for 60 seconds and 60°C for 60 seconds. The primer and probe sequences for BoHV-1 are presented in Table 1.

3.3.2 RNA Viral Multiplex Assay (BCG, Bpest, BRSV, & BPIV3).

The optimisation of the RNA virus multiplex was conducted using cell culture samples that were positive for both BVDV and BPIV3. BRSV primers and probe were not included in the assay optimisation because the lack of a positive control meant that the assay could not be validated. The optimisation included evaluating the concentration of primers and probe and experimenting with different real-time PCR matermixes to ensure maximal sensitivity and reproducibility of the assay. Only one clinical sample was available that was positive for both BPIV3 and BVDV, therefore the majority of testing was upon cell culture material. The BCG was not included in this multiplex due to the unavailability of a pre-developed TaqMan® 18S rRNA assay that contained probe labelling that was compatible with the other probes in this assay.

The RNA viral multiplex real-time PCR was carried out and optimised using the Corbett Rotor-Gene™ 3000. The PCRs were conducted in a 20 µl reaction mix containing 1 µl of the nucleic acid sample, 400 nM of each primer, 200 nM of each probe, 1x QuantiTect® Multiplex RT-PCR Mastermix (Qiagen), 1x QuantiTect® Multiplex RT Mix (Qiagen), and sterile deionised water. The cycling parameters were as follows: 50°C for 20 minutes and 95°C for 15 minutes, followed by 40 cycles of 94°C for 45 seconds and 60°C for 45 seconds. The primer and probe sequences for BPIV3 and Bpest are presented in Table 1.

3.3.3 Bacterial Multiplex Assay (BCG, Mh, Pm, & Hs).

A bacterial multiplex real-time PCR assay was developed to detect the three most important bacteria (*Mannhaemia haemolytica, Histophilus somni* and *Pasteurella multocida*) involved in the development of BRD. DNA extractions from bacterial cultures were used as positive control material for the optimisation of the individual assays. The reaction was optimised by the evaluation of numerous primer sets, commercial mastermixes, reagent concentrations and cycling parameters. Clinical samples that were previously identified as co-infected with two or more bacteria were tested with the bacterial multiplex to ensure that the assay could detect multiple bacteria without a reduction in assay sensitivity. Comparisons with the individual reactions were conducted to ensure that the detection sensitivity was not reduced by the multiplexing of the four reactions. The final bacterial multiplex real-time PCR assay is outlined below.

The bacterial multiplex real-time PCR was carried out and optimised using the Corbett Rotor-Gene™ 3000. The PCRs were conducted in a 20 µl reaction mix containing 1 µl of the nucleic acid sample, 200 nM of each primer, 200 nM of each probe, 1x pre-developed TaqMan® 18S rRNA assay (Applied Biosystems), 1x QuantiTect® Multiplex PCR Mastermix (Qiagen) and sterile deionised water. The cycling parameters were as follows: 95°C for 15 minutes, followed by 40 cycles of 94°C for 60 seconds and 60°C for 90 seconds. The primer and probe sequences for each bacterium are presented in Table 1.

3.3.4 BRD Viral Multiplex Assay (BoHV-1, BVDV, BPIV3 & BRSV)

A multiplex real-time PCR assay was developed to detect the four most important viral agents (BoHV-1, BVDV, BPIV3 and BRSV) involved with the development of BRD. The BRSV primer and probe set was included in the assay optimisation to ensure that they did not interfere with the other primers and probes in the multiplex. The reaction was optimised by the evaluation of numerous primer sets, commercial mastermixes, reagent concentrations and cycling parameters. Clinical samples that were previously identified as co-infected with two viruses were tested with the viral multiplex to ensure that the assay could detect multiple viruses without a reduction in assay sensitivity. In addition, mock samples were prepared by combining infected cell culture supernatants from the three viruses (BoHV-1, Bpest and BPIV3). Serial dilutions of each viral supernatant in solution with the other two viruses were tested to ascertain if the assay could detect low concentrations of one virus while also detecting high concentrations of the other two viruses without a reduction in assay sensitivity. The individual reactions were incorporated into these experiments as a baseline comparison for the multiplex reaction. The final viral multiplex real-time PCR assay is outlined below.

The viral multiplex real-time PCR was carried out and optimised using the Corbett Rotor-Gene™ 3000. The PCRs were conducted in a 20 µl reaction mix containing 1 µl of the nucleic acid sample, 200 nM of each primer, 200 nM of each probe, 1x QuantiTect® Multiplex RT-PCR Mastermix (Qiagen), 1x QuantiTect® Multiplex RT Mix (Qiagen), and sterile deionised water. The cycling parameters were as follows: 50°C for 20 minutes and 95°C for 15 minutes, followed by 40

cycles of 94°C for 45 seconds and 60°C for 75 seconds. The primer and probe sequences for each virus are presented in Table 1.

