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Prepared by: A Lew[1], B Corney[1], V Doogan[1], G Fordyce[1], J Bertram[1], R Holroyd[1], L McMillen[1], L Turner[1], L Smythe[2], S Fenwick[3], E Taylor[3], P Moolhuijzen[3], M Bellgard[3]

[1]Department of Primary Industries & Fisheries, Queensland

[2]Queensland Health

[3]Murdoch University

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Improved diagnosis of reproductive disease in cattle

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Abstract

Sensitive and specific molecular assays for the improved diagnosis of bovine campylobacteriosis, trichomoniasis, ephemeral fever and leptospirosis were extensively evaluated using spiked clinical specimens, infection trials, multicentre testing and by screening 400 field samples. For bovine venereal diseases and leptospirosis, 3 case study herds were repeatedly sampled to determine the impact of these pathogens on herd fertility and reproductive outcome. An unexpectedly higher prevalence of *Campylobacter fetus* subspecies *venerealis* prompted a preliminary investigation of virulence factors in field isolates. The results suggest that less pathogenic strains may exist in the bull prepuce and further research is required to confirm this observation to determine the consequences on venereal disease. The impact of these improved diagnostic tools will permit the implementation of appropriate management tools to minimise losses due to herd reproductive diseases.

Executive Summary

Sensitive molecular probe based polymerase chain reaction (PCR) assays were developed and evaluated for the improved detection for four bovine pathogens implicated in the following reproductive diseases: trichomoniasis, campylobacteriosis (formerly vibriosis), leptospirosis and ephemeral fever (3-day sickness). Fluorogenic probe based PCR assays, also called real time PCR, 5' *Taq* nuclease assays, quantitative PCR assays, or TaqMan® assays, have been successfully developed for the diagnosis of human venereal diseases, animal and human viral diseases and human leptospirosis. These assays are more sensitive, more specific and less labour intensive than conventional PCR assays and the traditional diagnostic techniques used to diagnose these bovine reproductive diseases. The research undertaken in this project was divided into stages for the venereal diseases and leptospirosis to enable the establishment of laboratory and animal sampling methods, extensive field sampling and case study analysis using the newly developed techniques. The bovine ephemeral fever research included assay development and laboratory evaluation using previously screened diagnostic samples.

Infections caused by both *Campylobacter fetus* subspecies *venerealis* and *Tritrichomonas foetus* lead to conception failure and embryonic loss. Bulls are asymptomatic carriers of these pathogens and female cattle remain immune following exposure to *C. fetus* subsp. *venerealis* whereas in the case of *T. foetus* infections can recur. Both pathogens appear to be cleared by most female cattle, thus bull screening is the most effective measure in determining the presence of either pathogen in a herd. The causative agents of bovine venereal diseases, trichomoniasis and campylobacteriosis (vibriosis), are currently diagnosed using traditional culture techniques which are compromised by poor sensitivity and specificity leading to a high incidence of false negative and positive results. Often in extensively grazed production regions, the presence of either disease, using the current diagnostic tools, can proceed undetected and the cause of delayed conceptions or reproductive failures not determined.

Conventional polymerase chain reaction (PCR) methods have previously been developed to identify *C. fetus* subsp. *venerealis* and *T. foetus* respectively, but have not been applied successfully for laboratory diagnosis. This project developed sensitive 5' *Taq* nuclease assays based on previously identified unique regions present in the DNA sequence of both pathogens. Fluorescently labelled probes were designed to specifically detect each pathogen during a PCR reaction using TaqMan®Minor Groove Binder (MGB) probe technology. These assays are also quantitative in that the diagnostician can determine the pathogen load within the diagnostic specimen (cells per ml). The *C. fetus* subsp. *venerealis* 5' *Taq* nuclease assay is 250 fold more sensitive than selective culture and the *T. foetus* assay is 2500 fold more sensitive than microscopic examination of the enrichment medium currently used. Both assays were also more sensitive than conventional PCR and did not require the preparation of pure DNA prior to testing. Both assays reliably detected 1 single cell equivalent in laboratory spiked smegma (bull prepuce) and mucus (female cervical vaginal). An animal sampling tool based on an Argentinean design demonstrated better isolation of *C. fetus* subsp. *venerealis* from smegma as detected by culture and 5' *Taq* nuclease assay in this study. This tool will soon be available for distribution (DPI&F) and improves the ability to collect samples from both bulls and female cattle for venereal disease diagnosis. Advice from collaborators at the Instituto Nacional De Tecnología Agropecuaria (Argentina) assisted in the adoption of appropriate animal sampling protocols.

Results from screening 341 cattle revealed strong evidence (P<0.0011) that the *C. fetus* subsp. *venerealis* 5' *Taq* nuclease assay (16% positive) produced more positive results than culture (5% positive). Similarly the *T. foetus* 5' *Taq* nuclease assay (9% positive) detected a higher proportion of positive results compared with culture (2% positive), (P=0.006, 361 cattle). Case study herds for each pathogen were selected based on the detection of *C. fetus* subsp. *venerealis* and *T. foetus* and samples were screened pre- and post- matings. Mating groups with uninfected and infected bulls were established however we could not confirm the definitive impact of *T. foetus* in particular. Evidence for delayed conception and lowered pregnancy rates could be attributed to *C. fetus* subsp. *venerealis* in one of the mating groups.

The unexpected high level of *C. fetus* subsp. *venerealis* detected during the field sampling stage prompted an investigation to determine the presence of virulence genes in field isolates collected during this research. We capitalised on recently available *C. fetus* subsp. *fetus* and *C. fetus* subsp. *venerealis* genome sequence data (Universidad Nacional De San Martin, Argentina) and the available literature describing virulence or pathogenicity genes present in more characterised *Campylobacter* species. A bioinformatics resource to enable the selection of genes for this study was established by collaborators at Murdoch University (WA's Centre for Comparative Genomics). The preliminary virulence gene screen revealed that it is feasible that *C. fetus* subsp. *venerealis* isolates are diverse and that perhaps less pathogenic strains can occupy the bull prepuce. However, we do not know whether these potentially less pathogenic *C. fetus* subsp. *venerealis* isolates are capable of causing campylobacteriosis.

Sensitive and specific techniques to detect bovine ephemeral fever virus are required to assist producers to confirm the presence of the pathogen and to subsequently implement vaccination and management strategies. Loss of production due to ephemeral fever can also impact on the reproductive health of an affected herd due to fever and lameness. We developed a sensitive and specific 5' *Taq* nuclease reverse transcriptase assay capable of detecting 10-100 fold more virus than conventional RT-PCR methods and far more sensitive than virus isolation methods. Importantly, the assay also did not detect the closely related non-pathogenic ephemeroviruses commonly detected in BEFV serology. The screening of 191 diagnostic specimens demonstrated definitive correlation with the previously used methods.

Bovine leptospirosis can result in stillbirths, abortion, weak calves and infertility. The members of the genus *Leptospira* is a complex of antigenically related serovars which are further classified as either saprophytic or pathogenic. These serovars are grouped into species which do not correspond to the serogroups. There are over 200 pathogenic serovars known. Hardjo and Pomona are the most common serovars present in Australian cattle and a killed bivalent vaccine is available. Leptospirosis is also a significant occupational hazard for workers in the dairy and beef industries with approximately 21% of human cases attributed to these groups. A human vaccine for leptospirosis is not currently available, thus accurate detection of the pathogenic *Leptospira* spp. in animal reservoirs is a high priority.

Leptospira are usually cultured from urine or kidneys however culture isolation is labour intensive and can take up to three months. The serological test (microscopic agglutination test) is difficult to interpret and anergic chronically infected animals can be missed. It is thus difficult to diagnose leptospiral induced sub-fertility with any degree of certainty. We adapted a real time assay developed for the diagnosis of human pathogenic *Leptospira* spp. for use with bovine diagnostic samples.

The human *Leptospira* assay was successfully adapted for use with bovine specimens with significant improvement over traditional diagnostic methods. Urine and tissue samples from female cattle were suitable for the modified real time assay. Urine samples were successfully prepared for assay using both a commercial kit and crude heat lysis extracts. Field screening of 222 animals resulted in 27 real time positives whereas no positive *Leptospira* cultures were obtained. A case study herd (*Hardjo* confirmed by serology) suggested that calving failure in the herd was partially if not exclusively due to the presence of *Leptospira* in this group. The leptospirosis component of this project was undertaken in collaboration with WHO/FAO/OIE Collaborating Centre for Reference and Research on Leptospirosis and Murdoch University's School of Veterinary and Biomedical Sciences.

Cattle industries will benefit from the wide adoption of the 4 newly described sensitive real time PCR assays to assist in the identification of pathogens responsible for herd reproductive loss. All methods improved substantially upon the currently available tools for the diagnostic detection of *C. fetus* subsp. *venerealis*, *T. foetus*, BEFV and the pathogenic *Leptospira*. It has been estimated that up to 66% gross margin losses can be attributed to campylobacteriosis and 50% to trichomoniasis. For the pathogenic *Leptospira*, improved detection in cattle will benefit human health by reducing the risk of disease transmission in meat industry workers.

The benefits of implementing these improved bovine reproductive disease assays include:

- Improved management of diseases through the implementation of appropriate management protocols such as vaccination.
- Improved understanding of disease epidemiology.
- Decreased economic loss.
- Improved trade and market access.
- Ability to ensure disease free status of semen and breeding stock.

Recommendations resulting from this research include:

- Widespread adoption of the new diagnostic assays, including the use of a new venereal disease sampling tool 'tricamper' (multicentre evaluations have included DPI&F, DPI-NSW, DPI-VIC and DAWA).
- Further communication strategies to promote uptake of the available assays.
- International evaluations of the new diagnostic techniques.
- Undertake controlled trials to further study the herd impact of campylobacteriosis, trichomoniasis and leptospirosis.
- Facilitate trials with vaccine manufacturers to determine the effectiveness of vaccination for both campylobacteriosis and leptospirosis.
- Economic cost benefit analyses to determine the effectiveness of improved diagnostic detection in decreasing reproductive losses.
- Further collaborative research to confirm the presence of less pathogenic *C. fetus* subsp. *venerealis*.

Contents

1 Background

1.1 PCR and *Taq* Nuclease Assays

Sensitive methods for the detection of pathogens in clinical samples have been simplified through the application of polymerase chain reaction (PCR) methods. One of the problems restricting the diagnostic value of PCR is the difficulty confirming specific PCR products. Nested PCR reactions (where PCR product is re-amplified to obtain required sensitivity) have a high risk of contamination and the amplification of non-specific products can become problematic. Post-PCR processing methods such as specific probe hybridisation can be time consuming. 5' *Taq* nuclease assays, often also referred to as fluorogenic probe assays or real time PCR or TaqMan® assays are increasingly applied for the detection and identification of pathogens and do not require post PCR electrophoresis or processing steps (4, 11, 65, 88, 90, 106, 110, 146, 153, 176). These assays exploit the 5' nuclease activity of *Taq* DNA polymerase cleaving a dual labelled fluorescent probe which has annealed to a specific sequence between two primers (104). Real time PCR has engendered wider acceptance of PCR due to its improved rapidity, sensitivity, reproducibility and the reduced risk of carry-over contamination (106). In our experience, TaqMan® assays are as sensitive or more sensitive than nested PCR assays, and at least 100 fold more sensitive than conventional PCR assays. In addition 3' minor groove binder (MGB) probes (TaqMan®MGB) provide improved specificity and sensitivity in comparison to commonly used TaqMan® dual-labelled probes (95, 102). ARI acquired Corbett Rotorgene real time amplification system equipment enabling these assays to be conducted on site. This research program exploited the application of *Taq* nuclease assays for the diagnosis of reproductive disease pathogens.

1.2 Bovine Ephemeral Fever

Bovine ephemeral fever virus (BEFV) is the causative agent of three-day sickness characterized by periodic summer epizootics of fever, respiratory distress, listlessness, stiffness, lameness and sometimes paralysis (89). BEFV is an insect-transmitted single stranded RNA virus of the genus *Ephemerovirus* in the family Rhabdoviridae. There are 2 vaccines available for control of BEFV: a live vaccine which provides about 12 months protection and a killed vaccine providing about 6 months protection. Vaccination strategies vary depending on the needs of producers ie. vaccination of valuable animals, dairy herds (milk production drops if infected), steers close to finishing weights and animals in certain regions where BEF outbreaks are predictable. Other serologically related Ephemeroviruses present in Australian cattle do not cause disease (Adelaide River, Berrimah and Kimberley) and are also insect transmitted (47, 70, 71). The serological methods used in most laboratories are not specific for BEFV and detect all *Ephemerovirus* species. As neutralizing antibodies can persist for years in an animal, these methods do not determine current infection status.

Virus isolation in cell culture has been applied to confirm the presence of virus yet this approach is not always successful (89). Furthermore, virus isolation takes over two weeks to complete. Recently a reverse transcriptase (RT)-PCR BEFV specific test was developed by Biosecurity DPI&F (based on primers designed by CSIRO) for BEFV detection. Although PCR assays can be quite sensitive, the risk of contamination and the amplification of non-specific products can become problematic. It was thus proposed to develop a real time or 5' *Taq* nuclease assay for the detection of BEFV.

1.3 Bovine Venereal Diseases

The primary feature of campylobacteriosis and trichomoniasis is considered failure to conceive and embryo loss; normal fertility resumes after immunity is attained for Camp whereas Trich infections can re-occur (female cattle) (33, 100). Bulls are carriers of these diseases and are thus asymptomatic. Any agent, such as infectious disease, that causes delayed conceptions, even by a week, will reduce the size of weaners in the following year and reduce the opportunity for cows to reconceive at an optimum time. Figure 1 illustrates an 'ideal' cumulative pregnancy% pattern over time in a mating group with no fertility problems.

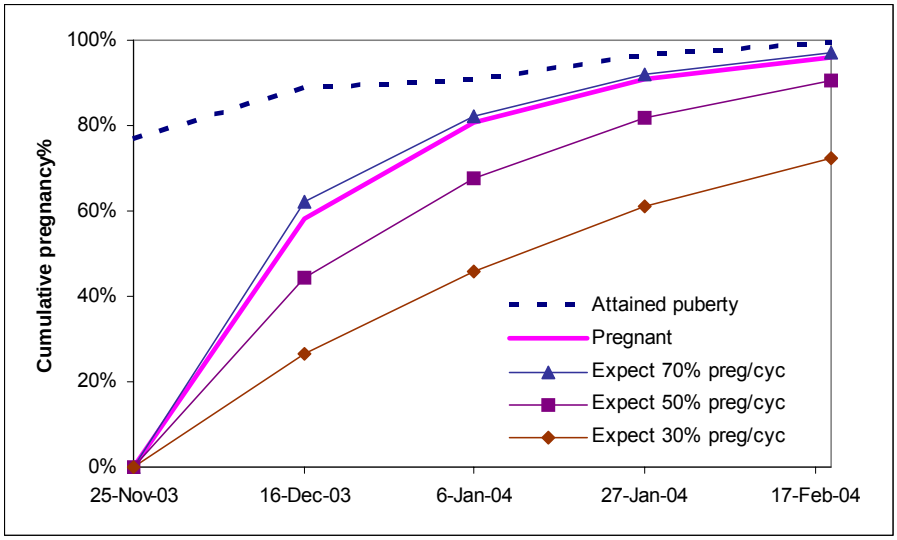


Figure 1. An example of pregnancies in a herd without problems

1.3.1 Campylobacteriosis/Vibriosis

Subspecies identification

Campylobacter fetus, the main *Campylobacter* species associated with bacterial bovine reproductive problems, contains two subspecies that are genetically almost identical (173). Subspecies *venerealis* causes venereal campylobacteriosis (vibriosis) in cattle, characterised by infertility and abortion. Subspecies *foetus* has a broader host range, is transmitted orally, occasionally causes abortion in cattle and sheep, and can cause systemic disease in humans (13). In bulls, *C. fetus* subsp. *venerealis* is confined to the glans penis, prepuce and distal portion of the urethra. Infection is not associated with either histological changes or deterioration in seminal quality. Semen is contaminated during ejaculation and transmission of the organisms to females occurs by coitus or by artificial insemination with contaminated semen. McCool et al., 1988 (112) found that campylobacteriosis in herds in the Northern Territory reduced branding rates by 30-50% compared to non-infected herds producing an annual loss of approximately 9,600 calves through reduced heifer fertility.

Conventional methods isolating the bacteria by culture on laboratory media are used to detect *C. fetus* subsp. *venerealis* in semen or preputial smegma (82, 97). These methods are time consuming and laborious and require the use of selective enrichment media and sufficient technical experience to differentiate *C. fetus* subsp. *venerealis* from non-venereal Campylobacters and other

microorganisms present in bull semen and preputial smegma. Also, low levels of this bacteria are present in semen samples and the presence of antibiotics and inhibitors in diluted semen can limit the sensitivity of culture. One PCR method claims to detect as little as 3 cells of *C. fetus* subsp. *venerealis* in semen (56). In Australia, bovine semen is screened for venereal diseases using conventional culture methods. The most widely utilised PCR method is that described by Hum *et al*. (83) which differentiates *C. fetus* subsp. *venerealis* from *C. fetus* subsp. *fetus* and has been favoured for this reason. This method has largely been applied to differentiate these species post-culture. There are no reports in the literature concerned with adopting PCR detection of these organisms directly in preputial washes, even though it has been recognised that failure to isolate *C. fetus* subsp. *venerealis* is due to the growth of contaminants from the washes (82). There have been a number of PCR methods developed which detect *C. fetus* without differentiating the subtype (14, 27, 127).

Other molecular methods to compare Campylobacters have been applied. These include amplified fragment length polymorphism (AFLP) (174), pulsed gel electrophoresis (PFGE) (67, 126) and the Hum et al 1997 (83) subtyping test described above. As the phenotypic method based on glycine tolerance has been unreliable (174), these methods have largely been developed to differentiate the 2 subtypes. Differences in electrophoretic profiles of the surface array protein (SAP) for isolates of *C. fetus* subsp*. venerealis* have been demonstrated (50). Additionally, the SAP genes have been proven to be effective as a DNA fingerprinting tool for *C. fetus* (53, 68). To date, there are no reports comparing isolates within the subtype for epidemiological studies.

Control of *C. fetus* subsp. *venerealis* in Australia is possible through vaccination consisting of killed bacteria (Vibrovax, Pfizer) (36, 86). The vaccine is not used in areas where *C. fetus* subsp. *venerealis* is not routinely isolated and improved methods of detecting the pathogen may indicate a need to expand vaccination programs.

***Campylobacter fetus virulence* genes**

During the course of this project, our new molecular real time assay (113), based on the Hum PCR target (83), identified the presence of *C. fetus* subsp. *venerealis* in Qld bulls (carriers) at a much higher rate than expected. There is also growing evidence that in some countries, the Hum PCR target is not a reliable gene target for subspecies identification, suggesting that this gene is not present in all isolates of *C. fetus* subsp. *venerealis* (170, 179). If this were the case in Australia, our assay would have potentially missed isolates suggesting that we have actually underestimated the presence of this pathogen. Until recently, no suitable subspecies gene sequences were available for exploitation as targets for specific molecular assays.

An abundance of *C. fetus* subsp. *venerealis* could be explained by the fact that field isolates may vary in virulence. A number of PCR-based studies have been published which compare the detection of virulence genes in other *Campylobacter* species:

- *C. coli* vs *C. jejuni* – turkey, chicken and human origin (122); pig and cattle origin (8).
- *C. jejuni* isolates originating from human, poultry meat and broiler, and bovine faeces (48); turkeys (7); human cases (93).

A *C. jejuni* study demonstrated that certain genes e.g. two-component regulatory systems were important for human disease pathogenicity whereas the role of other putative virulence genes was 'ambiguous' e.g. type IV secretory system *VirB11*, usually present on plasmids (93). However, *in vitro* experiments have demonstrated that a *VirB11* mutant resulted in a 6-fold reduction in adherence and an 11-fold reduction in invasion compared with the wild-type *C. jejuni* (5). A number

of comprehensive studies have confirmed the following processes as contributing to the virulence of these bacteria: adherence, motility, two-component systems, toxin production (cytolethal distending toxin), resistance, and membrane proteins (19, 25, 92, 108, 118, 166, 182).