Table 1. The primer and probe sequences from all of the validated real-time PCR assays

Primer/Probe Designation	Primer/Probe Sequence	Target Species	Gene
BHVfwd	ATGTTAGCGCTCTGGAACC	Bovine herpesvirus 1	gC
BHVrev	CTTTACGGTCGACGACTCC		
BHVprobe	ACGGACGTGCGCGAAAAGA		
BVDVfwd	TGGATGGCTTAAGCCCTGAGTA	Bovine pestivirus	5'UTR
BVDVrev	CCTCGTCCACGTGGCATC		
BVDVmgb	AGTCGTCAGTGGTTCGA		
BPI3fwd	TGTCTTCCACTAGATAGAGGGATAAAATT	Bovine parainfluenza virus 3	Matrix protein
BPI3rev	GCAATGATAACAATGCCATGGA		
BPI3mgb	ACAGCAATTGGATCAATAA		
Mhfwd	AAGGCGATGATATTCTCGATGGT	*Mannheimia haemolytica*	*lktA*
Mhrev	TACCATCGCCTTTACGGTGAA		
Mhprobe	TATCGATGGCGGTAAAGGCAACGACCTA		
Pmfwd	CGCAGGCAATGAATTCTCTTC	*Pasteurella multocida*	16S rRNA
Pmrev	GGCGCTCTTCAGCTGTTTTT		
Pm	ACTGCACCAACAAATGCTTGCTGAGTTAGC		
Hsfwd	AGGAAGGCGATTAGTTTAAGAGATTAATT	*Histophilus somni*	16S rRNA
Hsrev	TCACACCTCACTTAAGTCACCACCT		
Hsprobe	ATTGACGATAATCACAGAAGAAGCACCGGC		

3.4 Identification of Two Distinct Bovine Parainfluenza Virus 3 Genotypes

3.4.1 Electron Microscopy and Fluorescent Antibody Analysis

Electron microscopy of our BPIV3 isolates was initiated after the BPIV3 real-time PCR failed to detect four of our isolates that had been presumptively identified as BPIV3. Viral pellets from CRIB-1 infected cell culture were examined with a transmission electron microscope (Jeol TEM 1010) using standard protocols.

The presumptive BPIV3 isolates were further analysed by fluorescent antibody analysis of infected CRIB-1 cells. Anti-BPIV3 polyclonal antiserum (VRMD), conjugated to fluorescein isothiocyanate (FITC), was added to the cells according to the manufacturer's instructions. Cells were examined for fluorescence with a Zeiss Axiovert 200 inverted microscope using band pass excitation at 450-490 nm and long pass emission filter at 515 nm.

3.4.2 Universal Paramyxovirus Primers

Universal primer sets specific for individual genera of the *Paramyxoviridae* (Halpin, 2000) were utilised to confirm the genera of the viral isolates. All of the PCRs were conducted using the same reaction protocol and cycling conditions. Briefly, viral RNA was reverse transcribed in a 20 µl reaction mix, containing 5 µl of the nucleic acid sample, 1x StrataScript® buffer (Stratagene), 20U StrataScript® RT (Stratagene), 5U RNasin (Promega), 0.5mM dNTPs (Promega), 1 µM of each primer, and sterile deionised water, at 42°C for 60 minutes followed by 90°C for 5 minutes. PCR amplification was carried out in a 50 µl reaction volume, containing 3 µl of cDNA, 400 nM of each primer, 25 µl of HotStarTaq® Mastermix (Qiagen), and sterile deionised water. The PCR cycling parameters were: 95°C denature for 15 minutes, followed by 30 cycles of 95°C for 1 minute, 50°C for 1 minute and 72°C for 1 minute, and a final extension cycle at 72°C

for 10 minutes. All reactions were performed on a Corbett PCR machine (model: GG1-96). The amplification products were resolved by 1% (w/v) agarose gel electrophoresis.

3.4.3 Matrix Gene Sequencing

The reverse primer MR2 was designed downstream of the RspV1 primer to increase the product size available for sequencing of the M-protein coding region. The MR2 primer was designed in a conserved region that was identified from sequence alignments using BPIV3 sequences from GenBank. The PCR protocol for the RspV1-MR2 PCR was as described above (section 3.4.2). Amplification products were ligated into the pGEM®-T Vector System (Promega) and subsequently cloned according to the manufacturer's instructions. The nucleotide sequences of plasmid inserts were determined by automated DNA sequencing using BigDye® Terminator chemistry (ABI BigDye® Terminator version 3.1).