Specific studies to analyse virulence gene homologues in *C. fetus* subsp. *venerealis* have not been undertaken. Although *C. jejuni, C. coli, C. lari, C. upsaliensis* and *C. fetus subsp. fetus* pathogens colonise the intestine and are not usually implicated in venereal disease (119), studies of these more characterised species can provide background knowledge potentially relevant in the virulence of *C. fetus* subsp. *venerealis*. A comparative genomic analysis of *C. jejuni* (2 strains), *C. coli*, *C. lari* and *C. upsaliensis* recently provided a summarised list of virulence genes for these species (66). Genome sequencing of *C. fetus* subsp. *venerealis* was undertaken by an Argentinean group which produced approximately 72% of the genome sequence (Unpublished, Prof Daniel Sanchez, Universidad Nacional de San Martin, Argentina). In January 2006, a *C. fetus* subsp. *fetus* genome sequence was released in a public database (http://www.ncbi.nlm.nih.gov/). No publications studying these 2 *C. fetus* sequences/partial genomes have been released to date and this sequence data will provide valuable exploitable knowledge about these pathogens.

1.3.2 Trichomoniasis

Trichomoniasis is a venereally transmitted reproductive disease of cattle caused by the protozoan parasite *Tritrichomonas foetus*. Infection can be in apparent in bulls but can cause early embryonic death and abortion in cows. While cows clear the infection, bulls remain as carriers and can re-infect previously infected cows (177). Trichomoniasis produces a range of infertility syndromes similar to Campylobacteriosis, and indeed under extensive management conditions, the effects of each disease are indistinguishable (112). A 3-year controlled experiment undertaken in Victoria demonstrated that cows kept with bulls infected with trichomoniasis produced 17.6% fewer calves than cows kept with non-infected bulls (37). It can be difficult to relate figures where management systems and nutrition are in marked contrast to controlled experiments. Nevertheless, using these figures, it was estimated that trichomoniasis is causing an annual loss in production of approximately 25,500 calves in remote regions of the Northern Territory (112).

T. foetus is confirmed by microscopic examination of the enrichment/transport medium (139). As *T. foetus* is easily recognised without the need for further selective isolation (as for *C. fetus*), there is less literature on *T. foetus* isolation than for *C. fetus*. However, it is thought that the growth of *T. foetus* can be inhibited by other microbes in the transport medium and thus fail to reach adequate detectable numbers. Survival has been improved using commercial culture systems such as the InPouch™ (21). PCR tests developed to target the ribosomal RNA genes (62, 123) have been found to be effective when testing transport medium (131). Another study employed a novel DNA purification step prior to PCR directly from spiked preputial washes, however the researchers did not present a field application of the method (31). Most laboratories appear to be using the PCR to detect *T. foetus* following transport in medium and have not reported direct detection in washes. No reports on semen testing, using PCR, have been published. PCR testing following transport in media has disadvantages given contaminants also grow. DNA from these contaminants (also present in an extract) would also compromise specific PCR amplification. It has also been reported that an intestinal *T. foetus*-like protozoan was isolated from preputial smegma of virgin bulls and that it is apparently identical in appearance to *T. foetus* under light microscopy (18). It is not certain whether this organism is present in animals here in Australia, however the *T. foetus* PCR does not amplify this related organism (18). It has also been confirmed that *Tritrichomonas suis* and *T. foetus* are in fact the same organism (162). Although the ingestion of aborted foetuses is a likely means of

transmission for *T. foetus* from cattle to pigs, it does not seem likely for tritrichomonads to transmit from pig intestines (via faeces) to venereal infections in cattle. However, Tachezy *et al*. 2002 (162) thought that the existence of a 'natural' reservoir (feral pigs) and the demonstration of infectivity of porcine tritrichomonads for cattle, points to a possible risk of reintroduction of trichomoniasis to bovines.

PCR based methods have been investigated to compare *T. foetus* strains and polymorphisms are evident (141). These methods have not been applied in Australia and may be useful in tracing cases to particular bulls by matching genotypes. It may be possible to genotype Australian *T. suis* and *T. foetus* isolates to confirm Tachezy *et al*. 2002 observations (162).

There is no treatment for trichomoniasis. A vaccine based on killed *T. foetus* appeared to effectively control infection particularly in bulls ≤5 years old (38). The use of a vaccine to control trichomoniasis in Australia however, has never been adopted. Preventing the transmission of the disease through culling practices relies on the ability of a test to accurately identify infected animals. Improving detection may identify the need for a trichomoniasis vaccine.

Preliminary experiments by DPI&F indicated a significant improvement in detecting both *C. fetus* subsp. *venerealis* and *T. foetus* using PCR directly on preputial washes compared with conventional culture methods. As discussed above (PCR and *Taq* nuclease assays) conventional PCR techniques for routine diagnostic testing are not ideal. This project aimed to develop real time or 5' *Taq* nuclease PCR assays for the detection of *T. foetus* and for the detection of *C. fetus* subsp. *venerealis* directly from diagnostic specimens.

1.4 Leptospirosis

Members of the genus *Leptospira* are differentiated antigenically so that the basic taxonomic unit of the genus is the serovar. Antigenically related serovars are combined into serogroups, which are further classified as saprophytic or pathogenic. Serovars are also grouped into species based on DNA homologies (136, 137, 181). These species do not correspond with serogroups, but generally consist entirely of either saprophytic or pathogenic serovars. Over two hundred pathogenic serovars are known (61).

Hardjo and Pomona are the most common serovars in Australian cattle. Serovars Australis (23), Zanoni (111), Celledoni (45), Grippotyphosa (1) and Topaz (Corney et al, manuscript in preparation), have also been isolated from Australian cattle.

In cattle, leptospirosis may result in agalactia, hemoglobinuria, stillbirths, abortion, weak calves and infertility. Animals are infected by inhalation, via cuts and abrasions, venereally or through the conjunctiva. Infection is followed by a brief bacteremia, after which the *Leptospira* persist in various organs including the proximal renal tubules, the genital tract, the brain and the anterior chamber of the eye. *Leptospira* are excreted in urine for prolonged periods after infection, for example Thiermann (165) reported excretion in urine past 542 days after infection. Infected urine (either from cattle or from other reservoir animals) is the chief source of infection. Seroprevalence data for leptospirosis (for example, approximately 40% in central Queensland beef cattle) (12) indicates widespread exposure which is consistent with a significant role in sub-fertility.

In Australia, killed bivalent Hardjo-Pomona vaccines are available for use in cattle. Infected cattle may be cleared of infection by treatment with streptomycin, amoxicillin, oxytetracycline, tilmicosin or ceftiofur (2, 72, 73, 152).

Leptospirosis is a significant occupational hazard for workers in the dairy and beef industries including farm and meat workers. Of 197 human notifications Australia-wide in 2001 for which occupation data are available, 25 were meatworkers/inspectors/butchers, 10 were graziers, and 7 were station hands (154). These groups represent 21% of the leptospirosis notifications for that year. The main avenue of transmission is through contact with urine from infected animals. In humans leptospirosis is frequently debilitating and occasionally fatal.

Suitable vaccines against leptospirosis are not available for use in humans. Therefore the ability to better detect the presence of *Leptospira* (for example, by PCR) in infected and carrier domestic animals would greatly assist in reducing the disease burden on workers in allied farming and processing industries dependant on cattle and other domestic animals. For persons who contract the disease there is not only the physical suffering but its impact on their ability to attend work due to hospitalisation, the debilitating effects of the disease and even long term chronic manifestations (lasting months or even years). This places a significant burden on employers and the public health system.
The definitive serological test for leptospirosis is the microscopic agglutination test (MAT) (30). However, MAT results are often difficult to interpret, and anergic chronically infected animals are often missed by the MAT (30). The IgM ELISA may offer some advantages over the MAT in differentiating between recent infections and residual titres from earlier infections (46).

Leptospira is usually cultured from urine or kidneys. *Leptospira* may also be isolated from milk (165) the reproductive tract (58, 59) and aborted foetuses (165). However, although isolation of *Leptospira* provides indisputable evidence of infection, culture is labour intensive and takes up to three months, and is therefore unsuitable as a diagnostic test. For these reasons, it is often very difficult to diagnose leptospiral sub-fertility with any degree of certainty. PCR offers a sensitive and rapid alternative to culture for unequivocally demonstrating current infection with *Leptospira*.

Although many PCRs for pathogenic *Leptospira* are described in the literature, only a few have been used on clinical or veterinary samples (6, 114, 115, 140). Some have been used successfully for developing treatment regimens for leptospirosis (2, 72, 73). Also, PCR-based typing procedures have been developed to simplify serovar identification and taxonomy (9, 20, 43, 44). These PCRs involve amplification followed by a product detection step such as gel electrophoresis.

A more recent refinement of PCR in which product accumulation is monitored during amplification (real-time PCR) has also been applied to diagnosing leptospirosis (155). This has potential as a simple, rapid, and sensitive test for detecting pathogenic *Leptospira* in a range of veterinary samples, for use in disease diagnosis, and elucidating the pathogenesis and epidemiology of bovine leptospirosis. This research aimed to adapt a real time assay developed for the diagnosis of human pathogenic *Leptospira* spp. for use with bovine diagnostic samples.

2 Project Objectives

By 30 September 2004: Evaluate and test PCR tests in laboratory samples including standardisation of methods (500 samples in total) and select herds for case studies.

By 30 March 2005: Complete evaluation of field samples (400 animals in total) and multicentre laboratory evaluation of selected test samples.

By 30 November 2005: Evaluate case study herds evaluated and tests completed (one herd per pathogen) and communication material delivered.

By 31 May 2006: Preliminary screen of virulence genes within Australian isolates of *C. fetus* subsp. *venerealis*.

3 Methodology

The project was divided into stages mostly relevant to stage the laboratory development, field evaluation and case study analyses for the detection assays developed for *C. fetus* subsp. *venerealis*, *T. foetus* and the pathogenic *Leptospira*. The methodology described here outlines general research plan. Detailed methods will be described together with the results and discussion separately for each pathogen as chapters within section 4.

3.1 Stage 1 (6 months) – Assay Development

3.1.1 Campylobacteriosis/Vibriosis and Trichomoniasis

Design fluorogenic probe PCR assays based on sequences used for specific conventional PCR assays for *T. foetus* and *C. fetus* subsp *venerealis*.

Compare sensitivity of PCR probe assays with conventional culture methods using spiked preputial washings and vaginal scrapings/swabs. Confirm specificity of PCR.

Determine appropriate sampling procedures comparing different rods for sampling bulls and comparing rinses with mucus swabs for heifer sampling.

Determine efficacy of PCR vs culture using spiked samples, under different storage conditions, to emulate transport to the laboratory.

Investigate DNA genotyping methods for strains and isolates of both *T. foetus*, *C. fetus* subsp. *venerealis* (compare field with vaccine strains). This method will investigate the use of SYBR green - labelling and/or enzyme digestion of PCR products.

3.1.2 Bovine Ephemeral Fever

Confirm gene target for fluorogenic probe PCR assay through sequencing of relative regions in related viruses (Berrimah, Kimberley and Adelaide River viruses) and by aligning available sequences.

Design assay specific for BEFV.

Prepare cloned positive controls for the BEFV assay.

Evaluate assay using BEFV control samples (including available field samples). Compare sensitivity of RT-PCR assay with the TaqMan assay using plasmid samples and *in vitro* cultured virus samples of BEFV and related viruses (Adelaide River virus, Kimberley virus and Berrimah virus).

3.1.3 Leptospirosis

Develop sample preparation methods for urine, vaginal mucus, semen and tissues.

Compare sampling methods on experimentally infected animals.

Examine effect of transport and storage conditions on samples for PCR.

Confirm sensitivity and specificity of PCR.

3.2 Stage 2 (1 year) – Field Sampling

Collect samples from bulls and cows in northern herds for testing and screening in *T. foetus*, *C. fetus* subsp. *venerealis*, and pathogenic *Leptospira* assays in comparison with culture.

Multicentre evaluation of *Leptospira* assay involving Murdoch University, DPI-NSW (Elizabeth Macarthur Agricultural Institute) and Department of Agriculture Western Australia (DAWA).

Multicentre evaluation of *T. foetus* and *C. fetus* subsp. *venerealis* involving DAWA, DPI-NSW (EMAI), DPI-VIC and DPI&F.

*The *Leptospira* culturing component will be undertaken by Lee Smythe of the WHO/FAO/OIE Collaborating Centre for Reference and Research on Leptospirosis in Queensland Health Scientific Services (QHSS) of the Queensland Health Department.

3.3 Stage 3 (1 year)

Case Study herd screen for the each of the 3 pathogens - *T. foetus*, *C. fetus* subsp. *venerealis* and pathogenic *Leptospira* (3 herds). A herd identified in Stage 2 is selected for each case study experiment. The samples from animals are screened in all 3 assays. It is envisaged this will demonstrate the usefulness of these assays in confirming the cause of losses or lower calving rates in each of the herds. Sampling strategies were calculated based on 500 head herds of cattle and 30% prevalence.

COMMUNICATION ACTIVITY	KEY MESSAGE	RESPONSIBLE PERSON AND DATE
Diagnostic laboratories	New diagnostic methods - uptake into Australian diagnostic laboratories	Bruce Corney, Ala Lew Jan-July 2005 Conferences 2004 - Ala Lew, Bruce Corney
Producer information	New diagnostic procedures to assist the investigation of herd sub-fertility	John Bertram, Geoffry Fordyce, Mick Sullivan, Dick Holroyd Jan-July 2005

3.4 Project extension (6 months)

Preliminary investigation of the variation in virulence genes in Australian *C. fetus* subsp. *venerealis* isolates. Access to unpublished 'incomplete' *C. fetus* subsp. *venerealis* genome data from the Universidad Nacional de San Martin (UNSAM, Argentina) allowed the selection of putative virulence genes based on homology with previously identified genes in characterised *Campylobacter* species. Collaboration with WA's Centre for Comparative Genomics (Murdoch University) enabled the bioinformatics analysis of this genome data. Our aim was to apply approximately 20 PCR assays targeting 20 virulence genes to study up to 50 *C. fetus* subsp. *venerealis* preparations isolated from different locations in Qld.

3.5 Staff Resources

DEPARTMENT OF PRIMARY INDUSTRIES & FISHERIES (QLD)

Project Leader, VD and BEFV Sub-projects
Dr Ala Lew (Principal Research Scientist), Emerging Technologies (ET)

Leptospirosis Sub-project and Biosecurity Technology Transfer
Dr Bruce Corney (Principal Scientist), Biosecurity

Scientific Staff
Dr Lyle McMillen (Molecular Microbiologist), ET (employed by AHW.036 Oct 2003 – April 2005), coordination of field sampling, development of VD assays
Vivienne Doogan (Senior Biometrician), Animal Science, DPI&F
Barry Rodwell (Principal virologist), leptospirosis & technology transfer
Ibrahim Diallo (Virologist), leptospirosis, BEFV technology transfer

Technical Staff
Catherine Minchin (ET), BEFV assay screening
Bronwyn Venus (Animal Science), VD field sampling and case study screening
Bartosz Wlodek (ET, employed Dec 2005-February 2006) – *C. fetus* subsp. *venerealis* virulence gene screening
Michael Lawrie (work for the dole program, June-August 2004) – ET, DPI&F
Lucia Wright (Biosecurity), leptospirosis assay screening
Glen Hewitson (Biosecurity), leptospirosis assay screening
Mark Kelly (Biosecurity), leptospirosis serology

Veterinary and Field Sampling Staff (Animal Science, DPI&F)
Geoffry Fordyce (North West), VD infection trial
Dr Richard Holroyd (North Coast)
John Bertram (South)
Mick Sullivan (North West)
Lex Turner, leptospirosis infection trial
Geoff Dawson, leptospirosis infection trial
Andrew Kelly, leptospirosis infection trial

QUEENSLAND HEALTH, Brisbane, Qld
WHO/FAO/OIE Collaborating Centre for Reference and Research on Leptospirosis
Lee Smythe
Michael Dohnt
Meegan Symonds
Lee Barnett

MURDOCH UNIVERSITY, Murdoch, WA
School of Veterinary and Biomedical Sciences (leptospirosis case study)
Dr Stan Fenwick
Eric Taylor
Peter Wai'in
Patchara Phuektes

Dr Simon Reid
WA Centre for Comparative Genomics (*C. fetus* bioinformatics analyses)
Prof Matthew Bellgard
Paula Moolhuijzen

DPI-NSW
Dr Tracey Berg (Multicentre evaluations)

DPI-VIC
Rachel Auld (Multicentre evaluations)

DAWA
Anabel Vivas-Marfisi (Multicentre evaluations)

INSTITUTO NACIONAL DE TECNOLOGÍA AGROPECUARIA
Dr Carlos Campero (VD infection trial)

UNIVERSIDAD NACIONAL DE SAN MARTIN
Prof Daniel Sanchez (*C. fetus* subsp. *venerealis* incomplete genome data)

4 Results and Discussion

4.1 Bovine venereal diseases

4.1.1 Introduction

The aim of the research described here was to develop real time probe-based assays for the detection of *C. fetus* subsp. *venerealis* and *T. foetus* based upon previously identified specific gene targets. The sensitivity of the assays was confirmed using serial dilutions of cells and the specificity of the assays was tested by screening closely related species and subspecies. These assays were further tested under a variety of storage conditions to determine the best methods for sample transport and processing for real time PCR diagnosis of clinical specimens (mucus, smegma and urine). Additional aims included the evaluation of published protocols to genotype isolates of Australian *C. fetus* and *T. foetus*, and to undertake a preliminary screen of putative virulence genes in Australian isolates of *C. fetus* subsp. *venerealis*.

4.1.2 Methods

4.1.2.1 ASSAY DEVELOPMENT – Stage 1

Taq nuclease assays

Reference Strains. Isolates of several *Campylobacter* species were obtained from the Animal Research Institute (ARI, DPI&F, the American Type Culture Collection (ATCC) and the National Collection of Type Cultures (NCTC) (Table 4.1.1). *Campylobacter* strains were grown at 37^0C in Brain-Heart Infusion broth (Oxoid), 0.2 % yeast extract, 0.07 % Bacto agar for between 1 and 3 days. *T. foetus* was grown at 37^0C in 1.25 % neutralized liver digest, 0.5 % tryptose, 0.15 % bacto agar, 50 % sterile heat-inactivated bovine serum, 0.1 % P+S solution (0.75 % penicillin, 0.082 % streptomycin). *Pseudomonas aeruginosa* and *Proteus vulgaris* were grown at 37^0C on blood agar plates for 24 h. *Neospora caninum* tachyzoites were cultured in Vero cells as previously described (49). DNA for initial assay evaluation was prepared from all reference strains using QIAamp DNA kits (QIAGEN) as described by the manufacturer.

Sequence analysis. The 142 bp *C. fetus* subsp. *venerealis* specific amplicon (83) from strain 98-118432 and the 347 bp *T. foetus*-specific amplicon (62) from strain YVL-W were both ligated into cloning vectors (TOPO-TA cloning kit, Invitrogen Corporation) as described in the manufacturer's protocol. Plasmids with inserts were sequenced using the T7 and M13 Reverse primers, and BigDye Terminator Mix (Applied Biosystems Inc.), following the manufacturer's protocols. Sequences were analysed by the Griffith University DNA Sequencing Facility (School of Biomolecular and Biomedical Science, Griffith University Nathan QLD 4111 Australia) using an ABI 377 DNA Sequencer.

Assay development. 5' *Taq* nuclease (TaqMan®MGB) assays for *C. fetus* subsp. *venerealis* (based on an unknown *C. fetus* subsp. *venerealis* specific fragment (83)) and *T. foetus* (based on the internal transcribed spacer region 1) were developed using MGB probe technology (Applied Biosystems/AB) (Table 4.1.2) designed using Primer Express Ver. 2 (AB). Assay conditions described by the manufacturer for either the Platinum Quantitative PCR SuperMix-UDG (Invitrogen Life Technologies) or RealMasterMix Probe mix (Eppendorf) were initially attempted using the Corbett RotorGene 3000 (as recommended by the manufacturer). Initially 18 μM of each primer and 5 μM of each probe was used in the 2-step real time PCR cycling protocol (standard assay

conditions). Assay sensitivity was evaluated against diluted genomic DNA, and specificity evaluated using the genomic DNA preparations from closely related organisms and other bovine venereal microorganisms (Table 4.1.1).