3.4.4 Sequence and Phylogenetic Analyses

Continguous nucleotide sequences were assembled using the computer software Sequencher (GeneCodes) and all nucleotide positions were confirmed by three or more independent sequencing reactions in both directions. Putative amino acid sequences were generated following BLASTX search routines (Altschul *et al*., 1990). BPIV3 sequences were retrieved from GenBank for the compilation of nucleotide and amino acid alignments using the computer program GeneDoc (Nicholas and Nicholas, 1997).

Nucleotide and putative amino acid alignments that were generated using GeneDoc were subsequently used for phylogenetic analyses using the computer program Mega 3.1 (Kumar *et al*., 2004). Phylogenetic trees were determined by bootstrap analysis (500 replicates) using the neighbour-joining program with the Kimura-2 parameters method for nucleotide data analysis and Poisson correction for amino acid data analysis.

3.4.5 Complete Genome Sequence of Strain Q5592

Twenty-two overlapping primer sets were designed to amplify fragments of DNA to represent the entire genome of BPIV3. The primer sets were designed by targeting conserved regions from an alignment of all the available BPIV3 and HPIV3 genome sequences. The PCR amplification (Section 3.4.2), sequencing (Section 3.4.3) and phylogenetic analysis (Section 3.4.4) of the Q5592 genome were conducted using the protocols described above.

4 Results and Discussion

4.1 Real-time PCR Assays for Viral Components

4.1.1 Real-time PCR Assay for Bpest

The Bpest real-time PCR assay was initially optimised using infected cell culture. However, the assay was further evaluated using clinical material including nasal and eye swabs, and trachea, lung, heart and bronchial lymph node samples collected from clinical cases of BRD. Bpest was successfully detected in infected nasal swabs and trachea, lung and bronchial lymph node tissue. During the course of this project we identified Bpest infection in 5 of the 55 clinical samples tested. In addition, the Bpest real-time PCR was used for the confirmation of three persistently infected (PI) animals.

The Bpest real-time PCR assay is of great benefit for the detection of Bpest infection in feedlot cattle. Infection with this virus is often subclinical and difficult to detect using other methods. In addition, early detection of PI animals by real-time PCR screening can have significant effects on the health of other cattle on the lot (Fulton *et al*., 2006).

4.1.2 Real-time PCR Assay for BoHV-1

BoHV-1 was the most frequently detected viral pathogen from BRD clinical samples. This virus was detected by real-time PCR in 32 of the 55 clinical samples tested. This finding is comparable with previous studies that have identified BoHV-1 as one of the most important viral agents associated with the clinical presentation of BRD (Lovato *et al*., 2003). However, it should be noted that serological studies have shown that the highest activity of BVDV, BRSV and BPIV3 is before and soon after induction to the feedlot (DAN.064 and FLOT.203). The significance of the activity of these viruses at induction and during the early period on fed is yet to be established in regards to the long-term outcomes of the cattle in developing BRD.

Two BoHV-1 cell culture samples, for which the $TCID_{50}$ had previously been ascertained, were utilised to determine the detection limit of the BoHV-1 real-time PCR. The optimised real-time PCR could detect approximately 5 copies of DNA from both samples. The detection of BoHV-1 by real-time PCR was also compared to viral isolation in CRIB-1 cell culture using standard methods. Five samples were identified where BoHV-1 was detected by real-time PCR but not isolated from cell culture.

The BoHV-1 real-time PCR method was used to test nasal swab samples from the V155 Rhinogard pen trial conducted in May 2002 as part of FLOT.203. Using the real-time PCR it was possible to monitor the levels of BoHV-1 throughout the pen trial. BoHV-1 was initially detected in the vaccinated samples on day 7 following vaccination. Due to challenge with a different strain of BoHV-1 (Q3932) on day 14, all of the day 21 samples were positive for BoHV-1. However, levels of BoHV-1 were lower in the vaccinated group, suggesting that virus clearance was quicker in these cattle due to the vaccination.

4.1.3 Real-time PCR Assay for BCG

Real-time PCR detection of the BCG was successfully completed in a variety of materials including cell culture and clinical material.

4.1.4 Real-time PCR Assay for BRSV

A large number of BRD clinical samples from feedlots were tested using the BRSV real-time PCR. However, no positive samples were detected in the various sample types that were

analysed. Numerous primer sets, fluorescent antibodies and cell culture isolation techniques were all used in an attempt to identify a clinical sample infected with BRSV. The lack of a BRSV positive control made it difficult to be confident with the negative results that were obtained from all of the assays. However, serological studies have shown that the majority of BRSV activity occurs around induction of the cattle to the feedlot. During this study only cattle displaying clinical signs of BRD were tested with the various assays. Therefore, the non-detection of BRSV is not particularly surprising considering this is not traditionally when BRSV activity is observed.

A human respiratory syncytial virus (HRSV) isolate (A2) was provided by Dr. Paul Young (University of Queensland) and used as a positive control for all of the BRSV assays. The HRSV isolate was satisfactory as a positive control for the cell culture and fluorescent antibody techniques. However, the nucleotide differences between BRSV and HRSV meant that it was not suitable as a positive control for the real-time PCR methods.

4.1.5 Real-time PCR Assay for BAV-3

A large number of clinical samples were tested using the BAV-3 real-time PCR, cell culture and fluorescent antibody techniques. However, no positive samples were detected in the various sample types that were analysed.

An unknown BAV isolate was identified in our historical collection that had not been typed (prior to the identification of this isolate we were not aware of any BAV isolates in Australia). The BAV-3 real-time PCR did not react with this isolate. A conventional consensus PCR for BAV, followed by nucleotide sequencing, determined that this isolate was a BAV-4 genotype, thus explaining the negative result with the BAV-3 real-time PCR.

4.1.6 Real-time PCR Assay for BPIV3

The BPIV3 real-time PCR was redesigned to detect all of the BPIV3 isolates in our collection (see Section 4.4 for the results and discussion concerning the identification of two distinct genotypes of BPIV3). The assay was evaluated by comparison with conventional RT-PCR assays, cell culture isolation and fluorescent antibody techniques. BPIV3 was detected in 2 of the 55 clinical samples using the real-time PCR. BPIV3 was not detected by conventional RT-PCR and could not be isolated from cell culture passage from any of these clinical isolates.

The BPIV3 real-time PCR was also used to retrospectively detect BPIV3 infection in three of the cattle from a vaccine pen trial conducted in May 2002 as part of FLOT.203. These viruses could not be detected by conventional RT-PCR or isolated from the nasal swab samples using cell culture.

The titre for two BPIV3 cell culture samples, representing genotypes A and B, were determined using $TCID_{50}$ calculations. Serial dilutions of these samples revealed that the BPIV3 real-time PCR assay could detect approximately 10 copies of viral RNA.

4.2 Real time PCR assays for bacterial components

4.2.1 Real-time PCR Assay for Mh

The Mh real-time PCR method produced positive results for all of the available Mh strains. Mh was also detected in 9 of the 55 clinical specimens from BRD cases. No cross reactivity was observed with other bacterial strains tested.

4.2.2 Real-time PCR Assay for Pm

The Pm real time PCR assay was evaluated by testing on Pm isolates (provided by Dr. Pat Blackall) and from our own collection of BRD clinical samples. Pm was detected in 30 if the 55 clinical samples. No cross reactivity was observed with other bacterial strains tested.

4.2.3 Real-time PCR Assay for Hs

The Hs real-time PCR assay was evaluated using clinical material including nasal swabs and lung, trachea and bronchial lymph node tissues from feedlot cattle. Hs was detected in 26 of the 55 clinical samples tested. No cross reactivity was observed with other bacterial strains tested.

4.3 Development of multiplex assays

4.3.1 DNA Viral Multiplex Assay

The sensitivity of the DNA viral multiplex assay was compared to the individual assays using cell culture and various clinical materials. The simultaneous detection of BoHV-1 and BCG using this assay did not decrease the sensitivity of the assay.

4.3.2 RNA Viral Multiple Assay

The RNA viral multiplex real-time PCR was used to detect Bpest and BPIV3 simultaneously in the same reaction. The sensitivity of this assay was compared to the individual assays using mock samples from infected cell culture material. The CT values from the multiplex reactions were actually lower than those produced by the individual reactions when testing the same samples. This was unexpected, because the competition for reagents in the multiplex reaction was expected to reduce the sensitivity for individual viruses. The increased sensitivity of the multiplex is believed to be due to the QuantiTect Multiplex RT-PCR Mastermix.

A nasal swab sample from a PI animal was received that was co-infected with BPIV3. This sample was tested using the RNA viral multiplex to determine if the assay could simultaneously detect both viruses from a clinical sample without a decrease in sensitivity. Both viruses were detected without a reduction in sensitivity.

4.3.3 Bacterial Multiplex Assay

All three bacterial species and the BCG were detected using the multiplex real-time PCR assay without a reduction in sensitivity when compared to the individual assays. In addition, reaction sensitivity was not reduced when samples that were co-infected with two or three of the bacteria were tested.

It is difficult to interpret the significance of detecting the bacterial pathogens in BRD clinical samples. All three of these bacterial species are commonly found in the respiratory tracts of healthy cattle. It is only during episodes of high stress and viral infection that they act as opportunistic pathogens to cause disease. Therefore, the detection of these bacteria does not necessarily indicate a productive infection. Although detection of these bacterial species does not necessarily indicate an involvement in the clinical signs of the cattle, this assay is useful for epidemiological studies and the detection of these bacteria following pen trial challenge experiments.