Table 4.1.1. Species and strains used for 5'*Taq* nuclease assay development

Species	Strain	Source
Tritrichomonas foetus	YVL-W	Field Isolate (DPI&F, QLD)[1]
T. foetus	BP-4 (30003)	ATCC[2]
Tetratrichomonas gallinarum	TP-79 (30097)	ATCC
Pentatrichomonas hominis	hs-3 (30000)	ATCC
Trichomonas vaginalis	C-1 (30001)	ATCC
Campylobacter coli	11353	NCTC[3]
C. fetus subsp. *venerealis*	98-109383	Field Isolate (DPI&F, QLD)
C. fetus subsp. *venerealis*	19438	ATCC
C. fetus subsp. *venerealis*	biovar *venerealis*	Pfizer
C. fetus subsp. *venerealis*	biovar *intermedius*	Pfizer
C. fetus subsp. *fetus*	98- 118432	Field Isolate (DPI&F, QLD)
C. fetus subsp. *fetus*	15296	ATCC
C. jejuni subsp. *jejuni*	11168	NCTC
C. hyointestinalis	N3145	Field Isolate (DPI&F, QLD)
C. sputorum subsp. *bubulus*	Y4291-1	Field Isolate (DPI&F, QLD)
Pseudomonas aeruginosa	27853	ATCC
Proteus vulgaris	6380	ATCC
Neospora caninum	50843	ATCC
Leptospira borgpetersenii serovar Hardjobovis	93/94451/3	Field isolate (DPI&F, QLD)
L. interrogans serovar Pomona	Pomona	CCRL[4]

[1]Animal Research Institute, Department of Primary Industries and Fisheries, Queensland, Australia
[2]American Type Culture Collection, Virginia, USA
[3]National Collection of Type Cultures, London, United Kingdom
[4]WHO/FAO/OIE Collaborating Centre for Reference and Research on Leptospirosis, Australia and Western Pacific Region

Table 4.1.2. 5'*Taq* nuclease assay primers and probes for *T. foetus* and *C. fetus* subsp. *Venerealis*

Primer/probe	5'→3' sequence	Oligo type
T. foetus assay:		
TFF2	GCG GCT GGA TTA GCT TTC TTT	Forward primer
TFR2	GGC GCG CAA TGT GCA T	Reverse primer
TRICHP2	**6FAM**-ACA AGT TCG ATC TTT G	MGB probe
C. fetus subsp. *venerealis* assay:		
CFVF	CCC AGT TAT CCC AAG CGA TCT	Forward primer
CFVR	CGG TTG GAT TAT AAA TTT TAG CTT GGT	Reverse primer
CFVP1	**6FAM**-CAT GTT ATT TAA TAC CGC AA	MGB probe

Assay sensitivity and specificity. Detection limits of the 5' *Taq* assays were compared to culture-based diagnosis (gold-standard) and the conventional PCR assays previously developed for *C. fetus* subsp. *venerealis* and *T. foetus* (62, 83) using serial dilutions of purified gDNA and DNA prepared from spiked smegma and mucus. Culture based diagnosis for *C. fetus* subsp. *venerealis* was

conducted by inoculation of 5 ml modified Weybridge media (97) with 0.5 ml of preputial smegma or vaginal mucus in saline (usually used for transport to the laboratory). One hundred μl of the inoculated modified Weybridge media was plated onto Skirrow's *C. fetus* selective medium (148) and incubated at 37°C in a microaerobic environment generated using an anaerobic jar and a Campygen sachet (Oxoid). The presence of *C. fetus* subsp. *venerealis* was indicated by the presence of small (about 0.5 mm diameter), smooth, translucent colonies arising after 48 to 72 h followed by microscopic confirmation of *Campylobacter*-like morphology. Culture-based diagnosis for *T. foetus* was conducted by inoculating InPouch™ TF media (Biomed Diagnostics) with 0.5 ml of preputial smegma or vaginal mucus in saline and incubated at 37°C. The media was examined microscopically every 24 hours for 5 days for the presence of motile trichomonads.

Infection trial

A group of 16 bulls and 16 heifers were inoculated intra-preputial and intra-vaginally (respectively) with laboratory-acclimated strains of both pathogens (*T. foetus* strain YVL-W; *C. fetus* subsp. *venerealis* strain 98-109383) as described previously (36, 38). Sampling methods were evaluated for ease of use by the veterinarian, lack of distress of the animal, and suitability of the sample for laboratory diagnosis. The trial was undertaken at Swans Lagoon (North Qld DPI&F research station) following animal ethics approval.

Preputial smegma samples were collected from 16 bulls (8 each for *C. fetus* subsp. *venerealis* and *T. foetus*) using sterile pipettes, swabs or bull raspers. The bulls were restrained in a veterinary crush during the collection procedures. A sterile pipette (10 mm internal diameter, with a beveled edge) was gently scraped along the surface of the penis and internal prepuce near the fornix, with gentle aspiration being applied with an attached bulb or syringe. The collected smegma was rinsed into approximately 5 ml sterile phosphate buffered saline (PBS) or physiological saline. A sterile McCullough uterine mare swab (Minitube Australia Pty Ld) was gently scraped along the surface of the penis and internal prepuce near the fornix. The collected smegma was expressed into approximately 5 ml sterile PBS or physiological saline. A bull rasper (Polyethylene, 60 cm long with a 75 mm long, 8 mm diameter corrugated scraper head with a 1.5 mm collection bore attached to 6 mm diameter tubing with a 1.5 mm internal diameter, similar to those produced by Elastecnica, Argentina; based on the original design described previously (159)) was gently scraped along the surface of the penis and internal prepuce near the fornix. No aspiration was necessary. The collected smegma was rinsed into approximately 5 ml sterile PBS or physiological saline.

Vaginal mucus samples were collected from 16 cows (8 each for *C. fetus* subsp. *venerealis* and *T. foetus*) using artificial insemination pipettes, swabs or bull raspers, during restraint in a veterinary crush. A sterile artificial insemination infusion pipette was inserted so that the anterior end reached the cervix. Gentle suction was applied using a rubber bulb while moving the pipette gently backwards and forwards. The pipette was removed and the collected mucus rinsed into approximately 5 ml sterile physiological saline. A sterile 15 cm swab, held by sterile forceps, was inserted so that the anterior end reached the cervix. The swab was gently moved backwards and forwards while rotating to saturate the head with mucus. The swab was removed and the collected mucus expressed into approximately 5 ml sterile physiological saline. A bull rasper was inserted so that the anterior end reached the cervix. The rasper was moved gently backwards and forwards. No aspiration was necessary. The rasper was removed and the collected mucus rinsed into approximately 5 ml sterile physiological saline.

Urine was investigated to determine its feasibility as an alternative clinical specimen for diagnosis of venereal *C. fetus* subsp. *venerealis* infection in bulls. Urine from two consecutive voids was collected in a series of sterile collection containers following the subcutaneous administration of a diuretic (Frusemide, Ilium Veterinary Products, Australia). The first container collected was discarded as being the most likely to be heavily contaminated with fecal material, hair, and other debris.

DNA extraction

QIAGEN QIAamp DNA extraction kits (as per manufacturer's instructions) and a heat lysis protocol were compared. One ml of preputial smegma/cervicovaginal mucous in saline, or urine were centrifuged at full speed for 2 minutes in a microcentrifuge. The pellet was resuspended in 500 μl sterile dH_2O, and heated at 95°C for 10 minutes in a heating block. The sample was then briefly centrifuged to pellet any particulate matter, and then assayed (5 μl). The 2 methods were compared using laboratory spiked and naturally infected smegma, mucus and urine samples.

Effects of storage

C. fetus subsp. *venerealis* strain 98-109383 cells from 2-day old fresh cultures were treated 1:1 with methanol to reduce cell motility and were counted using a haemocytometer. Serial log dilutions of the 2 day-old *C. fetus* subsp. *venerealis* cultures were prepared at 10^5 cells/ml and were serially diluted to 1 cell/ml. *T. foetus* strain YVL-W cells were counted using a haemocytometer and serial dilutions were prepared as described for *C. fetus* subsp. *venerealis*. These dilutions were inoculated into smegma, mucus and urine obtained from healthy animals which had previously tested negative for both *C. fetus* subsp. *venerealis* (by selective culture and 5' *Taq* nuclease assay), and *T. foetus* (microscopic examination of InPouch™TF cultures (Biomed Diagnostics)) and *Taq* nuclease assay. The viability of the *C. fetus* subsp. *venerealis* cells was determined by spreading 100 µl of each dilution onto pre-poured Colombia Sheep Blood Agar plates (Oxoid) and colonies were counted following 2 days incubation at 37°C in micro-aerobic environment (as described above). Modified Weybridge transport enrichment media (TEM) for *C. fetus* subsp. *venerealis* and InPouch™TF for *T. foetus* were inoculated with these laboratory-spiked specimens, as for diagnostic culture. DNA was also extracted from aliquots of inoculated TEM/InPouch™TF using a commercial kit (QIAamp DNA mini kit, Qiagen) and assayed by both 5' *Taq* nuclease assay and conventional PCR assay. Inoculated specimens were also prepared for 5' *Taq* nuclease assay by heat lysis. Selective media was inoculated from the TEM as for diagnostic culture for *C. fetus* subsp. *venerealis* and InPouch™TF were examined for motile trichomonads. Estimates of cell equivalents/assay or cells/inoculum were calculated from the enumerated spiked specimen by determining equivalent cell numbers contained in the final volume used as either template for PCR or as inoculum in cultures.

5' *Taq* nuclease assays were scored positive if the fluorescence (normalized to a no-template control) passed a threshold of 0.1. A positive conventional *C. fetus* subsp. *venerealis* PCR assay required the detection of both the 960 bp species-specific and the subspecies-specific 142 bp amplicons (83) following agarose gel electrophoresis. Cultured samples were scored positive on the presence of *Campylobacter*-like colonies, followed by microscopic confirmation of *Campylobacter*-like morphology and motility. The conventional PCR assay produced a 347bp amplicon following positive amplification of *T. foetus* (62). Cultured samples were scored positive on the presence of motile trichomonads with multiple anterior flagella, a posterior flagellum, a visible undulating membrane and characteristic jerky motility upon microscopic examination.

Sample transport was simulated by storing the inoculated TEM/InPouch™TF and animal samples at ambient temperatures for smegma and mucus, and at 4°C for urine for up to 5 days. Samples for 5' *Taq* nuclease assays, conventional PCR assays and selective culture were processed as described above at time 0, and after 2 and 5 days of storage. To examine changes in cell numbers under these storage conditions, quantified cell estimates were compared using 5' *Taq* nuclease assay results of the 10^4 cells/ml spiked samples.

4.1.2.2 FIELD SAMPLING – Stage 2

Field Sampling Training

Sampling methods were based on the outcomes of Stage 1. A workshop for the field sampling veterinarians was held on 21 June 2004 and was held at Animal Research Institute, Yeerongpilly, Brisbane. The training consisted of project summaries, milestones, animal sampling demonstrations and a demonstration of laboratory procedures. Prior to this meeting a field sampling instruction sheet was developed for discussion.

Sample Collection

The Argentinean designed (raspador) 'Tricamper' (Figure 2) was manufactured through Supaflow Engineering and a fusion welder was used to construct the tool. Field sampling kits and instructions (Appendix 1) were dispensed to staff to standardise sampling protocols. Samples were submitted using the IATA approved containers and sent to ARI via Lyle McMillen who then distributed samples to the relevant laboratory. Urines for *Leptospira* culture were sent to WHO/FAO/OIE Collaborating Centre for Reference and Research on Leptospirosis, Queensland Health Scientific Services, Brisbane (QHSS). See sample distribution sheet (Appendix 1). A summary table of results was stored on the ARI server to expedite result reporting and updating.

Figure 2. Manufactured 'tricamper'

Preputial smegma (n=228-248) and cervico-vaginal mucus (n=113) specimens were collected from 361 animals originating from 39 properties throughout northeastern Australia (Queensland) using the bull rasper and the above collection protocols. Specimens were assayed for *C. fetus* subsp. *venerealis* and *T. foetus* by both diagnostic culture and 5' *Taq* nuclease assay. Urine was also collected randomly from 71 animals (48 bulls, 23 cows) to assess the usefulness of urine for *T. foetus* and *C. fetus* subsp. *venerealis* 5' *Taq* nuclease assay screening. Mucus samples from female cattle were also tested using the *C. fetus* subsp. *venerealis* ELISA as described previously (84).

Statistical analyses

Fisher's exact test was used to compare the distribution of positive and negative results for the 2 methods of testing, culture and *Taq* assay. To assess whether there was evidence of differences in the proportion positive between regions a chi-square test was applied separately to *Taq* assay results for *C. fetus* subsp. *venerealis* and *T. foetus*.

Selection of case study herds

During the sampling phase of stage 2, we aimed to select possible case study herds with a high incidence of each of *T. foetus*, *C. fetus* subsp. *venerealis* and the pathogenic *Leptospira* for stage 3 of this project (2005).

MULTICENTRE EVALUATION Stage 2

Kits for the multicentre evaluations were prepared, and contained the appropriate forward and reverse primers, probe (at working concentrations) and sufficient real time PCR reagent premix (RealMasterMix probe mix, Eppendorf) to perform 100 of each assay. In addition, genomic DNA from the target species, a quantitative standards series for *C. fetus* subsp. *venerealis* and *T. foetus*, and a number of "blind" pre-prepared field specimens (previously assayed positive and negative specimens) were also sent to the collaborating laboratories. Instructions were provided for specimen processing, assay preparation and cycle conditions without including primer and probe sequence information. These kits were supplied to Rachel Auld – VIAS, DPI-VIC and Tracey Berg – EMAI, DPI-NSW. A *C. fetus* subsp. *venerealis* kit was also supplied to Dept of Agriculture WA (DAWA).

CT data or a positive/negative assay result was collected and compared to that obtained for the specimens during initial assay in our laboratory (DPI&F). Eight samples were tested by all 3 laboratories for *T. foetus*. For *C. fetus* subsp. *venerealis* 8 samples were tested by both DPI&F and DPI-Vic and a further 5 samples were tested by both DPI&F and DPI-NSW. The degree of agreement between the laboratories in categorising samples as positive or negative was assessed using the kappa statistic (Kappa coefficient of agreement for nominally scaled data).

4.1.2.3 CASE STUDIES – Stage 3

Case Study 1

The herd used in Case Study 1 was selected on the basis of the presence of *T. foetus*, although *C. fetus* subsp. *venerealis* was detected during pre-screening. All results for the case studies relied upon positive and negative detection by 5' *Taq* nuclease (real time TaqMan®MGB) assay methods developed in Stage 1. The herd grazed in relatively fertile country in NW Qld (e.g., steers gain an average of 190 kg/year), and calf output was usually quite high. The reason for including the herd in this study was that recently it appeared that conception rates per cycle were well below benchmark levels and both *C. fetus* subsp. *venerealis* and *T. foetus* has been detected. Data for the 2004 mating of maiden 2-year-old heifers weighing 350-450 kg are presented in Figure 5 (page 39). In 2004, no abortions from 135 pregnancies (3-10 weeks) were recorded between March and September. Pregnancies accumulated at a slower rate in this group compared to that in Figure 1 (page 10).

A group of 66 bulls was sampled in December 2004 and 20% of bulls were found to carry *C. fetus* subsp. *venerealis*, with a single bull having both *C. fetus* subsp. *venerealis* and *T. foetus* (Table 4.1.18). All 3-year-old and older bulls were given 5mL of VibroVax (campylobacteriosis vaccine, Pfizer). Selected bulls (n=40) were allocated to 5 mating groups (466 female cattle) in mid January 2005. Only 228 of the total 466 female cattle were pre-screened in our assays for *T. foetus*, *C. fetus* subsp. *venerealis* and *Leptospira*. All groups were mated from mid January 2005. Subgroups of females were pregnancy tested and sampled for *C. fetus* subsp. *venerealis, T. foetus* and pathogenic *Leptospira* spp in mid June 2005. Most bulls were also sampled for *C. fetus* subsp.

venerealis and *T. foetus* in mid June 2005. Allocation of bulls aimed to mate disease-free bulls in 3 of the mating groups. However, some movement of bulls between groups and changes in disease status of bulls prevented this.

Case Study 2

This herd was primarily chosen due to our detection of a significant number of *C. fetus* subsp. *venerealis* positive bulls. A group of 29 bulls from Station in South-west Qld were tested for *T. foetus* and *C. fetus* subsp. *venerealis* on 22/9/04 and 6 (21%) were found to be positive for *C. fetus* subsp. *venerealis*, one suspect and the remaining 22 negative. All bulls were negative for *T. foetus* thus we determined that we could confirm that decreases in reproductive rates may be attributed to the presence of *C. fetus* subsp. *venerealis*. Some of these *C. fetus* subsp. *venerealis* positive bulls were used in subsequent matings. Three mating groups were used in the study which included 312 female cattle and 17 bulls. All groups were mated from 1/12/04 to 23/2/05. Females were pregnancy tested and sampled for *C. fetus* subsp. *venerealis* and *T. foetus* on 19/4/05. Bulls that had been tested in September 2004 were not retested.

4.1.2.4 GENOTYPING

C. fetus subsp. *venerealis* genotyping

Genomic DNA was isolated from cultures of *C. fetus* subsp. *venerealis* or direct from field samples using QIAGEN QIAamp DNA minikits, and the 16S-23S rDNA Internal Spacer Region (ISR) amplified using the PCR primers CISR1 and CISR2 (Table 4.1.5). A variable region within the amplified ISR region was sequenced using CISR4 and CISR5 (Table 4.1.5), and the sequence data analysed for suitable restriction enzyme recognition sites for RFLP analysis. Two primers (CISR6 and CISR7, Table 4.1.5) were designed to amplify the 23S rDNA Internal Spacer Region (ISR), found to be variable between *C. fetus* subsp. *venerealis* isolates. The melting point of the amplicon was determined using SYBR green.

Primers and Fluorescence Resonance Energy Transfer (FRET) probes were designed to amplify and hybridise to the ISR region. The FRET probes included a reporter probe covering the variable region (CISRHP1) and an anchor probe (CISRHP2) identical to an adjacent conserved region. Field specimens were enriched for the target region by conventional PCR using CISR2 and CISR5 prior to FRET hybridisation analysis. PCR enrichment of field specimens was performed in 0.2 mL PCR tubes using the components as described in Table 4.1.3. The PCR assays were cycled following an initial denaturation at 94°C for 2 min; for 35 cycles at 94°C 20 s, 50°C 20 s, 72°C 2 min, and final extension at 72°C for 10 minutes. The expected band size from *C. fetus* subsp. *venerealis* is 289 bp and this product was used as template in the FRET hybridization analysis.

Table 4.1.3. Pre-mix components for PCR enrichment of *C. fetus* subsp. *venerealis* field extracts

Component	μL/reaction	μL/12 reactions
Taq buffer (10x)	1.5	18
dNTPs (2 mM)	1.5	18
CISR5 primer (5 μM)	1.5	18
CISR2 primer (5 μM)	1.5	18
sdH_2O	7.8	93.6
Taq polymerase (5 U/μL)	0.2	2.4
Template gDNA/crude lysates	1	-

FRET hybridization analyses were performed in triplicate in 0.1 mL thin-walled PCR tubes using a fluid liquid handling robot (Corbett CSA-1200) as described in Table 4.1.4 (72 reactions). Tubes were sealed and cycled using the Corbett RotorGene 3000 as follows: 50°C 2 min; 94°C 2 min; 45 cycles at 94°C 20 s, 58°C 20 s, 68°C 20 s; Melt analysis: 48°C to 65°C, 60 seconds on the first step, 0.5°C/step, 5 seconds per step; and a final hold at 30°C for 1 min. Acquisition occurred at the end of each 68°C annealing step, and at the end of each 5 second melt step. Excitation wavelength was 495 nm (FAM), while emission was 670 nm (Cy5).

Table 4.1.4. Pre-mix components for *C. fetus* subsp. *venerealis* FRET genotyping assay

Component	μL/reaction	μL/12 reactions	μL/72 reactions
RealMasterMix (2.5×)	10	120	720
CISR5 primer (18 μM)	1.25	15	90
CISR6 primer (18 μM)	1.25	15	90
CISRHP1 probe (5 μM)	0.625	7.5	45
CISRHP2 probe (5μM)	0.625	7.5	45
sdH_2O	9.25	111	666
DNA/ sdH_2O (NTC controls)	2	-	-

***T. foetus* genotyping**

Genomic DNA was isolated from cultures of *T. foetus* and from field specimens positive on the *T. foetus* assay, and a RAPD (Random Amplification of Polymorphic DNA) procedure performed using the primers TAP5 and TAP6 (142). The amplification pattern was analysed by electrophoresis on 2% agarose in TBE. The RAPD procedure was modified to enable melting curve analysis of the RAPD amplification products (McRAPD). Amplification conditions were as the RAPD procedure, with SYBR green used to monitor the denaturation of the amplification products through a 50°C to 97°C melting cycle. A 500 bp product from the RAPD procedure, common to *T. foetus* isolates was further analysed by sequencing using BigDye terminator mix as described in Section 4.1.2.1. The Internal Transcribed Spacer (ITS) regions flanking the 5.8S rDNA gene of isolates of *T. foetus* were sequenced using TFR3 and TFR4 (62) described in Table 4.1.5.

Table 4.1.5. Primers used in genotyping methods

Primer	Sequence (5'→3')
CISR2	GCTCAATAGCTTGTGATG
CISR5	CACACTTGTTTAGGTTTGAGGG
CISR6	GACTTGTGACTTTTAACAATG
CISR7	CGCCTGCTTTGCACGC
CISRHP1	GAAAAGTTTAATTAGAAAGCTTG -6-FAM
CISRHP2	Cy5-TTTTATATTTAAACTTTCTCATTAGAC
TFR3	CGGGTCTTCCTATATGAGACAGAACC
TFR4	CCTGCCGTTGGATCAGTTTCGTTAA
TAP5	ATGTTCTATCTTTTCA
TAP6	ACCACCTTAGTTTACA

4.1.2.4 *C. fetus* subsp. *venerealis* VIRULENCE GENE AMPLIFICATION

Bioinformatics

Bioinformatics services were outsourced through WA's Centre for Comparative Genomics (CCG) at Murdoch University. *C. fetus* subsp. *venerealis* genomic sequence data was transferred under a confidential data transfer agreement with Prof Daniel Sanchez from Universidad Nacional de San Martin, Argentina to DPI&F (2005). DPI&F have a Confidentiality Agreement with Murdoch University's CCG. The *C. fetus* subsp. *venerealis* transferred sequence data represented approximately 72% of the entire genome within 273 contigs at a total of approximately 1.3 Mb of DNA sequence. The CCG undertook predicted protein sequence homology searches (full non-redundant NCBI amino acid and amino acid patent databases) of the 1370 putative genes identified in the 273 contigs. *C. fetus* subsp. *fetus* data became publicly available (http://www.ncbi.nlm.nih.gov/) early 2006 and thus a comparison of the available sequence data from both *C. fetus* subsp. *fetus* and *C. fetus* subsp.*venerealis* was also undertaken. Details of methods used in these analyses are provided in Appendix 2.

Assays for putative virulence genes

C. fetus subsp. *venerealis* protein homologues for virulence determinants identified in related *Campylobacter* species (66) were selected for further study. Single and nested PCR assays were designed for each gene using Primer Express Ver. 2 (AB) and Primer 3 programs. Primer sequences and assay conditions are described in Appendix 3. Primers were screened against the *C. fetus* subsp. *venerealis* (Argentina strain) and *C. fetus* subsp. *fetus* (strain 82-40) genome data and public sequence databases to confirm assay specificity. Assays were conducted in 20 μl reaction volumes, using 10nM of each forward and reverse primer, 1× PCR reaction buffer with 25mM Mg^{2+} (HotMaster *Taq* Buffer, Eppendorf), 200 μM dNTPs, 1 U HotMaster™ *Taq* DNA polymerase and 1 ng of *C. fetus* DNA or 2-5μl of smegma preparations (identified as positive in field collections). The reactions were cycled in a Gradient Palm Cycler (Corbett Research), using the following temperature profile: an initial denaturation at 94°C for 2 min, followed by 35 cycles of denaturation at 94°C for 20 s, annealing at 45 to 55°C (depending on primer pair, Appendix 3) for 10 s, and extension at 72°C for 30 s, including a final single extension for 7 min at the end of the profile. Amplification products were separated in 2% TBE (89 mM Tris borate, 2 mM EDTA, pH 8) agarose gels using 100bp ladder (Invitrogen) and were visualized under UV illumination by ethidium bromide staining. DNA preparations from reference strains were screened in all assays, Table 4.1.6.

Table 4.1.6. References strains tested in putative *C. fetus* subsp. *venerealis* virulence PCR assays

Species	Strain	Source[1]
C. fetus subsp. *venerealis*	98-109383	Field Isolate (DPI&F, QLD)
C. fetus subsp. *venerealis*	19438	ATCC
C. fetus subsp. *venerealis*	Argentina	UNSAM, Argentina[2]
C. fetus subsp. *venerealis*	Biovar *venerealis*	Pfizer Animal Health
C. fetus subsp. venerealis	Biovar *intermedius*	Pfizer Animal Health
C. fetus subsp. *fetus*	98- 118432	Field Isolate (DPI&F, QLD)
C. fetus subsp. *fetus*	15296	ATCC
C. coli	11353	NCTC
C. jejuni subsp. *jejuni*	11168	NCTC
C. hyointestinalis	N3145	Field Isolate (DPI&F, QLD)
C. sputorum subsp. *bubulus*	Y4291-1	Field Isolate (DPI&F, QLD)
Pseudomonas aeruginosa	27853	ATCC
Proteus vulgaris	6380	ATCC
Neospora caninum	50843	ATCC
T. foetus	YVL-W	Field Isolate (DPI&F, QLD)

[1]See Table 4.1.1 for origin of all reference strains except *C. fetus* subsp. *venerealis* Argentina
[2]DNA imported from Prof. Daniel Sanchez, Universidad Nacional de San Martin, Argentina

Smegma preparations (n=27) identified as *C. fetus* subsp. *venerealis* positive using the 5' *Taq* nuclease PCR assay were screened in these assays using nested PCR format. 3-5μl of the first PCR reaction was added to the second reaction containing nested forward and reverse primers utilising the same conditions as described above for PCR. Primer sequences and specific assay conditions are described in Appendix 3.

4.1.3 Results

4.1.3.1 ASSAY DEVELOPMENT – Stage 1

***Taq* nuclease assays**

Optimal conditions for the *T. foetus* assay: 25 μL reaction volume with 250 nM primers TFF2 and TFR2, and 80nM TRICHP2 using the following thermal profile: 50°C for 2 mins, 95°C for 2 mins, and 40 cycles of denaturation at 95°C for 20 s and annealing/extension at 60°C for 45 s. An initial set of *T. foetus* primers (TFF1 & TFR1) and probe (TRICHP1) was not suitable due to low levels of non-specificity detected when testing un-related species (not shown). The assay in Table 4.1.2 was subsequently developed as the preferred assay.

Optimal conditions for the *C. fetus* subsp*. venerealis* assay: 25μL reaction volume with 250 nM CFVF and CFVR primers, and 170 nM CFVP1 probe. The standard 2 step real time PCR profile did not adequately amplify *C. fetus* subsp. *venerealis* and thus the assay was optimised using a 3-step protocol: 50°C for 2 mins, 95°C for 2 mins, and 45 cycles of denaturation at 95°C for 20 s, annealing at 50°C for 20 s, and extension at 72°C for 20 s.

The optimised assays reliably detected genomic DNA at concentrations of 3 pg/reaction for *T. foetus* and 6.3 pg/reaction for *C. fetus* subsp. *venerealis* (not shown). Both assays (*T. foetus* and *C. fetus* subsp. *venerealis*) were able to detect a single cell equivalent in an assay following extraction using

the QIAamp kit. Detection was reliable at 10 cell equivalents per assay. Spiked smegma, mucus and urine yielded higher sensitivity detection using *Taq* assays when compared with diagnostic culture, (Table 4.1.7). All non-target organisms tested did not yield positive *Taq* nuclease assay results, including the closely related *C. fetus* subsp. *fetus* (listed Table 4.1.1).

Table 4.1.7 Cell number detection limits of culture and *Taq* nuclease assays for *T. foetus* and *C. fetus* subsp. *venerealis* (DNA prepared using commercial kit)

Sample type	*T. foetus*		*C. fetus* subsp. *venerealis*	
	Culture	*Taq* assay	Culture	*Taq* assay
Smegma	10000	1	1000	1
Mucus	25000	1	>25000	1
Urine	500	1	500	10

Culture based detection of *C. fetus* subsp. *venerealis* from vaginal mucus was extremely problematic due to a high incidence of overgrowth by non-*Campylobacter* species. In addition, one false positive was observed demonstrating the growth *Campylobacter*-like colonies which were later identified as *Weissella confusa*. This sample was confirmed as negative in the *C. fetus* subsp. *venerealis Taq* nuclease assay.

Infection Trial

While the inoculations of the 32 animals did not lead to detectable infection, two bulls were *C. fetus* subsp. *venerealis* positive by culture and *Taq* nuclease assay prior to inoculation, and a third infected bull was detected during the course of the trial. No test animal was positive for *T. foetus* at any stage of the trial. Veterinarian's advice (Geoffry Fordyce, see Figure 3) concerning the ease of sampling indicated a preference for the raspador, and no adverse effect on the animals was noted using any technique. It is recommended to cut off the end of the raspador into the saline to avoid potential sample cross contamination. The cutting tool should be disinfected between samplings. Figure 3 shows examples of the use of different sampling tools during this trial. Urine sampling from bulls proved difficult but could not be a viable sampling technique for heifers. All sample types proved suitable for detection of the naturally occurring *C. fetus* subsp. *venerealis* infections, with the raspador providing the greatest consistency between culture and *Taq* nuclease assay results. Quantitation of the number of organisms detected in the assay indicated the raspador provided the highest number of *C. fetus* subsp. *venerealis* cells/ml in the sample (Table 4.1.8).

Figure 3 – Sampling tools and trial – Swans Lagoon Station, DPI&F

Table 4.1.8. Diagnostic assay results from successive testing of naturally infected bulls using different specimen collection tools

Bull	Collection tool	Selective culture[1]	Quantitative 5'*Taq* nuclease assay[2]
1	Pipette	-	1430
	Rasper	+	2258
	Swab	-	85
2	Pipette	+	72
	Rasper	+	1501
	Swab	-	148
3	Pipette	-	2876
	Rasper	+	8118
	Swab	+	2910

[1]+: positive culture result; -: negative culture result
[2]Quantitative 5' *Taq* nuclease assay estimates are given in cells/ml which is an average value calculated from duplicate results.

Template preparation

The heat lysis method for sample preparation for *Taq* nuclease assay proved to be a suitable alternative to QIAGEN's QIAamp DNA extraction kit for both laboratory spiked and naturally infected samples. Processing time is significantly reduced, with sensitivity being improved upon that obtained using the QIAamp kit, allowing reliable detection of a single cell equivalent per assay (Table 4.1.9).

Table 4.1.9. Reliable detection limits in cells/assay or cells/inoculation

Sample type	Storage time[1]	*Taq* nuclease assay		Culture2
		QIAamp	Heat Lysis	
T. foetus:				
Smegma	0 days	1	0.1	>25000
	2 days	1	1^3	2500
	5 days	1	0.1	>25000
Mucus	0 days	1	0.1	25000
	2 days	1	0.1	250
	5 days	1	0.1	25000
Urine	0 days	1	0.1	5000
	2 days	1	1	500
	5 days	1	0.1	ND
C. fetus subsp. *venerealis*:				
Smegma	0 days	10	1	>25000
	2 days	10	1	25000
	5 days	1	10	2500
Mucus	0 days	10	10^3	>25000
	2 days	10^3	1	>25000
	5 days	10	1^3	>25000
Urine	0 days	10	100^3	500
	2 days	10	1	ND
	5 days	10	1	ND

[1]Smegma and mucus samples for *Taq* nuclease assay were stored at ambient temperatures in physiological saline. Urine samples were stored at 4°C. Samples for culture were stored in transport medium (InPouch TF test kits, or modified Weybridge medium) at ambient temperatures.
[2]Preliminary results obtained for urine culture only.
[3]Positive assay results were possible for the log dilutions below this concentration, but were not reliably obtained.

Effects of storage

Comparison of diagnostic culture, conventional PCR and *Taq* nuclease assays on stored spiked clinical specimens (transport media and saline) demonstrated a higher sensitivity of *Taq* nuclease assays. Direct amplification from mucus or smegma in saline following transport at ambient temperatures for up to 5 days provided better sensitivity than PCR of DNA from transport medium (Tables 4.1.10, 4.1.11). A comparison of quantitative average cell/ml estimates in both 5' *Taq* nuclease assays of stored smegma, mucus and urine inoculated with 10^4 cells is presented in Tables 4.1.12 and 4.1.13. Heat lysis proved to be more sensitive method for the preparation of clinical samples for *Taq* nuclease amplification compared to QIAamp kit extracts, however conventional PCRs did not effectively amplify crude cell lysates requiring purified kit extracts (results not shown). Urine samples were transported chilled with ice bricks in an insulated container in accordance with the *Leptospira* sample transport conditions. The standard transport media supported the growth of *C. fetus* subsp. *venerealis* and *T. foetus* from spiked urine samples. These samples were successfully amplified in the relevant *Taq* nuclease assays.

Table 4.1.10. Detection limits for *C. fetus* subsp. *venerealis* from stored smegma, mucus and urine using selective culture, conventional PCR assay (83) and 5' *Taq* nuclease assay

Sample type	Storage time[1]	Selective culture (cells/ inoculum)[2]	Assay post enrichment culture[3]		Conventional PCR	5' *Taq* nuclease assay	
			5' *Taq* nuclease assay	Conventional PCR	QIAamp	QIAamp	Heat lysis
Smegma	0 days	>25000	500	5000[4]	>1000	10	1
	2 days	25000	500	5000[4]	100	10	1
	5 days	2500	5000	>5000	100	1	10
Mucus	0 days	>25000	50	50	100	10	10^4
	2 days	>25000	500	500[4]	10	10^4	1
	5 days	>25000	500	5000	10	10	1
Urine	0 days	500	ND[5]	ND	ND	10	100[4]
	2 days	>25000	ND	ND	ND	10	1
	5 days	>25000	ND	ND	ND	10	1

[1]Smegma and mucus samples for 5' *Taq* nuclease or conventional PCR assay were stored at ambient temperatures in either PBS or physiological saline. Urine samples were stored at 4°C. Samples for culture were stored in modified Weybridge medium (TEM) at ambient temperatures.
[2]Sensitivity limits for both conventional PCR and 5' *Taq* nuclease assay are given in cell equivalents/assay. Selective culture sensitivity limits are indicated as the minimum number of cells/inoculum in TEM required to produce *Campylobacter*-like colonies on subsequently inoculated selective media. Standard deviations on viable cell counts for each dilution are presented in Table 3.
[3]DNA was extracted from TEM for 5' *Taq* nuclease and conventional PCR assay using a commercial kit (QIAamp).
[4]Positive assay results were possible for the log dilutions below this concentration, but were not reliably obtained.
[5]Not Done

Table 4.1.11. Detection limits for *T. foetus* from laboratory-spiked stored smegma, mucus and urine using microscopy following selective culture, conventional PCR assay (62) and 5' *Taq* nuclease assay

Sample type	Storage time[1]	Selective culture/microscopy (cells/inoculum)[2]	Assay post enrichment culture[3]		Conventional PCR	5' *Taq* nuclease assay	
			5' *Taq* nuclease assay	Conventional PCR	QIAamp	QIAamp	Heat lysis
Smegma	0 days	>25000	50	500	1	1	0.1
	2 days	2500	50	500	1	1	1[4]
	5 days	>25000	50	5000	1	1	0.1
Mucus	0 days	25000	50	500	1	1	0.1
	2 days	250	50	500	1	1	0.1
	5 days	25000	50	500	1	1	0.1
Urine	0 days	5000	ND[5]	ND	ND	1	0.1
	2 days	500	ND	ND	ND	1	1
	5 days	500	ND	ND	ND	1	0.1

[1]Smegma and mucus samples for 5' *Taq* nuclease assay were stored at ambient temperatures in physiological saline. Urine samples were stored at 4°C. Samples for culture were stored in InPouch™ TF test kits at 37°C.
[2]Sensitivity limits for both conventional PCR and 5' *Taq* nuclease assay are given in cell equivalents/assay. Selective culture/microscopy sensitivity limits are indicated as the minimum number of cells/inoculum of InPouch™ TF media required for motile *Tritrichomonas*-like protozoans to be visible on subsequent microscopic examination.
[3]DNA was extracted from InPouch™ TF media for 5' *Taq* nuclease and conventional PCR assay the QIAamp kit.
[4]Positive assay results were possible for the log dilutions below this concentration, but were not reliably obtained.
[5]Not Done.

Table 4.1.12. A comparison of quantitative average cell/ml estimates for *Campylobacter fetus* subsp. *venerealis* by 5' *Taq* nuclease assay of stored smegma, mucus and urine inoculated with 10^4 cells

Sample type	Storage time[1]	5' *Taq* nuclease assay post enrichment culture[2, 3]	5' *Taq* nuclease assay	
			QIAamp	Heat Lysis
Smegma	0 days	15510	379	1259
	2 days	3380	94	1836
	5 days	0	47	183
Mucus	0 days	7905	996	5282
	2 days	5948	253	1838
	5 days	2764	206	810
Urine	0 days	ND[4]	976	693
	2 days	ND	414	426
	5 days	ND	393	776

[1]Smegma and mucus samples for 5' *Taq* nuclease or conventional PCR assay were stored at ambient temperatures in either PBS or physiological saline. Urine samples were stored at 4°C. Samples for culture were stored in modified Weybridge medium (TEM) at ambient temperatures.
[2]DNA was extracted from TEM for 5' *Taq* nuclease assay using a commercial kit (QIAamp).
[3]Quantitative 5' *Taq* nuclease assay estimates are given in cells/ml.
[4]ND: Not Done.

Table 4.1.13. A comparison of quantitative average cell/ml estimates of *T. foetus* by 5' *Taq* nuclease assay of stored smegma, mucus and urine inoculated with 10^4 cells

Sample type	Storage time[1]	5' *Taq* nuclease assay post enrichment culture[2, 3]	5' *Taq* nuclease assay	
			QIAamp	Heat Lysis
Smegma	0 days	3431	5820	3435
	2 days	3353	664	2480
	5 days	5408	79	157
Mucus	0 days	1727	4667	7682
	2 days	2434	11755	21185
	5 days	148	6994	11554
Urine	0 days	ND[4]	3343	191
	2 days	ND	494	427
	5 days	ND	7957	1387

[1]Smegma and mucus samples for 5' *Taq* nuclease or conventional PCR assay were stored at ambient temperatures in either PBS or physiological saline. Urine samples were stored at 4°C. Samples for culture were stored in InPouch™ TF media at 37ºC.
[2]DNA was extracted from InPouch™ TF media for 5' *Taq* nuclease assay via a commercial kit (QIAamp).
[3]Quantitative 5' *Taq* nuclease assay estimates are given in cells/ml.
[4]ND: Not Done.

Recommended sampling protocol

Smegma and mucus: Collect using a raspador (preferred) or a wide-bore pipette, and resuspend physiological saline. Transport to the laboratory at ambient temperatures, and process for assay using either QIAGEN's QIAamp DNA mini kit or heat lysis. Smegma and mucus samples are preferred over urine.

Urine: Collect first pass urine and transport to the laboratory chilled by ice bricks in an insulated container. Process for assay using either QIAGEN's QIAamp DNA mini kit or heat lysis.

4.1.3.1 FIELD SAMPLING – Stage 2

Field Sampling Training

The training meeting in June 2004 was led by Ala Lew, Bruce Corney and Lyle McMillen. Animal sampling was demonstrated by Geoffry Fordyce (Figure 4). The meeting was attended by Geoffry Fordyce, Dick Holroyd, Lex Turner, John Bertram (veterinary staff), Vivienne Doogan (biometrician) and Bronwyn Venus (laboratory technician). Mick Sullivan (Mt. Isa) could not attend and was linked by teleconference to the group to assist in planning animal sampling for Stage 2. The major outcomes of this training were:

- Refined animal sampling protocol (Appendix 1) – coordinated for *C fetus* subsp. *venerealis*, *T. foetus* and *Leptospira*.
- Each field veterinarian was able to experience the application of the 'tricamper' (with the exception of Mick Sullivan who was later trained by Geoffry Fordyce).
- Understanding of new IATA approved containers for sample packaging and transport.
- Each officer agreed to sample approximately 80 animals. The aim was to also target animals with potential high risk eg. – bulls over 4 years, heifers empty after initial pregnancy testing. The goal was 40 positives by conventional and novel methods for each pathogen, from 400 animals total. Samples collected from multiple herds (approximately 40 herds, if possible).
- Lex agreed to sample abattoir animals (Dinmore) based on recommendations from John, Dick, Geoffry or Mick.