4.3.4 BRD Viral Multiplex Assay

A multiplex real-time PCR assay was developed to detect the four most important viral agents (BoHV-1, Bpest, BPIV3 and BRSV) associated with the clinical BRD. Although the BRSV assay

could not be validated due to the lack of a positive control, the primers and probe for this virus were included because serological evidence (DAN.064 and FLOT.203) suggests that BRSV is common in Australian feedlots.

The evaluation of this assay using co-infected clinical and mock samples determined that this assay could detect all three viruses (BoHV-1, Bpest and BPIV3) simultaneously without a reduction in sensitivity when compared to the individual reactions.

The common viral multiplex assay reported here is believed to be the most important test developed in this study. The simultaneous detection of all four of the major viruses involved in BRD will prove time and cost effective when compared to traditional detection methods.

4.4 Identification of Two Distinct Bovine Parainfluenza 3 Genotypes

4.4.1 Identification of BPIV3 Isolates

All of the BPIV3 isolates examined using electron microscopy exhibited typical BPIV3 morphology with spherical particles of approximately 100 nm and large external spikes measuring approximately 10 nm (Figure 1).

Subsequent incubation of infected CRIB-1 cells with anti-BPIV3 polyclonal antibodies conjugated to FITC demonstrated strong and specific binding of anti-BPIV3 to all clinical isolates tested, when compared to uninfected cells (Figure 2). No differences in the cellular distribution or intensity of fluorescence were observed between isolates.

Both morphology and anti-BPIV3 antibody recognition confirmed that all isolates were BPIV3. Subsequently, all BPIV3 isolates were tested by RT-PCR using a panel of universal paramyxovirus primers. The BPIV3 isolates all produced a DNA amplicon consistent with the expected size of 178 bp using the *Respirovirus* primer set. No amplicons were produced using the universal primer sets for *Morbillivirus, Rubulavirus* or *Pneumovirus* genera (data not shown).

4.4.2 Matrix Gene Sequencing and Phylogenetic Analysis

Phylogenetic analysis of the BPIV3 M-protein coding region using the computer software MEGA 3.1 revealed that there were two distinct genotypes of BPIV3 (Figure 3). Genotype A contained all of the isolates previously sequenced including BPI3 (JCU) and 06-121612. Genotype B has never been described before and was comprised by the isolates Q5592, 04-15885, 04-169206 and 06-12889. The sequence variation displayed by BPIV3 genotype B explains the inability of the original BPIV3 real-time PCR assay to detect the members in this group.

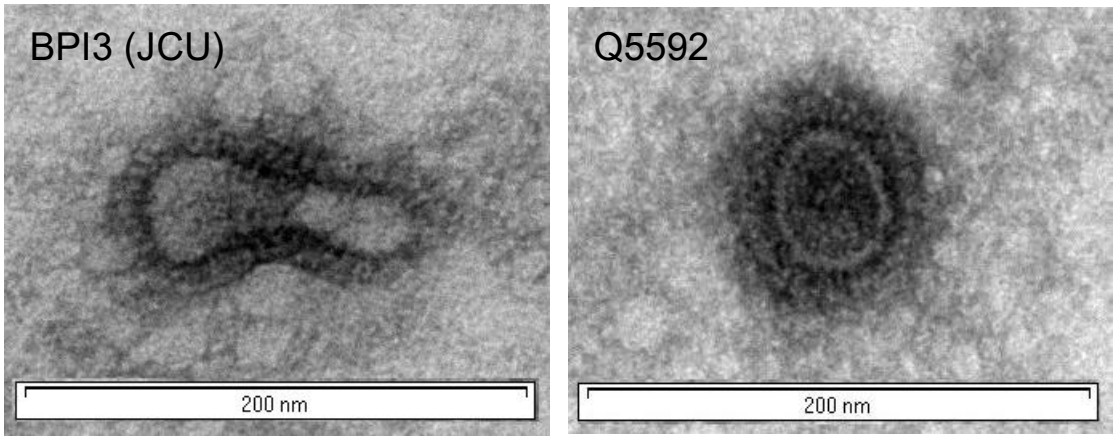

Figure 1. Electron microscopy of the BPIV3 isolates BPI3 (JCU) and Q5592. Both viruses display typical paramyxovirus morphology with spherical particles of approximately 100 nm and large external spikes measuring approximately 10 nm.

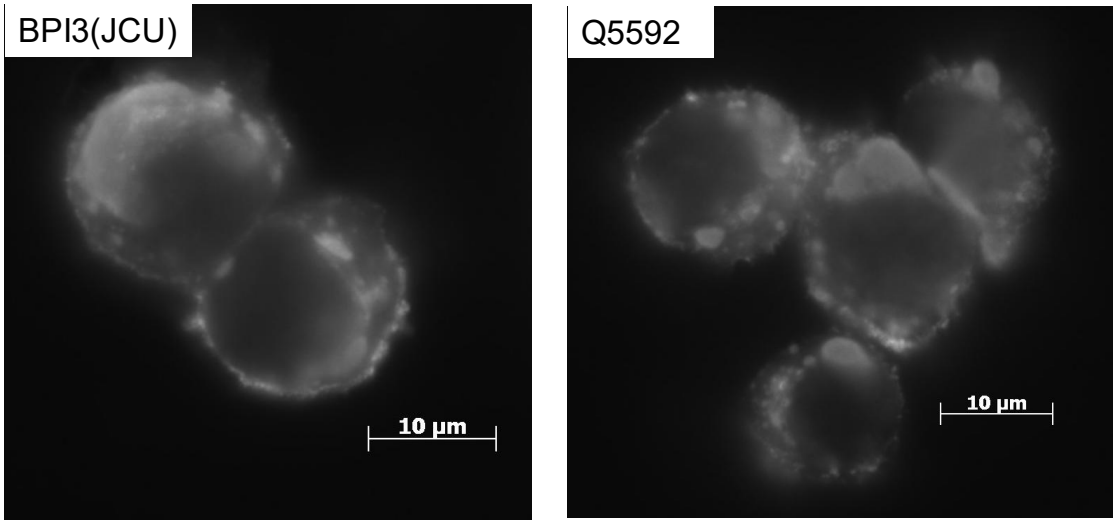

Figure 2. Fluorescent antibody staining of BPIV3-infected CRIB-1 cells with anti-BPIV3 polyclonal antibodies conjugated to FITC.