- Animal ethics approval for sampling in Qld and for Northern Territory animals was required.
- Decision to use arbitrary herd labels to keep the data confidential.
- Continual updating of results required to both enable samplers to focus on new herds in different regions (if necessary) and to determine whether a candidate case study herd could be identified.

Figure 4 – Field sampling training, Animal Research Institute, June 2004

Summary of sampling results

Table 4.1.14 summarises the results obtained from different Qld regions for each pathogen by both culture and *Taq* nuclease assay.

Table 4.1.14 Summary of field sampling data[1] for Stage 2

Region	No. of properties		*C. fetus* subsp. *venerealis*		*T. foetus*	
			Culture	*Taq* assay	Culture	*Taq* assay
South	8	bulls	10/56	11/56	0/56	0/56
		heifers	0/16	0/16	0/16	0/16
Central coast	8	bulls	1/53	11/53	1/53	5/53
		heifers	0/30	0/30	0/30	1/30
North coast	15	bulls	2/59	11/64	0/59	2/64
		heifers	0/39	0/49	0/39	0/49
North west	7	bulls	0/55	6/55	1/55	4/55
		heifers	0/18	0/18	0/18	0/18
South East[2]	1	bulls	ND	ND	1/20	2/20
Total	**39**		**13/326**	**39/341**	**3/346**	**14/361**

[1]Data is presented as: number of positive (*C. fetus* subsp. *venerealis* and *T. foetus)* / (total number of samples tested).
[2]Submitted to Toowoomba diagnostic laboratory, source most likely central coast.

Data was collected from conventional and *Taq* nuclease assay of specimens (smegma or vaginal mucus, urine) obtained from 341 animals. As a consequence of sampling in the June to November period, the likelihood that females would have spontaneously cleared any infection was high. In view of this, 228 (248 for *T. foetus*) of the 341 animals tested were bulls. A single heifer was

positive for *T. foetus*, with none positive in the *C. fetus* subsp. *venerealis Taq* nuclease assays. No female cattle were positive for either pathogen using selective culture. Vaginal mucus specimens from 106 female cattle were tested using the established *C. fetus* ELISA, with 14 providing positive results. None of these 14 ELISA positive were also positive for *C. fetus* subsp. *venerealis* using either selective culture or *Taq* nuclease assay, however 4 positives originated from herds with *C. fetus* subsp. *venerealis Taq* assay positive bulls. The remaining 10 ELISA positives originated from herds where bulls were not tested. Due to practical constraints on some properties, culture specimens and urine samples were not submitted for all samples corresponding to all *Taq* assay screened smegma/mucus samples. Of the 16 *C. fetus* subsp. *venerealis Taq* assay smegma positive animals, only 2 corresponding urine samples were positive, and all *T. foetus* tested urines were negative however no correlating *T. foetus* smegma positive urines were screened. The difficulties inherent in the collection of urine from bulls and the poor performance of urine as a *C. fetus* subsp. *venerealis* or *T. foetus* assay specimen suggested that urine is likely to be unsuitable as a specimen for routine diagnostic assay. Data on the use of Vibrovax *Campylobacter* vaccine was collected for 20 properties, 7 of which maintained a vaccination program as part of routine disease management. Of the 20 properties, *C. fetus* subsp*. venerealis* infected animals were detected on 12 properties, of which 6 maintained a vaccination program. It is not known whether vaccination guidelines were strictly adhered to.

Statistical analysis

For *C. fetus* subsp. *venerealis*, no female cattle were positive for either *Taq* nuclease assay or culture, thus statistical analyses were applied to data collected from bulls only. A comparison of the properties of positive results provided strong evidence (P<0.001) that the *Taq* assay (16% positive) produced more positive results than culture (5% positive) for *C. fetus* subsp*. venerealis* (Table 4.1.15).

Table 4.1.15. Comparison of *C. fetus* subsp. *venerealis* diagnostic culture and 5' *Taq* nuclease assay results from smegma specimens collected from northern Australian properties

	5' *Taq* nuclease assay:		
Culture:	Positive	Negative	**Total**
Positive	9	[1]4	**13**
Negative	30	206	**236**
Total	**39**	**210**	**249**

[1]Confirmed that the 4 5' *Taq* nuclease assay negative-culture positive isolates were non-specific positives

Further examination (metabolic profiling, 16S rRNA gene sequencing) of the *Campylobacter*-like colonies did not confirm the presence of *C. fetus* subsp. *venerealis.* None of the 4 *Taq* assay negative cultures were identified as *C. fetus* subsp. *venerealis* by conventional PCR.

For *T. foetus*, the number of positive results recorded was low for both culture and *Taq* assay methods (Table 4.1.16). However, a comparison of the properties positive supports the conclusion that the *Taq* assay (9% positive) is producing a higher proportion of positive results than culture (2% positive) (P=0.006).

Table 4.1.16. Comparison of microscopy following selective culture and 5'*Taq* nuclease assay diagnosis of *T. foetus* infection from culture positive herds from north eastern Australia

	Selective culture/microscopy[1]		
5'Taq nuclease assay	Positive	Negative	**Total**
Positive	3	11[2]	**14**
Negative	0	145	**145**
Total	**3**	**156**	**159[2]**

[1]For one herd group of 20 animals, the selective culture/microscopy was performed in an accredited veterinary diagnostic laboratory during routine herd management.
[2]A total of 30 female cattle (129 bulls in total) were tested, none of which were culture/microscopy positive, one cow tested *Taq* nuclease positive

Comparison of Qld regions: The percentage of positive results for *C. fetus subsp. venerealis* using the *Taq* assay did not differ (χ^2 = 2.24; P=0.524) across regions with percentages positive being 20.8, 19.6, 17.2 and 10.9% for central coast, south, north coast and north west, respectively. However, there was evidence (χ^2 = 8.05; P=0.045) of regional differences for *T. foetus*, with percentages positive being 11.3, 7.3, 3.1 and 0.0% for central coast, north west, north coast and south regions, respectively.

Selection of case study herds

A *C. fetus* subsp. *venerealis* case study herd was identified first in the south-west region of Qld. Negotiation with the producers secured their understanding and cooperation, and pre-mating screening of the property's bulls was completed by November 2004. Mating commenced within the month following.

Positive results for *T. foetus* were obtained in north-west Queensland, and a Station agreed to participate in the case study analysis. Pre-mating tests of bulls were conducted late November-early December, 2004.

4.1.3.2 MULTICENTRE EVALUATION – Stage 2

Table 4.1.17 summarises the results obtained by different laboratories processing the same specimens for each assay evaluation.

Table 4.1.17. Comparison of C_T values or positive/negative results from *C. fetus* subsp. *venerealis* and *T. foetus* in multi-centre evaluations (DPI&F, DPI-VIC, DPI-NSW)

Centre	*C. fetus* subsp. *venerealis* specimens[1]												
	345	409	521	535	537	591	592	1024	606	607	608	616	617
DPI&F	26.98	28.9	28.21	-	-	31.93	-	29.59	-	29.33	26.88	24.63	-
DPI-VIC	27.61	28.06	24.84	-	-	27.67	-	27.10	NT	NT	NT	NT	NT
DPI-NSW	NT	NT	NT	NT	NT	NT	NT	NT	-	30.83	30.18	28.89	-

[1]NT, not tested

Centre	*T. foetus* specimens							
	345	409	521	535	537	591	592	1024
DPI&F	-	-	35.41	32.62	37.11	-	-	-
DPI-VIC	-	-	34.62	32.02	34.31	-	-	-
DPI-NSW	-	-	-	33.9	37.71	-	-	-

DPI-VIC: Rachel Auld adapted the *C. fetus* subsp*. venerealis* and *T. foetus* assays readily to the Roche Lightcycler used at VIAS (DPI-VIC) for *Taq* nuclease PCR assays. Perfect positive/negative results were obtained between the samples. While interested in adopting the new assays, VIAS performs *C. fetus* subsp*. venerealis* and *T. foetus* assays principally for export testing, and will not be able to adopt the novel assays until they are approved for export testing.

DPI-NSW: Tracey Berg conducted the *C. fetus* subsp*. venerealis* assay on a Corbett RotorGene 2000, similar to the RotorGene 3000 used to develop the assays, and experienced no difficulties in optimising the assay. High correlations with our results were obtained on assay of the provided specimens, and higher sensitivity was obtained when compared to the PCR-based assay developed at EMAI. Tracey has since sought a confidentiality agreement to enable EMAI to obtain its own stocks of primers and probes, and have adopted the assays as routine diagnostic tests.

There was excellent agreement between the 3 laboratories for categorisation of samples as positive or negative for *T. foetus* (kappa = 0.812; P<0.001). For *C. fetus* subsp*. venerealis*, there was complete agreement between DPI&F and DPI-Vic (kappa = 1.000; P=0.016) and between DPI&F and DPI-NSW (kappa = 1.000; P=0.016).

CASE STUDIES – Stage 3

Case Study 1

Bulls were in store condition in December 2004, and forward store to prime condition in June 2005. Table 4.1.18 presents results of sampling for *C. fetus* subsp. *venerealis* and *T. foetus* in December 2004 and June 2005. By June 2005:

- One bull treated with antibiotics in December remained *C. fetus* subsp. *venerealis* positive.
- Of 6 untreated *C. fetus* subsp. *venerealis* positive bulls in December, 4 were negative; the other 2 were not tested.
- One bull previously *T. foetus* positive was negative.
- Three bulls previously *C. fetus* subsp. *venerealis* negative were positive.

Table 4.1.18. Disease status of bulls (number and distribution) in December 2004 and June 2005

December 2004		June 2005			
Code	Description	Neg	C	nt	**% excluding nt**
Neg	*C. fetus* subsp. *venerealis* & *T. foetus* negative (Camp & Trich)	32	3	16	**78%**
C	Camp positive & Trich negative	4		1	**9%**
Ce	Camp pos, Trich neg & EM[1]	4	1	2	**11%**
Csus	Camp suspect			1	
T	Trich positive	1			**2%**
CT	Camp & Trich positive			1	
nt	Not tested	7			
% excluding nt		**91%**	**9%**		

[1]Treated with erythromycin at 200 mg per 50 kg im daily for 3 days

Chi-square tests indicated bull age was unrelated to infection with either *C. fetus* subsp. *venerealis* or *T. foetus* (Table 4.1.19). None of the females sampled were *C. fetus* subsp. *venerealis* positive. Of the 228 females sampled, 5 (2%) were *T. foetus* positive and 3 of these were not pregnant in June 05 (Table 4.1.20). A comparison of proportions test indicated no significant difference between the pregnancy rate in *T. foetus* infected and uninfected females (P=0.136). Concurrent urine sampling identified 2 and 6 heifers that were positive and suspect for pathogenic *Leptospira* spp., respectively, and all eight were pregnant, indicating no adverse effect of *Leptospira* spp. on pregnancy rate (Table 4.1.21).

Table 4.1.19. Bull age and disease status (number and distribution)

	December 2004			June 2005		
Bull age (Years)	No. of bulls	Camp pos or suspect	Trich pos	No. of bulls	Camp pos	Trich pos
2	25	12%	0%	14	7%	0%
3	20	30%	0%	19	11%	0%
4-7	21	24%	10%	19	5%	0%

Table 4.1.20. Disease status and pregnancy rates of females excluding *Leptospira* spp. suspect positive and positive results

	Camp&Trich negative	Camp positive	Trich positive	Camp&Trich positive
No.	213	0	5	0
% of group	98%		2%	
% pregnant	71%		40%	

Table 4.1.21. Disease status and pregnancy rates of females excluding *C. fetus* subsp. *venerealis* positives and *T. foetus* positives

	Lepto negative	Lepto suspect & positive
No.	149	8
% of group	95%	5%
% pregnant	73%	100%

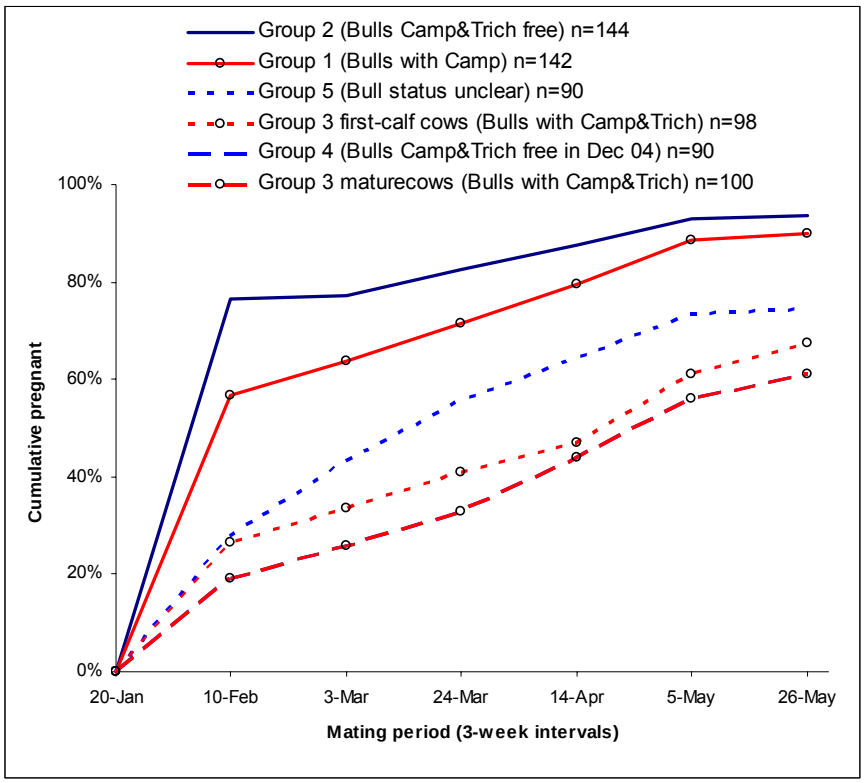


Figure 5. Pregnancy rates – Case study 1 mating groups

Mating Group 1

142 maiden heifers were mated to 7 bulls starting in January 05. All 7 bulls were tested prior to mating in December 2004 and 2 bulls were positive for *C. fetus* subsp. *venerealis* and all were *T. foetus* negative. Bulls were not treated with erythromycin prior to mating. Six of the 7 bulls and 68 of the females were tested for *C. fetus* subsp. *venerealis* and *T. foetus* in June 05 and tested negative in both assays. In addition, 52 of the 68 females were also tested for *Leptospira* spp. and 5 were 'suspect' positive. An extra 'immigrant' bull 'emerged' in the group in June 05. This bull was negative for *C. fetus* subsp. *venerealis* and *T. foetus* in Dec 04 and June 05. Overall pregnancy% achieved was 90%. Figure 5 above shows the conception pattern of all the groups (based on the stage of pregnancy estimated in mid June 2005).

Summary:

- *C. fetus* subsp. *venerealis* was present in bulls at the start of mating but no evidence of *C. fetus* subsp. *venerealis* or *T. foetus* in June 05 in bulls or heifers. Infected bulls were not treated for *C. fetus* subsp. *venerealis*.
- Possibility of *Leptospira* spp. in females (5 suspect samples).
- Overall pregnancy% of 90% is reasonable.
- Reasonable proportion of early conceptions – 57% in the first 3 weeks of mating.

Mating group 2

144 maiden heifers were mated to 4 bulls starting in January 05. All 4 bulls tested negative for *C. fetus* subsp. *venerealis* and *T. foetus* prior to mating in December 2004. Bulls were not treated

with erythromycin prior to mating. All the bulls and 66 of the heifers were tested in June 05 and all were negative for *C. fetus* subsp. *venerealis*, while 3 heifers were positive for *T. foetus*. In addition, 39 of the 66 females were also tested for *Leptospira* spp. and 2 were found to be positive and 1 suspect. Overall pregnancy% achieved was 94%.

Summary:

- All bulls clean for *C. fetus* subsp. *venerealis* and *T. foetus* before and after mating.
- No evidence of *C. fetus* subsp. *venerealis* in the group.
- Evidence of *Leptospira* spp. in females.
- Overall pregnancy% of 94% is good.
- High proportion of early conceptions – 76% in the first 3 weeks of mating.

Mating group 3

198 females comprising 98 3-year-olds and 100 mature cows (≥4 year-old) were mated to 12 bulls starting in mid January 05. Five of the 12 bulls were tested in December 2004 and one was positive for *C. fetus* subsp. *venerealis* and one *T. foetus* respectively. All of the bulls and 46 of the cows were tested in June 2005 and all were negative for *C. fetus* subsp. *venerealis* while 2 cows were positive for *T. foetus*. In addition, 27 of the 46 females tested negative for *Leptospira* spp. Overall pregnancy% achieved by mid June was 67% for the 3-year-olds and 61% for the cows.

Summary:

- Evidence of *C. fetus* subsp. *venerealis* and *T. foetus* in bulls prior to mating
- No evidence of *C. fetus* subsp. *venerealis* or *Leptospira* spp. in the group in June 2005.
- Evidence of *T. foetus* in the cows in June 05.
- Overall pregnancy% of 67% and 61% are low.
- Low proportion of early conceptions – 27% and 19% in the first 3 weeks of mating for the 3-year-olds and mature cows respectively.

Mating group 4

90 cows were mated to 19 bulls starting in mid January 2005. All bulls were tested in December 2004 and four were positive for *C. fetus* subsp. *venerealis* whereas all were negative for *T. foetus*. The bulls with evidence of *C. fetus* subsp. *venerealis* were treated with erythromycin in January 2005 prior to mating. All bulls and 46 of the cows were tested in June 2005 and were negative for *T. foetus* while 2 bulls were positive for *C. fetus* subsp. *venerealis*. These 2 previously tested negative for *C. fetus* subsp. *venerealis* when tested in December 2004. The 4 that were positive for *C. fetus* subsp. *venerealis* in December 2004 and treated in January 2005 were negative for *C. fetus* subsp. *venerealis* when tested in June 2005. In addition, 43 of the 46 females tested negative for *Leptospira* spp. Overall pregnancy% achieved by mid June was 49%.

Summary:

- No evidence of *T. foetus* or *Leptospira* spp. in the group in June 05.
- Evidence of *C. fetus* subsp. *venerealis* in the bulls in June 2005.
- Infected bulls treated for *C. fetus* subsp. *venerealis* were negative at the subsequent testing.
- Overall pregnancy% of 49% is low.
- Low proportion of early conceptions – 13% in the first 3 weeks of mating.

Mating group 5

90 three-year-old cows were mated to 10 bulls starting in mid January 05. All bulls were tested in December 2004 and two were positive for *C. fetus* subsp. *venerealis* while all were negative for *T. foetus*. The bulls with evidence of *C. fetus* subsp. *venerealis* were treated with erythromycin in January 2005 prior to mating. All of the bulls but none of the cows were tested in June 2005 and 2 bulls were positive for *C. fetus* subsp. *venerealis*. One of these 2 was negative for *C. fetus* subsp. *venerealis* in December 2004 and had been treated with antibiotics. Overall pregnancy% achieved by mid June was 74%.

Summary:

- No evidence of *T. foetus* in the group in June 2005.
- Evidence of *C. fetus* subsp. *venerealis* in the bulls in June 2005 even after treating infected bulls before mating.
- One infected bull treated for *C. fetus* subsp. *venerealis* was positive at the subsequent testing.
- Overall pregnancy% of 74% is moderate.
- Low proportion of early conceptions – 28% in the first 3 weeks of mating.

Case Study 2

First calf cow mating group

145 first calf cows were mated to 11 bulls. All 11 bulls were tested prior to mating on 22/9/04 and one bull was positive for *C. fetus* subsp. *venerealis* while all were negative for *T. foetus*. All females were tested on 19/4/05 and 7 were found to be positive for *T. foetus* and negative for *C. fetus* subsp. *venerealis*. Of the 7 that were positive for *T. foetus*, 2 were not pregnant (71% pregnancy rate) and the remaining 5 were pregnant (3 conceived within the first 4 weeks of mating, while the remaining 2 conceived in week 8 and 12, respectively).

Overall pregnancy% achieved was 81%. Figure 6 below shows the conception pattern of the group (based on the stage of pregnancy estimated on 19/4/05). There was a steady increase in cumulative pregnancy% over the mating period. Only 35% of the group conceived in the first 4 weeks of mating. Pregnancy% of the *T. foetus* infected females (71%) was not significantly different to that of the remaining females (81%).