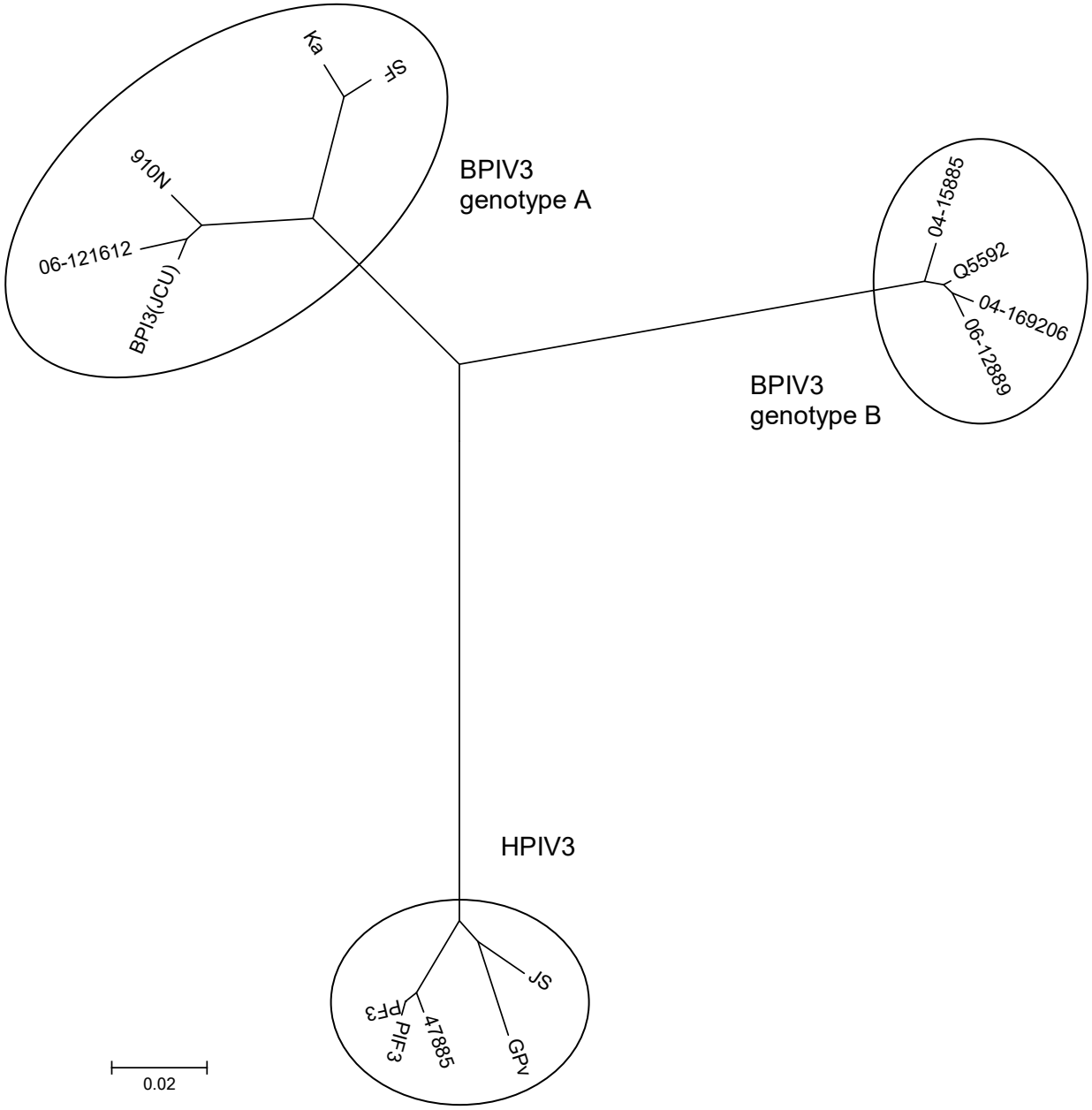

Figure 3. . Nucleotide phylogenetic tree (radiation style) of the M-protein nucleotide sequences of BPIV3 and HPIV3, displaying two distinct genotypes of BPIV3. The tree was prepared by bootstrap analysis (500 replicates) using the neighbour-joining method.

4.4.3 Complete Genome Sequencing of Strain Q5592

The complete genome sequence of the genotype B Q5592 strain was determined using overlapping amplification fragments. The analysis of the Q5592 genome revealed that this strain was much more variable compared to all of the previously reported strains of BPIV3 (Table 2). Strains of BPIV3 have previously been described with sequence polymorphisms (Coelingh *et al*., 1990; Swierkosz *et al*., 1995) and different characteristics in cell culture (Brekker-Klassen *et al*., 1996; Shibuta *et al*., 1983). However, the degree of variation reported did not identify any distinct groups of BPIV3 due to the low level of variation compared with that identified in the present study.

The recognition of sequence variability, and hence two distinct BPIV3 genotypes, is significant because current RT-PCR methods that have specifically been designed to detect BPIV3 may provide false-negative data. In addition, the high degree of variation displayed between the newly identified A and B genotypes may also have important implications to the design of subsequent anti-BPIV3 vaccines.

Table 2. Comparison of the Q5592 genome regions with the BPIV3 type strain (Ka) and the HPIV3 type strain (JS).

Coding regions	**Ka - identity score**		**JS - identity score**	
	Nucleotide (%)	Amino acid (%)	Nucleotide (%)	Amino acid (%)
Nucleoprotein	85	93	78	83
Phosphoprotein	83	76	70	59
Matrix protein	83	96	78	90
Fusion protein	82	85	76	80
Haemagglutinin/neuraminidase	81	87	72	75
Large protein	85	93	81	88
Complete nucleotide	83	N/A	75	N/A
Complete amino acid (coding regions)	N/A	89	N/A	82

5 Success in Achieving Objectives

5.1 Real-time PCR for BRD pathogens

- To develop real-time PCR assays for the viral and bacterial pathogens implicated in the bovine respiratory disease complex of feedlot cattle.

The project objective to develop real-time PCR assays for the viral and bacterial pathogens implicated in the bovine respiratory disease complex of feedlot cattle was achieved.

5.2 Evaluation of the role of pathogens in BRD

- Evaluate which pathogens are most important in the development of the bovine respiratory disease complex of Australian feedlot cattle using these assays.

The second objective of this project to evaluate which pathogens are most important in the development of the bovine respiratory disease complex of Australian feedlot cattle using these assays was also achieved. We have applied the single and multiplex assays for the various BRD pathogens on 55 clinical samples. These samples include nasal swabs (fresh and stored), tracheal rings, lung material (fresh and formalin fix), and sera samples. In most of these cases we have been able to confirm the presence of at least one of the pathogens implicated in the development of BRD.

5.3 Multiplex assay systems for BRD

- Develop multiplex assay systems to ensure rapid and economical application of these assays to clinical samples.

The third objective of this project to develop multiplex assay systems to ensure rapid and economical application of these assays to clinical samples was also achieved. Due to the large number of pathogens implicated in BRD a number of multiplex assays were developed. The first two multiplex assays developed were for the BRD virological pathogens based on the genetic composition of the viral genomes. While the third virus multiplex assay was developed to detect the four viruses most commonly associated with BRD:

Assay 1 for detection of viruses with DNA genomes (BoHV-1 and Bovine adenovirus).

Assay 2 for detection of viruses with RNA genomes (BVDV, BPIV3 and BRSV).
No differences were observed in the detection limits of the single assays compared to the virus multiplex assay.

Assay 3 for detection of viruses commonly associated with BRD (BoHV-1, BVDV, BPIV3 & BRSV).

One caveat that remains with the application of the viral assay is that we have not been able to identify a positive clinical sample containing BRSV.

The next multiplex assay (**Assay 4**) was developed to detect the most commonly associated bacterial components of BRD. Similar results were obtained when the single bacterial assays were also combined into a multiplex assay.

Based on these studies we are confident that these assays will be very useful for the rapid confirmation of these pathogens in clinical material.

6 Impact on Meat and Livestock Industry – now & in five years time

This project has the potential to have an immediate impact on the Australian feedlot sector if the tests developed are taken up by diagnostic service providers. The tests should provide rapid and accurate diagnosis of pathogens associated with clinical disease.

We also expect the project outcomes to have a significant impact in the next five years. We are currently in the final stages of developing a proposal to take a comprehensive look at the epidemiology of BRD pathogens in the Australian feedlot sector. As part of this study we aim to accurately define the association of the various pathogens associated with BRD and the incidence of BRD. In the past only BoHV-1 and BVDV have been regularly associated with BRD as these agents are most commonly isolated from cattle with clinical disease. However, it is well recognised that infection with both BPIV3 and BRSV are very common in feedlot cattle. One of the outcomes of defining the epidemiology of BRD more accurately may be that cattle with BPIV3 and BRSV infections at induction may be more likely to develop BRD.

The test developed as part of this project will be integral in assessing the infection status of newly inducted cattle and those cattle diagnosed with BRD in this important study. The major outcome of the proposed study will be the development of an industry best practice manual for the management of BRD in feedlot cattle. This study should be complete by the end of 2009.

7 Conclusions and Recommendations

7.1 General Conclusions

During the course of this project we have successfully developed real-time PCR assays for the major pathogens implicated in the BRD complex. The assays have been used to detect many of the pathogens in a variety of clinical samples demonstrating excellent specificity and sensitivity. Furthermore, we have been able to combine these tests in multiplex formats for optimal detection of pathogens in disease investigations.

We have also demonstrated the value of using techniques such as real-time PCR for investigating the presence of pathogens in clinical material. This includes high sensitivity as we were able to identify some viruses in samples that had previously been tested and has been positive for a single pathogen using standard techniques but were shown to contain multiple pathogens using the methodologies developed in this study. Further we were also able to demonstrate that these new assays can be used to speed up the identification of pathogens as we were able to complete the assessment of most material within 24 hours of receipt. In comparison isolation of pathogens, where successful, took a minimum of 1 to 2 week to complete. Another advantage the PCR assays was that in some cases the viral pathogens were detected but could not be isolated.

The value of using PCR technology was further illustrated by those cases where multiple viral pathogens were detected. These cases may be of particular importance with respect to the BRD complex as synergistic interactions of pathogens have been implicated in this syndrome. No such cases were identified using standard methodologies.

7.2 Future Studies

An unexpected finding of this study was the identification of two genotypes of BPIV3. These genotypes had not been previously described. The different genotypes could be significant in the context of BRD as the genotypes may have different clinical implications. For example, many genotypes of BVDV have been described but only a subset of these genotypes have been implicated in BRD development. Relevant to this, it will be important to determine the importance of these genotypes in the context of vaccination if BPIV3 vaccines were considered for use in the Australian cattle population.

One remaining issue is the lack of a positive control for use with BRSV detection systems. We were able to obtain some samples from a Victorian property that had a number of deaths caused by an undiagnosed agent. Pathology reports indicated that the most likely agent was BRSV. Unfortunately when we were contacted there was only formalin fixed material remaining. Staining of sections of this material with a BRSV specific fluorescently labelled antibody indicated that there was some specific staining present. However, without a known positive control we were not sure if the staining was real or not. As negative control we also stained sections using a BVDV fluorescently labelled antibody which was also positive. Subsequent real-time PCR analysis detected BVDV nucleic acid but not BRSV, though the absence of BRSV is still considered equivocal without adequate controls. We are planning to continue trying to obtain clinical BRSV material. As a possible source, we are continuing to investigate reports of classical BRSV clinical signs in cattle during the summer-autumn period so by focusing our sampling we may be able to obtain BRSV material.

We are continuing to try and import BRSV for use as positive control material, though the use of overseas viral strains as controls could be problematic. For example, in this study we have identified new genotypes of BPIV3 that required redevelopment of the original PCR assay that was based on data from overseas strains to detect both genotypes. In addition, we have also

identified an uncommon genotype of BVDV in Australia (FLOT.203; Mahony *et al*., 2005). On the basis of these results obtaining an Australian strain of BRSV for use as a control remains a priority to ensure high confidence in the application of any tests.

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