Summary: One of the 11 bulls used in this mating tested as positive for *C. fetus* subsp. *venerealis* and all bulls tested negative for *T. foetus* some 2.5 months prior to the start of mating. When females were tested 2 months after the end of mating, none tested positive for *C. fetus* subsp. *venerealis*, yet 7 were positive for *T. foetus*. We did not know the disease status of these females prior to mating. Possible conclusions:

- If the infected bull infected any of the females with *C. fetus* subsp. *venerealis* during mating then either the infection cleared within 2 months or the females were already immune to *C. fetus* subsp. *venerealis*.
- It is not known where the *T. foetus* present in 7 females 2 months after mating originated from. It is not known whether the females were infected prior to mating and remained positive, or whether they may have become infected during their first mating and then carried the infection through to the second mating. As we did not know the disease status and

reproductive history of these females we could not confirm the source of the *T. foetus* or the cause for the delayed conception pattern.

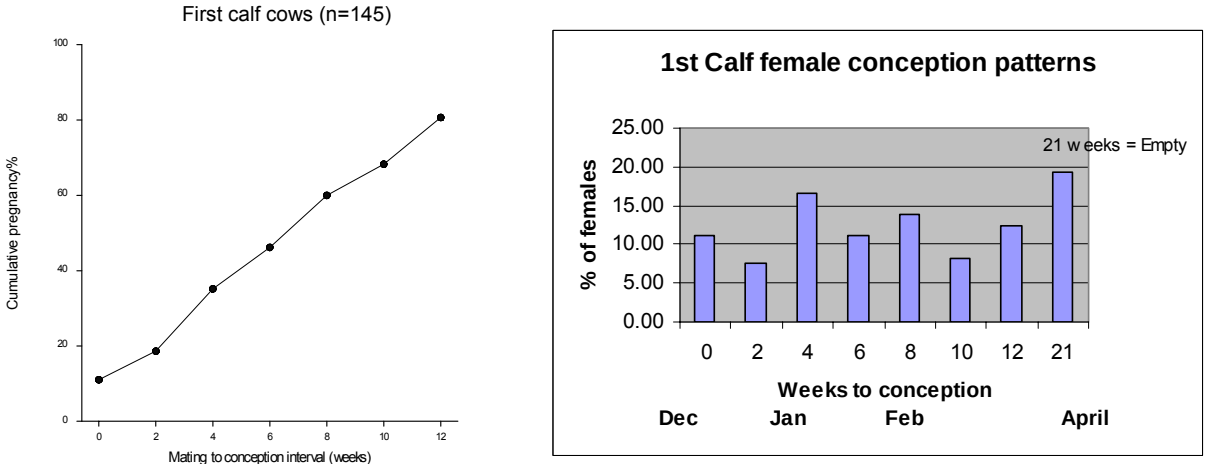


Figure 6. Pregnancy pattern of the first calf cow mating group

'Bought' maiden heifers mating group

84 bought Santa Gertrudis maiden heifers were mated to 3 bulls. Bulls were not tested for disease prior to mating. Two of the bulls were tested for *C. fetus* subsp. *venerealis* and *T. foetus* after mating and both were negative for both pathogens. The third bull was not tested either before or after mating.

All females were tested on 19/4/05 (two months after the end of mating) and 3 were positive for *T. foetus* and all were negative for *C. fetus* subsp. *venerealis*. All 3 that tested positive for *T. foetus* were pregnant on 19/4/05 and all 3 conceived in the first 4 weeks of mating. Pregnancy% of the *T. foetus* infected females (100%) was not significantly different to that of the remaining females (89%). Overall pregnancy% achieved was 89%. Figure 7 below shows the conception pattern of the group (based on the stage of pregnancy estimated on 19/4/05). There was a rapid increase in pregnancy% in the first 4 weeks of mating, then a slower increase for the remainder of the mating period.

Summary: We did not know the disease status of the bulls prior to mating and thus it is difficult to confirm transmission. However, the pattern of conceptions is markedly different to that for the first calf cows, with 65% of bought maidens conceiving in the first 4 weeks of mating compared with 35% of first calf cows.

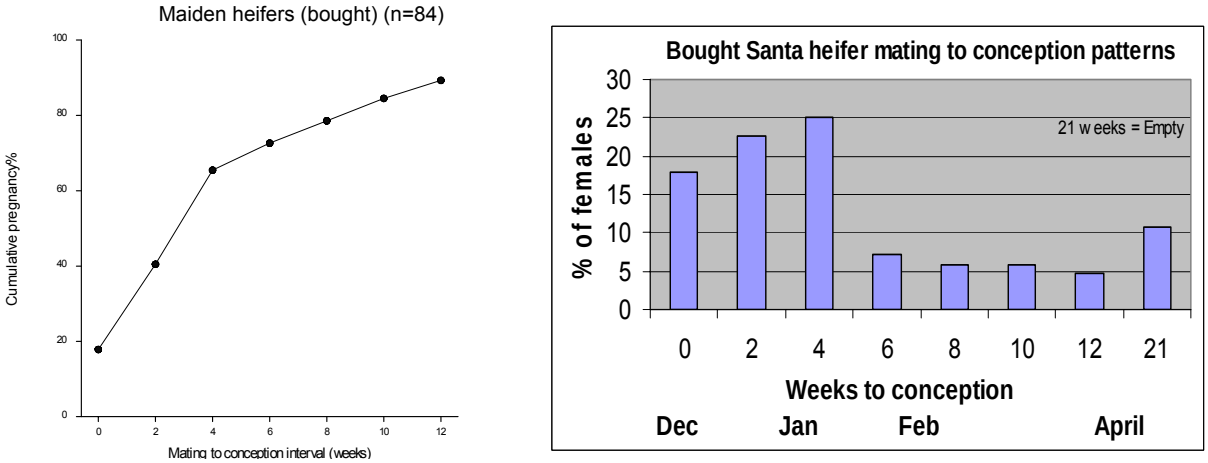


Figure 7. Pregnancy pattern of the bought maiden heifer mating group

Home-bred maiden heifers mating group

83 home-bred maiden heifers were mated to 3 bulls. Only one of the bulls was tested for *C. fetus* subsp. *venerealis* and *T. foetus* prior to mating on 22/9/04 and was negative for both. The other 2 bulls were tested after mating on 20/4/05 with one bull being negative for both *C. fetus* subsp. *venerealis* and *T. foetus* and the other positive for *C. fetus* subsp. *venerealis*. All females were tested on 19/4/05 (2 months after the end of mating) and 5 were positive for *T. foetus* whereas they were all *C. fetus* subsp. *venerealis* negative. Four out of these 5 were pregnant on 19/4/05 (all conceived within the first 6 weeks of mating), while stage of pregnancy was not assessed for the 5th heifer. Pregnancy% of the *T. foetus* infected females (100%) was not significantly different to that of the remaining females (81%). Overall pregnancy% achieved was 82%. Figure 8 below shows the conception pattern of the group (based on the stage of pregnancy estimated on 19/4/05). The increase in cumulative pregnancy% slowed down over time. Forty-eight% of pregnancies occurred in the first 4 weeks of mating.

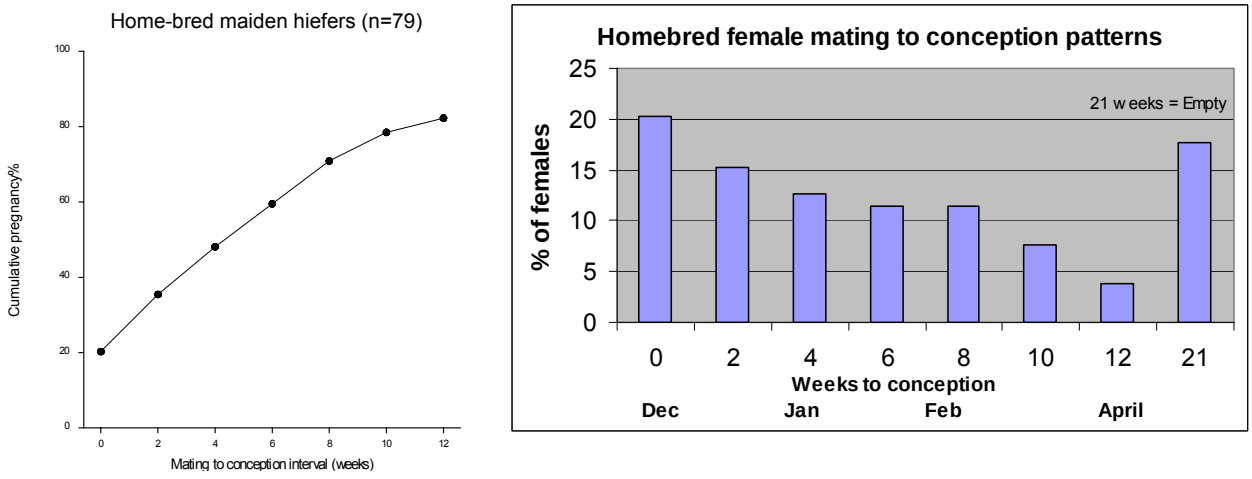


Figure 8. Pregnancy pattern of homebred female mating group

Summary: The disease status of all of the bulls prior to mating was unknown, but there is evidence of *C. fetus* subsp. *venerealis* in one bull 2 months after mating. No females were positive for *C. fetus* subsp. *venerealis* 2 months after the end of mating, but 5 were positive for *T. foetus*. It is not known where the *T. foetus* originated from. If any females did become infected with *C. fetus* subsp. *venerealis*, it appears that they cleared the infection by the time of sampling.

4.1.3.2 GENOTYPING

C. fetus subsp. _venerealis_ genotyping

The initial melting curve analysis approach for *C. fetus* subsp. *venerealis* genotyping encountered problems with specificity, generating T_M data for *C. fetus* subsp. *fetus* in addition to *C. fetus* subsp. *venerealis*. The T_M estimates did not differ between the isolates and field specimens sufficiently to allow discrimination between strains.

The addition of a PCR reaction using CISR2 and CISR5 as primers to enrich field specimens for the hybridisation assay target allowed reliable data to be generated from heterogenous gDNA. The resulting melting point data however, did not differ and thus did not discriminate between *C. fetus* subsp. *venerealis* isolates from Australia and the USA.

T. foetus genotyping

Culture of field strains for diagnostic comparison (Table 4.1.16) only yielded 3 positive cultures from 361 specimens. While capable of discriminating between the two pure strains of *T. foetus* available at this time, the RAPD was not suitable for use on DNA extracted from clinical specimens. Sequence data of the TFR3-TFR4 amplicon from four of our isolates of *T. foetus* were compared with *T. foetus* published GenBank accessions and no variation in this ITS region was identified.

The RAPD procedure generated significant variation in banding patterns, both between isolates and within repeats of the same isolate. Attempts to improve reproducibility of the amplification pattern through modification of the annealing temperature and primer concentration were not successful.
The McRAPD analysis generated consistent T_M data between isolates, and thus did not allow discrimination between isolates. Sequence analysis of the 500 bp RAPD product confirmed sequence homogeneity between the American Type Culture Collection strain 30003 and three Australian isolates.

4.1.3.3 *C. fetus* subsp. *venerealis* VIRULENCE GENE AMPLIFICATION

Bioinformatics

Appendix 2 describes the bioinformatics approaches used to prepare these analyses and also provides examples to demonstrate the database resources developed for this project by the CCG (Murdoch University).

Prior to the release of the *C. fetus* subsp. *fetus* genome data, 199 of the 1370 *C. fetus* subsp. *venerealis* predicted gene sequences did not match significantly (with an expectation value less than 1e-05) with any protein sequences in the global protein search. The *C. fetus* subsp. *venerealis* search against the global protein database search revealed 61 contig regions with significant matches outside the predicted *C. fetus* subsp. *venerealis* gene regions.

On the release of the *C. fetus* subsp. *fetus* sequence, re-analysis of the *C. fetus* subsp. *venerealis* gene sequences against global protein sequences was conducted. These results are summarised as:

- 17 *C. fetus* subsp. *venerealis* predicted genes with no protein hits with any known sequences.
- 10 *C. fetus* subsp. *venerealis* predicted genes with low significant protein alignments (>1e-05) with *C. fetus* subsp. *fetus.*
- 55 *C. fetus* subsp. *venerealis* predicted genes with low significant protein alignments (>1e-05) with non-*C. fetus* subsp. *fetus* proteins.

Therefore a total of 82 *C. fetus* subsp. *venerealis* predicted genes had no significant protein alignments to any species based on the expectant value of >1e-05.

1194 and 94 *C. fetus* subsp. *venerealis* predicted genes had significant alignments (<1e-05) to *C. fetus* subsp. *fetus* and non-*C. fetus* subsp. *fetus* proteins, respectively. Regions from 40 *C. fetus* subsp. *venerealis* contigs had significant matches outside predicted gene regions. Table 4.1.22 lists a number of putative virulence genes identified from these searches.

Table 4.1.22. A list of predicted *C. fetus* subsp. *venerealis* (Cfv) genes with significant matches to putative virulence genes

Virulence determinant	No. of Cfv genes	Description of most significant match	Species
Motility	7	flagellar basal-body rod proteins	*C. fetus* subsp. *fetus*
	1	flagellar biosynthesis protein	*C. fetus* subsp. *fetus*
	2	flagellar export protein	*C. fetus* subsp. *fetus*
	5	Flagellar hook & hook associated proteins	*C. fetus* subsp. *fetus*
	6	flagellar ring proteins	*C. fetus* subsp. *fetus*
	6	flagellar motor switch proteins	*C. fetus* subsp. *fetus*
	5	Flagellins	*C. fetus* subsp. *fetus*
	2	Other flagellar proteins	*C. fetus* subsp. *fetus*
Two component system	7	Response regulator	*C. fetus* subsp. *fetus*
	10	Sensor histidine kinase	*C. fetus* subsp. *fetus*
Cytolethal distending toxin	2	Toxin subunit A	*C. fetus* subsp. *fetus*
	1	Toxin subunit B	*C. fetus* subsp. *fetus*
	2	Toxin A/C family	*C. fetus* subsp. *fetus*
Chemotaxis	3	Chemotaxis proteins CheA, CheV, CheW	*C. fetus* subsp. *fetus*
	1	Chemotaxis regulatory protein	*C. coli*
	7	Methyl accepting chemotaxis proteins	*C. fetus* subsp. *fetus*
haemolysin	3	Haemolysin secretion/activation protein	*C. fetus* subsp. *fetus*
Iron transport and metabolism	6	Iron transporter proteins	*C. fetus* subsp. *fetus*
	4	Ferric receptors	*C. fetus* subsp. *fetus*
	5	Iron-sulfur cluster-binding proteins	*C. fetus* subsp. *fetus*
	3	Iron permeases	*C. fetus* subsp. *fetus*
	2	Ferrochetalases	*C. fetus* subsp. *fetus*
Type IV secretion systems	2	Type IV secretion system proteins VirB4	*C. upsaliensis*
	2	VirB6 Plasmid conjugal transfer proteins	*C. lari; C. upsaliensis*
	1	Type IV secretion system proteins VirB9	*C. coli*
	2	Type IV secretion system proteins VirB10	*C. lari*
	3	Type IV secretion system proteins VirB11	*C. upsaliensis; C.coli*
	1	Type IV secretion system proteins VirD4	*C. lari*
Adherence	4	Outer membrane proteins (OMP), outer membrane lipoproteins, membrane protein yebN	*C. fetus* subsp. *fetus*
	2	fibronectin type III domain protein; outer membrane fibronectin binding protein	*C. fetus* subsp. *fetus; C. jejuni*
	2	PEB1/amino acid ABC transporter	*C. jejuni*
ABC transporters efflux pumps	12	multi-drug efflux transporter, cation efflux family protein, macrolide-specific ABC-type efflux carrier, outer membrane efflux protein	*C. fetus* subsp. *fetus*

Separate alignments screening *C. fetus* subsp. *venerealis* genes with *C. fetus* subsp. *fetus* revealed that 1219 of the 1370 *C. fetus* subsp. *venerealis* genes aligned to 1059 of the 1720 *C. fetus* subsp. *fetus* proteins (<1e-05). In addition, 250 of the 273 *C. fetus* subsp. *venerealis* contigs aligned to 1165 of the 1720 *C. fetus* subsp. *fetus* protein sequences (<1e-05). Approximately 94 predicted genes had 74 significant predicted proteins identified as unique to the *C. fetus* subsp. *venerealis* sequence (not present in *C. fetus* subsp. *fetus* but with significant matches to other species, Appendix 4, Table 1). Five genes were selected for the development of new 5' *Taq* nuclease assays for the specific detection of *C. fetus* subsp. *venerealis*. These were designed as described previously using Primer Express Ver. 2 (AB) and are provided in Table 2 in Appendix 4 (assays not evaluated).

Assays for putative virulence genes

A selection of gene targets were chosen from the list in Table 4.1.22 and PCR assays were designed. For specific primer sequences, PCR annealing temperatures and amplicon sizes see Appendix 3. Available reference strains and field isolate extracts were screened in these assays and results are summarised in Table 4.1.23.

Two assays targeting Type IV secretion genes (Contigs1023 gene 1 and 733 gene 3) were specific for *C. fetus* subsp. *venerealis* Argentina strain which was the source of the *C. fetus* subsp. *venerealis* sequence data used here. Another assay based on a gene in contig 1023 amplified both *C. fetus* subsp. *venerealis* Argentina and *C. fetus* subsp. *venerealis* biovar *intermedius* only. An additional 4 assays targeting Type IV secretion systems amplified all *C. fetus* subsp. *venerealis* reference strains except for *C. fetus* subsp. *venerealis* biovar *intermedius.* The 2 *C. fetus* subsp. *fetus* reference strains did not amplify in Type IV secretion system assays. Interestingly, 2 assays targeting flagellin amplified *C. fetus* subsp. *venerealis* Argentina, *C. fetus* subsp. *venerealis* biovar *intermedius*, and the *C. fetus* subsp. *fetus* strains and not the 3 remaining *C. fetus* subsp. *venerealis* reference strains tested.

Table 4.1.23 Amplification of virulence genes in *C. fetus* subsp. *venerealis* real time PCR positive field smegma lysates

Virulence gene assays (*C. fetus* subsp. *venerealis* gene number)[3]	Reference strains							Field samples (n= 21-27)[2]		
	C. fetus subsp. *venerealis*[1]					*C. fetus* subsp *fetus*				
Nested PCR assays:	DPI	ATCC	Arg	biovar veneralis[4]	biovar intermedius[4]	DPI	ATCC	pos	Suspect[5]	Pos/ Total
Cytolethal distending toxin subunit A (927g1)	+	+	+	+	+	+	+	15	1	16/21
Cdt subunit C (927g2)	+	+	+	+	+	+	+	13	4	17/27
Cdt subunit B (927g3)	+	+	+	+	+	+	+	7	2	9/21
Iron uptake ABC transport system (1095g2)	+	+	+	+	+	+	+	25	0	25/27
ABC transporter multidrug efflux pump (1172g12)	+	+	+	+	+	+	+	0	4	4/27
Type IV secretion system VirB6 (875g2)	+	+	+	+	-	-	-	18	3	21/27
Type IV secretion system VirB10 (1023g1)	-	-	+	-	-	-	-	0	0	0/27
Type IV secretion system VirB9 (1165g2)	+	+	+	+	-	-	-	20	0	21/24
Type IV secretion system VirD4 (1165g6)	+	+	+	+	-	-	-	18	2	20/24
Histidine kinase (1083g1)	+	+	+	+	+	+	+	18	0	18/27
Sensor histidine kinase (1047g1)	+	+	+	+	+	+	+	18	0	18/27
Two-component sensor (1034g7)	+	+	+	+	+	+	+	19	4	23/27
Two- component sensor (1040g1)	+	+	+	+	+	+	+	16	6	22/27
Regulator (995g4)	+	+	+	+	+	+	+	2	3	5/27
Flagellar export protein (1172g7)	+	+	+	+	+	+	+	7	1	8/27
FlhF (1013g1)	+	+	+	+	+	+	+	7	0	7/27
C. fetus subsp. *fetus* FlhA (ZP01072457.1)	+	+	+	+	+	+	+	4	2	6/27
Chemotaxis protein (1179g4)	+	+	+	+	+	+	+	10	2	12/27
Single PCR assays (not nested):										
Type IV secretion system VirB4 (1120g2)	+	+	+	+	-	-	-	6	1	7/27
Type IV secretion system VirB4 (733g3)	-	-	+	-	-	-	-	ND	ND	ND
Type IV secretion system VirB11 (1023g2)	-	-	+	-	+	-	-	ND	ND	ND
FlgH – flagellar L-ring protein (878g1)	+	+	+	+	+	+	+	1	3	4/27
Flagellin (1155g3)	-	-	+	-	+	+	+	0	0	0/21
Flagellin (1155g2)	-	-	+	-	+	+	+	ND	ND	ND
FlhB (999)	+	+	+	-	-	+	+	0	2	2/21
2 component regulator (1034g9)	+	+	+	+	+	+	+	2	8	10/21
Response regulator (1179g1)	F+	ND	F+	ND	ND	ND	ND	5	2	7/21
Sensor (995g5)	+	+	+	+	+	+	+	14	2	16/27
Haemolysin activation (1037g1)	+	+	+	+	+	+	+	0	3	3/27
Outer membrane protein (988g1)	+	F+	+	+	+	+	+	1	0	1/23
Membrane protein (1006g4)	+	+	+	+	+	+	+	3	2	5/23
Outer membrane protein (1143g5)	+	F+	+	Vf+	+	+	+	ND	ND	ND
Fibronectin type III domain protein (992g5)	+	+	+	+	+	+	+	2	0	2/23

TABLE LEGEND

[1] All negative control DNA stocks tested: *C. coli*, *C. jejuni* subsp. *jejuni*, *C. hyointestinalis*, *C. sputorum* subsp. *bubulus*, *Pseudomonas aeruginosa, Proteus vulgaris*, *Neospora caninum*, *T. foetus* - were negative in each of the assays.

[2] 21-27 field isolates were tested as stocks were depleted during the course of these experiments.

[3] Primer sequences in Appendix 3

[4] Pfizer *C. fetus* subsp. *venerealis* vaccine strains

[5] Suspect positive results describe very faint positive bands

For most assays the amplification of field isolates improved in the nested format. Assays which targeted two-component sensor (or histidine kinase) genes appeared to amplify field isolates more successfully than two-component regulator-based assays. Assays which had poor success amplifying field isolates were based on the following genes: haemolysin activation protein, flagellar proteins, response regulators, ABC transporter multidrug efflux pump and cytolethal distending toxin subunit genes.

Not one field isolate amplified successfully in all of the nested assays (n=17, excluding the nested assay specific for *C. fetus* subsp. *venerealis* Argentina), however the number of genes and the specific genes which amplified varied greatly for each isolate. In summary only one isolate amplified in 16 of the 17 assays; 2 isolates – 14/17; 3 isolates - 13/17; 4 isolates - 11/17; 4 isolates - 10/17; 4 isolates - 9/17; 3 isolates - 8/17; 4 isolates - 7/17 and 2 isolates - 5/17 (results not shown). It was thought perhaps that some of the extracts which demonstrated low PCR assay reactivity may have degraded however strong positive results in other assays discounted this possibility.

4.1.4 Discussion

4.1.4.1 ASSAY DEVELOPMENT Stage 1

Real time PCR-based techniques such as 5' *Taq* nuclease assays have been applied for the clinical diagnosis of a wide range of pathogens from a variety of sources, including *C. jejuni* from human stools (85, 124) and *T. vaginalis* from female genital secretions (88). These assays provide improvements in sensitivity and specificity compared to selective culture and direct microscopic examination, and diagnoses can be obtained in significantly less time. These techniques are also less labour intensive than conventional PCR-based assays as there is no need for electrophoretic analysis and the use of specific probes offers improved assay sensitivity and specificity (178). MGB probe technology can provide better specificity and sensitivity compared with common dual labeled probes utilized in these assays (95, 102). Real time assays can also provide quantitative measures of target organisms providing useful tools to clinicians and diagnosticians (51, 107, 160). The assays described here are the first real time probe based PCR assays developed for the specific detection and quantification of *C. fetus* subsp. *venerealis* and *T. foetus* to improve the identification of campylobacteriosis and trichomoniasis in bull carriers, respectively.

The 'gold standard' diagnostic test for trichomoniasis in either male or female cattle is the cultivation of live organisms from smegma or cervicovaginal mucus until parasites propagate to detectable numbers to allow the recognition of *T. foetus* organisms microscopically (39, 138). Traditional culture/microscopy diagnosis is more effective for smegma (81-91%) than mucus (58-75%) and PCR methods have been developed to differentiate *T. foetus* from non-pathogenic trichomonads (21, 24, 39, 62, 64, 105, 129, 138, 149, 150). Increased specificity and sensitivity for the detection of *T. foetus* infection in cattle will improve both the accuracy of prevalence studies and the understanding of disease epidemiology, thereby improving disease management practices (63, 101, 134).

The 5' *Taq* nuclease assay for *T. foetus* described here is based on the ITS-1 region shown previously to be conserved and reliable as a target for conventional PCR (41, 62, 79, 128). Previous reports using the Felleisen et al (62) *T. foetus* PCR assay have described the amplification of non-specific fragments (17). The ITS-1 based 5' *Taq* nuclease assay described here proved specific and robust with no non-specific reactions. The *in silico* matches of the primers and probe with ITS-1 sequences of *T. suis* and *T. mobilensis* are consistent with the current phylogenetic understanding of the Tritrichomonadinae subfamily which indicate that these organisms belong to the same species (63, 91, 162). The Felleisen (62) assay has also been

adapted to detect *T. foetus* in cat faeces (75), further demonstrating the specificity of this target to detect this species from a range of clinical sources.

Sensitivity of detection was markedly improved using the *Taq* nuclease assay compared to culture and microscopy, with the reliable detection of a single organism equivalent per assay from smegma, cervico-vaginal mucus or urine. Our observations concluded that *T. foetus* loses viability in culture, due to the absence of visible motile trichomonads in InPouch™ TF test kits within a few hours of inoculation. The reason for this death is unclear, although isolate-specific antimicrobial susceptibility may play a significant role. The low sensitivity of microscopic examination following selective culture indicates that this is not the most effective diagnostic tool for *T. foetus* infection, leading to false negative results. For example, a sheath scrape from an infected bull is reported to contain an average of 141 organisms/ml (120), which is well below the sensitivity limits of selective culture/microscopy. In addition, the presence of non-*T. foetus* trichomonads cultured from the preputial mucosa of bulls can confound positive identification of *T. foetus* (77). The poor sensitivity of microscopy following selective culture was confirmed in our field study where only 3 of the 14 infected animals that were detected by 5' *Taq* nuclease assay detected as positive by culture/microscopy. This study clearly demonstrates that PCR direct detection from clinical specimens is more sensitive compared with PCR following culture enrichment.

The 'gold standard' detection of *C. fetus* subsp. *venerealis* is the selective isolation of *Campylobacter* colonies from the transport medium. The 5' *Taq* nuclease assay provided several significant improvements over conventional culture diagnostic methods. Approximately one single target cell was sufficient for a positive result from smegma or cervico-vaginal mucus whereas culture-based diagnosis to isolate *Campylobacter*-like colonies required a minimum of 1000 cells. A method which can withstand prolonged transport conditions is critical for the sampling of animals from extensively grazed cattle regions. Prolonged transport results in poor culture isolation of *C. fetus* subsp. *venerealis* and it is recommended that for successful culture samples be transported for only up to 48 hrs prior to sub-culture onto selective medium (34). The slow growth and fastidious nutritional requirements of *C. fetus* subsp. *venerealis* allows rapid overgrowth by more vigorously multiplying contaminating organisms. The organism also maintains limited viability under normal levels of atmospheric oxygen, limiting its survival during transport (35). In addition, anti-microbial susceptibility varies between isolates of *C. fetus* subsp. *venerealis*, with a majority of isolates showing susceptibility to polymyxin B, which is used in most *C. fetus* selective media (87). The decline in quantitative 5' *Taq* nuclease assay estimates of *C. fetus* subsp. *venerealis* numbers during 5 days of simulated transport in modified Weybridge media illustrates the impact of these factors on the subsequent isolation of the pathogen. Therefore, these factors reduce the effectiveness of culture-based diagnosis, leading to false negative results for infected animals as confirmed by our field investigation.

The assay designed here was based on a sub-species specific PCR target shown to previously identify *C. fetus* subsp. *venerealis* successfully differentiating 99 strains of *C. fetus* subspecies (83). The sequence from this fragment is homologous to *Wollinella succinogenes* ParA protein which is associated with DNA segregation during prokaryotic cell division and appears to originate from a plasmid (54). Evidence for the origin of the *parA* sequence in *C. fetus* subsp. *venerealis* is currently unknown and a preliminary investigation indicates that it is perhaps not plasmid-borne (179). The same group in Europe reported that Hum PCR positivity did not match phenotypic discrimination of these 2 subspecies describing the isolation of 18 *C. fetus* subsp. *venerealis* which are negative in this assay (179). If this were the case here in Australia, our assay would have underestimated the presence of the pathogen. Nevertheless, evidence prior to the commencement of this research indicated that this PCR target was suitable for the sub-

species identification of *C. fetus* subsp. *venerealis* in Australia (121, 172), and was therefore considered a suitable target for the development of the 5' *Taq* nuclease assay described here. Phenotypic discrimination between *C. fetus* subsp. *fetus* and *C. fetus* subsp. *venerealis* can be determined using biochemical assays including H_2S, selenite reduction, growth at 42^oC, susceptibility to metronidazole and cefoperazone, basic fuchsin, $KMnO_4$ and glycine tolerance with the latter being the OIE recommended assay (99, 126). However, doubts for the stability of these markers have been noted (28, 29, 126, 170). Additionally, although the biochemical H_2S test is described to differentiate *C. fetus* subsp. *fetus* (positive) and *C. fetus* subsp. *venerealis* (negative), a strain subsequently named *C. fetus* subsp. *venerealis* biovar *intermedius* is positive in this assay (173). Our *Taq* nuclease assay detected all *C. fetus* subsp. *venerealis* reference strains tested here including *C. fetus* subsp. *venerealis* biovar *intermedius*. The inherent difficulty in isolating pure *C. fetus* subsp. *venerealis* colonies from the clinical specimens in this study did not allow us to confirm the biochemical phenotype of the *Taq* assay positive extracts. However, it may be pre-emptive to investigate new subspecies assays and given the release of *C. fetus* subsp. *fetus* and *C. fetus* subsp. *venerealis* genome data (incomplete), this is now possible (see later discussion).

Heat lysis techniques have been successfully applied for the isolation of template DNA from diagnostic specimens and thus offer considerable time and labour savings for the routine application of DNA based diagnostics (94, 103, 116, 133, 183). Although crude sample processing does not remove all potential PCR inhibitory substances, 5' *Taq* nuclease assays appear to be more robust enabling successful amplification of target material as demonstrated in this study in comparison with the traditional PCR methods used (62, 83). This also simplifies requirements for transport from the field to the laboratory, without the need for complex transport enrichment media.

Previous studies have demonstrated the bull rasper as an effective tool for the collection of venereal samples for diagnosis (164). The Argentinean 'raspadors' proved to be the best sampling tool for collecting both smegma and mucus and this tool is now being produced by DPI&F and are available for distribution. The animal ethics representatives specified that the term 'raspador' was not suitable and thus the group adopted the name 'tricamper'. By comparing quantitative 5' *Taq* nuclease assay results, we were able to confirm that specimens collected from infected bulls using the tricamper yielded higher estimates of *C. fetus* subsp. *venerealis* cells than other collection tools. This was also confirmed by a higher success of positive culture from some specimens. In addition, the tricamper led to marked improvements in the ease of specimen collection from both male and female cattle. Ease of use for the veterinarian, combined with improved specimen quality and no notable adverse impact upon the animal provided evidence to support the preferred use of the tricamper for the collection of genital specimens from cattle for the diagnosis of campylobacteriosis by either selective culture or 5' *Taq* nuclease assay. The samples collected using this tool were thus also used in trichomoniasis screening in this study.

4.1.4.2 FIELD SAMPLING Stage 2

Field sampling provided further evidence to support the effectiveness of the *Taq* nuclease assays over conventional culture and microscopy for the detection of *C. fetus* subsp. *venerealis* and *T. foetus* in clinical specimens. For *C. fetus subsp. venerealis* a higher correlation of culture with *Taq* nuclease results was obtained due to the higher detection rate of this pathogen compared with *T. foetus*. The identification using ELISA of female cattle positive for *C. fetus* subsp. *venerealis* antigens but bearing no detectible infection by selective culture or *Taq* infection supports the observation that females are likely to spontaneously clear an infection within a few months. This IgA immune response, detected by the ELISA, can persist for up to 10 months

after infection, long after the infection has been eliminated (84). The presence of infected cattle was evident in six of seven vaccinated properties. This may be due to a failure of the vaccine to elicit an effective immune response, or that producers may be using an efficacious vaccine in an ineffective manner, or that we have detected *C. fetus* subsp. *venerealis* organisms which are colonising pre-exposed and immune animals but which is not causing disease. The ELISA is unsuitable for use in diagnosis of bulls, due to a lack of sufficient antibody titres in preputial fluids (180). A relatively lower number of *T. foetus* positive results were observed possibly due to season or a low prevalence of *T. foetus.*

The 5' *Taq* nuclease assays described here suffered only minor inhibition in the presence of urine following crude cell lysis, and no significant loss of sensitivity or specificity in the presence of smegma or mucus, including specimens contaminated with the blood, faeces or semen as observed during this study. Urine is commonly used for the diagnosis of human venereal diseases in males but is less suitable for similar diagnoses in females (98, 145). Laboratory-spiked urine specimens were suitable for 5' *Taq* nuclease assay, but the suitability of urine as a clinical specimen proved less satisfactory than smegma during field testing. Similarly for mucus, although both organisms can be reliably detected in spiked laboratory studies, it is not possible to effectively screen female cattle given the fact that female cattle can clear the pathogens within 6-12 weeks (15). We have confirmed that preputial smegma collected from bulls continues to be the recommended most reliable clinical sample for the specific diagnosis of both *C. fetus* subsp. *venerealis* and *T. foetus* (15).

4.1.4.3 MULTICENTRE EVALUATION Stage 2

The multicentre evaluations (DPI&F, DPI-Vic, DPI-NSW) of the *C. fetus* subsp. *venerealis* and *T. foetus* assays, using the same diagnostic specimens, were successful with good correlation between laboratories. DAWA has previously undertaken a comparison of our *C. fetus* subsp. *venerealis Taq* nuclease assay with their SYBR green *C. fetus* subsp*. venerealis* assay (also based on the Hum PCR target) which demonstrated a higher sensitivity of our method (results not shown). Probe-based *Taq* nuclease assays are more specific than SYBR green assays (DNA calating dye requiring melting curve analysis to confirm the presence of target amplicon) and are best for routine application. DAWA will continue to use their SYBR green developed *Taq* assays (for *T. foetus* and *C. fetus* subsp. *venerealis*) until such time that animal diagnostic laboratory standards recommend an alternative. Our *Taq* assays for *T. foetus* and *C. fetus* subsp. *venerealis* have been adopted for routine application by the DPI&F's Biosecurity diagnostic testing laboratory at Yeerongpilly and are in the process of acquiring NATA accreditation. DPI-NSW has similarly adopted these assays.

4.1.4.4 CASE STUDIES Stage 3

The dynamics of *C. fetus* subsp. *venerealis* and *T. foetus* infections are clearly poorly understood. We selected 2 case study groups based on the identification of *C. fetus* subsp. *venerealis* and *T. foetus* during the field sampling stage of this project. However, both pathogens were present in both groups as confirmed during the course of the research.

Questions arising from Case Study 1 (Site A) include: Do bulls spontaneously clear *C. fetus* subsp. *venerealis* infections? Or, How did bulls acquire *T. foetus* infection during mating in a seasonally-mated herd with reasonable bull control, is it from long-term infection in cows? Despite raising new questions, this case study did demonstrate the expected effect of *C. fetus* subsp. *venerealis* infection on pregnancy status, ie, non-pregnancy. *Leptospira* spp. infection is associated with late-term abortion and weak calves, and thus a high pregnancy rate in *Leptospira* infected animals was not surprising.

Vaccination did not appear to eliminate *C. fetus* subsp. *venerealis* as indicated by the vaccine manufacturer. At this stage, there is no evidence of how efficacious the vaccine is in preventing clinical disease and this is a definite area of future research needing development.
The 2nd case study revealed that conceptions seem to be delayed and overall pregnancy% low in the first calf cow group and the home-bred heifers. This may be due to any combination of the following: disease causing abortions; delayed calving patterns for the previous calf for the first calf cows (we did not know the date of calving from the first mating); genotype (home-bred vs bought); or differences in nutrition between paddocks. As the mating groups were not controlled it was not possible to distinguish between these possible causes. There was no evidence that *T. foetus* infection in females had affected overall pregnancy %.

The spread of both *C. fetus* subsp. *venerealis* and *T. foetus* is known to be primarily venereal; this appears to include homosexual behaviour in bulls as previously reported (40, 130). It was previously thought that the incidence of both *C. fetus* subsp. *venerealis* and *T. foetus* was very low in bulls less than 4 years of age. Our studies using the *Taq* nuclease assays indicate that *C. fetus* subsp. *venerealis* infects bulls that are as young as 2 years of age, however perhaps the infection is more readily cleared from younger bulls as opposed to older bulls. There was no evidence of *T. foetus* in young bulls.

While the new *Taq* assays demonstrate significant improvements in sensitivity and specificity compared to conventional diagnostic culture, it is not known whether the detection of these target organisms indicates the potential for the development of clinical disease. As yet, we do not have sufficient information to indicate what factors trigger an asymptomatic infection to become clinical disease, nor do we know if the number of cells/ml estimated in a quantitative assay can predict clinical disease outcome. We have not identified the reservoir infecting young bulls with *C. fetus* subsp. *venerealis*, or whether *C. fetus* subsp. *venerealis* vaccination is an effective management practice (not in the scope of this study). We also considered that perhaps the *C. fetus* subsp. *venerealis* isolates detected may vary in virulence and for this reason we developed an additional milestone (see later discussion). This may help explain the widespread presence of the organisms consisting of strains perhaps of lowered virulence and pathogenicity. In addition, recent data confirming the uncertainty in using both biochemical and PCR methods to categorically identify venereal disease causing *C. fetus* subspecies (170, 179), has further strongly suggested that further study into subspecies differences is needed before a definitive method for detecting these pathogens can be devised.

These studies did indicate that the presence of both pathogens within herds can fluctuate and as the studies were limited it was difficult to determine relative reproductive failures due to the presence pathogens. Some of the issues encountered included:

- poor control of bulls and property barriers (and possible sources of infections),
- limited history of *Taq* nuclease positive status of all bulls and cows,
- uncertain knowledge in regard to *C. fetus* subsp. *venerealis* immunity of cows (previous exposure can provide immunity to subsequent infections),
- timing of sampling i.e. it may be best to sample animals weekly rather than at 4 or 6 monthly intervals, particularly in cows, who can clear these pathogens quickly (15).

There is a great deal of evidence that the presence of these pathogens in a herd does lead to reproductive failure as determined by infections and diagnosis using culture and microscopy detection. The diseases predominate in extensive cattle areas of northern Australia and in most regions that rely on extensive herd management and natural breeding including areas of USA, Canada, Spain, Africa, Asia and Latin America (10, 15, 16, 42, 60, 76, 96, 109, 112, 132, 135, 163). Thus it is evident that even though vaccines for *C. fetus* subsp. *venerealis* are used (36, 41), there is still widespread presence of the organisms and the resulting variable reproductive

problems indicate that either unknown pathogen reservoirs are transmitting the disease to cattle, or that low levels of pathogens are not being eliminated and can still cause disease. A vaccine for *T. foetus* is not available in Australia, however animals appear to naturally clear the pathogen (sooner than for campylobacteriosis), and less positive animals were detected in our studies.
These case studies were not intended to emulate controlled trials but have indicated that a balance between infection timing and herd immunity is important in forecasting the impact of *T. foetus* or *C. fetus* subsp. *venerealis* positive bulls on the reproductive performance of infected herds. Large 'controlled' infection trials are required to fully understand the dynamics of these pathogens in extensively grazed herds. Nonetheless, both pathogens were present in both case studies and thus the potential for disease and poor reproductive rates continues to be a threat for producers. These methods will be important tools for future studies under controlled conditions. Widespread adoption of these tests both nationally and internationally will also contribute to our understanding of the epidemiology of these pathogens and their relative impacts on bovine production.

4.1.4.5 Genotyping

The application and/or development of genotyping tools to study *C. fetus* subsp. *venerealis* and *T. foetus* field isolates were not successful. Although only a few *T. foetus* isolates were available for the investigation of these methods, the results confirmed that the species is highly conserved and the methods used did not discriminate Australian isolates from the American reference strain. The conservation of this species is congruent with observations in the related species *T. vaginalis*, where differences can be demonstrated using pulsed field electrophoresis analysis rather than at the individual gene level (167). Application of PFGE is not feasible given the difficulty encountered in culturing the field *T. foetus* isolates to levels required for such analyses. Thus until *T. foetus* genome data is available, the current available knowledge is insufficient for the development of a useful genotyping method.

Although methods such as amplified fragment length polymorphism (AFLP) have been developed to differentiate *C. fetus* isolates (170), this method also cannot be applied to field samples. Inherent difficulty experienced in isolating pure colonies of *C. fetus* subsp. *venerealis* from our field lysates undermines the usefulness of techniques such as AFLP. We developed a FRET assay targeting sequence differences in *C. fetus* subsp. *venerealis* ISR regions which failed to produce consistent melting profiles when applied to the field preparations. FRET assays are capable of identifying point mutations in genes of interest as demonstrated with the FRET assay for *Helicobacter pylori* gyrA gene (74), however it is likely that pure DNA preparations are also required for the success of these assays as contaminating DNA would interfere with the melting point analyses.

The application of AFLP and PFGE methods to study isolates of *C. fetus* have demonstrated that the genetic diversity in this subspecies is limited (170, 171). This is contrary to the observations in related *Campylobacter* species such as *C. jejuni*, *C. coli*, *C. lari* and *C. upsaliensis*, which demonstrate greater diversity in AFLP patterns within these species compared to *C. fetus* (55). The clonal nature of *C. fetus* has been further confirmed by multilocus sequence typing (169), indicating that at present development of *C. fetus* subsp. *venerealis* specific genotyping tools may also not be possible.

4.1.4.6 *C. fetus* subsp. *venerealis* virulence gene amplification

Regardless of the fact that there are concerns that the currently available tools used to differentiate the *C. fetus* subspecies are inadequate (170, 179), studies to identify the presence of virulence genes in *C. fetus* subsp. *venerealis* have also not been undertaken to date. We

capitalised on the recent availability of incomplete genome data for both *C. fetus* subsp. *venerealis* and *C. fetus* subsp. *fetus* and developed a number of assays targeting virulence factors previously identified in *C. jejuni, C. coli*, *C. lari* and *C. upsaliensis* genomes. The pathogenic mechanisms responsible for acute intestinal infections by *Campylobacter*, although poorly understood, are thought to involve adherence, cellular invasion, and toxin production (66). Motility is a major factor implicated during invasion and flagellar export systems have also been implicated in the secretion of bacterial and *Campylobacter* virulence factors (182). Carrillo et al (2004) provided compelling evidence that *flhA* in *C. jejuni* was responsible for the coordination of the regulation of late flagellar genes and or virulence factors in this organism i.e. cytolethal distending toxin, flagellin, two-component regulators, chemotaxis proteins, flagellar basal rod proteins (25). A homologue for *flhA* was not identified in the available *C. fetus* subsp. *venerealis* data, thus we designed an assay based on *C. fetus* subsp. *fetus flhA*. Although *flhA* was amplified in all *C. fetus* subsp. *venerealis* and *C. fetus* subsp. *fetus* reference strains, few positives were detected in the field extracts. Motility and chemotaxis genes have been found to be conserved among related *Campylobacter* species (66). Other flagellar genes amplified poorly in the majority of field isolates and one chemotaxis gene assay amplified approximately half of the field isolates.

Colonization of bacteria is a multifactorial process involving adaptation of the bacterium to different microenvironments. Two-component regulatory (TCR) systems are commonly used by bacteria to respond to specific environmental signals such as temperature (19). These systems depend on two families of proteins, the sensory histidine kinases and response regulators, which cooperate to transmit environmental signals to bacterial response machinery (19). Five TCR systems (pairs of adjacent histidine kinase and response regulator genes) have been identified as conserved across *Campylobacter* species (66). The high degree of conservation of ORFs suggests an importance in *Campylobacter* pathogenicity due to the likely exposure to temperature stress during the infectious process (66). We assayed a number of TCR genes and the field isolates screened appeared to have a high prevalence of the sensor genes compared with the regulator genes. The significance of this is unknown however both genes are required to elicit the appropriate survival response.

Adherence of other *Campylobacter* species to epithelial cells is mediated by multiple adhesins including CadF, PEB1, JlpA and a 43-kDa major outer membrane protein (66). *C. fetus* subsp. *venerealis* homologues for all of these genes were not identified indicating that perhaps different genes are involved in adherence to epithelial cells in the genitalia as opposed to intestinal cells common for other *Campylobacter* or that contigs containing these sequences are yet to be sequenced (only ~72% of *C. fetus* subsp. *venerealis* genome has been sequenced). Fibronectin (FN)-binding proteins have been found to be conserved among *Campylobacter* spp. and are also implicated in epithelial attachment (117). Field isolates amplified poorly in assays targeting fibronectin and outer membrane proteins however, we are not certain that the homologous *C. fetus* genes used to develop our assays were the most relevant genes potentially implicated in adhesion.

The three cytolethal distending toxin (*cdt*) subunits A, B and C are conserved across the four *Campylobacter* species (*C. jejuni, C.lari, C. coli, C. upsaliensis*) (66) and *C. fetus* (this study). Field isolates amplified moderately in these assays indicating that *cdt* production is not conserved in all of the field isolates.

C. coli, C. lari, C. upsaliensis and *C. jejuni* are known to harbour plasmids and evidence suggests that plasmids can play a role in pathogenesis (5, 66). One noted similarity in the list of genes absent in *C. fetus* subsp. *fetus* but present in *C. fetus* subsp. *venerealis* (Appendix 4) is that many of them are homologous for genes present on the plasmids of related *Campylobacter*.

This includes the Type IV secretory genes which can be involved in conjugative plasmid transfer or the secretion of virulence factors (32) and these were not detected in *C. fetus* subsp. *fetus* and *C. fetus* subsp. *venerealis* biovar *intermedius*. Two Type IV secretory genes were unique to *C. fetus* subsp. *venerealis* Argentina and one was unique to both *C. fetus* subsp. *venerealis* Argentina and *C. fetus* subsp. *venerealis* biovar *intermedius*. Two nested assays for 2 of Type IV secretory genes (VirB6 and VirB9) were specific for *C. fetus* subsp. *venerealis* excluding biovar *intermedius* and also subsequently amplified most of the field isolates tested.

C. fetus subsp. *venerealis* biovar *intermedius* is positive in the H_2S test usually negative for strains of *C. fetus* subsp. *venerealis* (173). Other than this difference, diagnosis, isolation and control follow that described for *C. fetus* subsp. *venerealis* even though it is not evident whether this biovar occurs only in the genital tract or in both the genital and intestinal tracts (22, 69). Currently the VibroVax vaccine (Pfizer) contains both *C. fetus* subsp. *venerealis* and *C. fetus* subsp. *venerealis* biovar *intermedius* (strains used for assay screening in this study). The Hum PCR assay and the assay we developed for the same gene target both amplify both strains/biovars (83, 113) yet a recently developed PCR assay (based on an AFLP fragment) is specific for *C. fetus* subsp. *venerealis* but does not amplify biovar *intermedius* (170). AFLP analysis differentiates *C. fetus* subsp. *fetus*, *C. fetus* subsp. *venerealis* and *C. fetus* subsp. *venerealis* biovar *intermedius*, whereas MLST typing does not (169, 170). In regards to the virulence gene assays used here, *C. fetus* subsp. *venerealis* biovar *intermedius* appeared to react similarly to *C. fetus* subsp. *fetus* rather than *C. fetus* subsp. *venerealis*.
It is thus evident that assays which react differently between subspecies and biovars may not be suitable as 'virulence gene' screening assays given that the basis of genetic differences between these *C. fetus* strains is currently uncertain. We also only included one *C. fetus* subsp. *venerealis* biovar *intermedius* isolate in our analyses and thus it is evident that wider screening of these assays with an increased number of references strains for all subspecies and biovars would be beneficial. Nevertheless, we identified a number of genes which may be specific to *C. fetus* subsp. *venerealis* (not including biovar *intermedius*) and virulence genes which are conserved in *C. fetus* were not detected in all of the field isolates. Thus it is feasible that less characterised and perhaps less pathogenic isolates of *C. fetus* subsp. *venerealis*-like organisms occupy the bull prepuce, complicating both the phylogeny and epidemiology of this pathogen and venereal disease, respectively.

4.1.5 Conclusions

- Increased sensitivity and specificity in the direct detection of both *T. foetus* and *C. fetus* subsp. *venerealis* was observed using the 5' *Taq* nuclease (TaqMan®MGB) assays developed and optimised in this research program.
- Bull smegma continues to be the preferred clinical specimen for the detection of these pathogens without the need for enrichment medium transport.
- Crude cell extracts prepared from clinical specimens is the preferred sample processing method for 5' *Taq* nuclease screening.
- The impact of trichomoniasis as detected by *Taq* nuclease assay was negligible with no recognisable impact on pregnancy rates.
- Presence of *C. fetus* subsp. *venerealis* appeared to be related to a decrease in pregnancy rates.
- Repetitive sampling and carefully controlled trials are required to confirm the above observations.
- A larger proportion of *C. fetus* subsp. *venerealis* -*Taq* assay positive results prompted the investigation of putative virulence genes leading to preliminary evidence for the presence of less pathogenic strains in the prepuce of bulls.

- The sensitive detection of these pathogens has revealed some gaps in our current knowledge of venereal disease epidemiology i.e. do young bulls readily clear venereal pathogens without treatment?

4.2 Bovine Ephemeral Fever

4.2.1 Introduction

The aim of this activity was to develop a probe based reverse transcriptase real time PCR assay (RT-*Taq* nuclease assay) for the specific detection of bovine ephemeral fever virus in RNA extracts prepared from blood.

4.2.2 Methods

Virus isolation. BEFV virus was grown in *Aedes albopictus* cells using MEM medium and 20% serum as previously described (168). To isolate virus from blood, the buffy coat is collected following the centrifugation of 10 ml EDTA blood and one wash in PBS. 0.1-0.2 ml of buffy coat was then added to a small culture flask containing seeded cells and medium, and cells were observed for cytopathic effect after 14 days incubation.

Conventional gel-based RT-PCR (Corney, unpublished). The 3 primer sequences are listed in Table 4.2.1 as glycoprotein gene conventional PCR assay. Two forward primers and one reverse primer are used in a semi-nested assay format. Reactions consisted of buffer and enzyme (Titan One Tube RT-PCR System, Roche Diagnostics), 400 μM DTT, 400 nM each primer, RNase Inhibitor (Roche) and 160 μM dNTPs mixed with RNeasy RNA templates (2μl) as described by the manufacturer. RNA transcription and cycling was undertaken in the Hybaid PCR Express Thermal Cycler using the following temperature profile: 50°C 30mins, 94°C 2 min, 10 cycles at 94°C 30s, 55C 30s, 68°C 1.5 min; 25 cycles at 94°C 30s, 55°C 30s 68°C 1.5min + 5s/cycle; final extension at 68°C 7min and hold at 4°C. PCR products were separated using standard agarose gel electrophoresis (ethidium bromide stained) and presence of positive bands were confirmed under UV illumination. Both virus isolation and the conventional RT-PCR were undertaken by DPI&F Biosecurity (Bruce Corney).

Taq nuclease RT-PCR development. Available BEFV sequences (GenBank accessions: NC002526, M94266, AF058325, AF058324, AF058323, AF058322, AF058321, AF234533) were aligned using a WEB ANGIS Pileup program and conserved regions determined. Primer and minor groove binder probe (MBG) sequences were then designed using Primer Express ver.2 and BLASTn searches undertaken to confirm sequence specificity. Two MGB *Taq* nuclease assays were initially designed, one targeting the conserved regions of the BEFV glycoprotein (G) gene and the other targeted the BEFV nucleoprotein (N), Table 4.2.1. A subsequent 2nd assay for the glycoprotein gene was designed (assay 2) following re-analysis of BEFV sequences (Biosecurity field isolate, unpublished) to confirm specificity to a range of BEFV sequences.

Optimal conditions for the BEFV Glycoprotein RT-*Taq* assay: 25 μL reaction volume with 200 nM each of primers and probe and 2μl of RNA prepared using RNeasy. The thermal profile is 50°C for 30 minutes, 95°C for 2 minutes, and 45 cycles of denaturation at 95°C for 20 s and annealing/extension at 60°C for 45 s. The assay was developed using the SuperScript III Platinum One-Step qRT-PCR System following conditions recommended by the manufacturer (Invitrogen).

Specificity and preparation of RNA templates. Control BEFV virus preparations were obtained from Bruce Corney (Biosecurity, DPI&F). Assay specificity was demonstrated using RNA prepared from the related ephemeroviruses (Berrimah, Adelaide River, and Kimberley) and from

other blood-borne pathogens, Table 4.2.2. RNA was prepared using RNeasy kits (QIAGEN) from 100μl whole blood following the manufacturer's instructions. This protocol followed the current method used in DPI&F's diagnostic laboratories to facilitate technology transfer of the new PCR method.

Table 4.2.1. Probes and primers used in *Taq* nuclease and conventional BEFV RT-PCR assays

Probe/primer	5'→3' sequence	Oligo type
Glycoprotein gene conventional RT-PCR assay:		
PA68F	CGG AAT GAT CTT CGT GGA ACC AAC	Forward primer
P787F	TTA CCA GAC TCG GGT AGA GTT TCC	Forward primer
P846R	TTG CAC CAA CAG CCC AAA TTG TCC	Reverse primer
Glycoprotein gene RT-*Taq* assay1:		
BEFVgF	TTT TAT CTg CTg TTg TAg gTT ggT	Forward primer
BEFVgR	AgC CCA AAT TgT CCA TCT TAC Tg	Reverse primer
BEFVgP	[1]**6FAM**- TAC AAg CAC ggC AAA	MGB probe
Glycoprotein gene RT-*Taq* assay2:		
BEFVgF2	TTT TAT CWg CTg TTg Tag gTT ggT	Forward primer
BEFVgR2	AAC AgC CCA AAT TgT CCA TCT T	Reverse primer
BEFVgP2	[1]**6FAM**-ACG GCA AAG GCA G	MGB probe
Nucleoprotein gene RT-*Taq* assay:		
BEFVnF	Agg ATg CCg CAg gAC TCA	Forward primer
BEFVnR	TCT CCA ATg gTC AAA CCA gTC A	Reverse primer
BEFVnP	[1]**6FAM**- CAT TAg gTC ATg CCT gTg AT	MGB probe

[1]The probe is labelled with 6-carboxyfluorescein (6-FAM) phoshoramidite as the 5' reporter dye and includes a non fluorescent quencher attached to the 3' minor groove binder moiety.

Table 4.2.2. Reference strains used to confirm specificity of RT-*Taq* nuclease assays for BEFV

Species	Source
BEFV	Biosecurity, DPI&F
Berrimah virus	Biosecurity, DPI&F
Adelaide River virus	Dept. of Business, Industry and Resource Development (NT)
Kimberley virus	Biosecurity, DPI&F
Aino virus	Biosecurity, DPI&F
Akabane virus	Biosecurity, DPI&F
mucosal disease virus	Biosecurity, DPI&F
infectious bovine rhinotracheitis (IBR) virus	Biosecurity, DPI&F
bovine leukemia virus	Biosecurity, DPI&F
Bovine enterovirus K2577	Biosecurity, DPI&F
Bovine enterovirus 66/27	Biosecurity, DPI&F
Bovine enterovirus SL305	Biosecurity, DPI&F
Anaplasma centrale	Tick Fever Centre, DPI&F
Leptospira borgpetersenii serovar Hardjobovis	Biosecurity, DPI&F
L. interrogans serovar Pomona	Biosecurity, DPI&F
Salmonella typhimurium	Biosecurity, DPI&F
Escherichia coli	Biosecurity, DPI&F
Babesia bovis	Tick Fever Centre, DPI&F

Assay Sensitivity. Assay sensitivity was determined using serial dilutions of viral RNA directly amplified in both the *Taq* nuclease and the conventional RT-PCR assays. Sensitivity was also determined by serially diluting fresh BEFV (from culture) in bovine blood and RNA was prepared from each dilution and subsequently tested in both assays.

Comparison of direct diagnostic methods. Sixty diagnostic samples (blood) were obtained from a BEFV viremia study to evaluate the *Taq* nuclease RT-PCR assay with conventional RT-PCR and virus isolation (culture) results previously undertaken to screen these samples. A further 131 RNA samples from DPI&F's diagnostic laboratory (Biosecurity) were screened (only 15 of these were previously cultured for virus isolation). All samples were tested in duplicate.

4.2.3 Results

The BEFV glycoprotein *Taq* assay 1 was 7-8 cycles more sensitive than the N protein gene *Taq* nuclease assay and thus was further developed and applied. The detection of all BEFV positive extracts by this assay was subsequently not successful and thus following sequence analysis (results not shown), we developed the BEFV glycoprotein *Taq* RT-PCR assay 2 which was the assay subsequently applied in this research.

Specificity of the *Taq* RT-PCR assay for BEFV was confirmed with negative results obtained for all related ephemeroviruses: Kimberley, Adelaide River and Berrimah virus RNeasy preparations and other blood borne viruses and pathogens listed in Table 4.2.2.

The *Taq* nuclease RT-PCR assay was approximately 10-100 times more sensitive than the conventional PCR assay detecting approximately 44pg of RNA (Table 4.2.3).

Results of *Taq* nuclease RT-PCR when screening diagnostic samples were compared with conventional RT-PCR and traditional virus isolation/culture (where available). Results are provided in detail in Appendix 5 and are summarised in Table 4.2.4 below.

Table 4.2.3. Comparison in assay sensitivity of BEFV *Taq* assay RT-PCR with the current conventional RT-PCR assay using RNA prepared from bovine blood spiked with serial dilutions of virus and with serial dilutions of RNA prepared from BEFV

Sample	Concentration	*Taq* RT-PCR C_T	Conventional RT-PCR[1]
Dilutions of virus in whole blood			
Virus undiluted		18.45	5+
Virus 10-1		21.00	5+
Virus 10-2		26.13	4+
Virus 10-3		29.39	1+
Virus 10-4		32.86	-
Virus 10-5		0.00	-
Virus 10-6		0.00	-
Virus neg (blood only)		0.00	-
Dilutions of BEFV RNA:			
RNeasy 10-1	22.17 ng/uL	29.39	4+
RNeasy 10-2	2.2 ng/uL	29.43	1+
RNeasy 10-3	220 pg/uL	32.67	-
RNeasy 10-4	22 pg/uL	35.74	-
RNeasy 10-5		0.00	-
RNeasy 10-6		0.00	-

[1]Intensity of bands in agarose gels were scored from 1+ (faint positive) up to 5+ for strong positives.

Table 4.2.4. Comparison of *Taq* nuclease RT-PCR with the conventional gel-based RT-PCR, summary of results presented in Appendix 5 (Tables 1&2)

Gel RT-PCR	5' *Taq* nuclease RT-PCR		**TOTALS**
	Negative	Positive	
Negative	63	0	**63**
Suspect	2	1	**3**
Positive	0	125	**125**
TOTALS	**65**	**126**	**191**

4.2.4 Discussion

The glycoprotein gene based *Taq* nuclease RT-PCR assay was more suitable as a gene target than the nucleoprotein gene. However, it was evident sequence variation was an issue in the first assay developed targeting the glycoprotein. The glycoprotein gene is more variable than the nucleoprotein gene (175), however, currently only 3 GenBank database sequences exist for this target, whereas 9 BEFV glycoprotein sequences were available to determine regions of conservation for the development of a diagnostic assay targeting this gene.

Results demonstrated an excellent correlation of the new glycoprotein gene *Taq* nuclease PCR compared with both virus isolation and the conventional (semi-nested) PCR. The *Taq* nuclease PCR demonstrated improved specificity eliminating false positive or suspect results. Furthermore BEFV specificity was demonstrated showing no amplification of related ephemeroviruses (47, 70, 71). This assay has been transferred to DPI&F biosecurity who are undertaking NATA accreditation of the assay.

Simultaneously while completing our research, reports describing the development of 2 new sensitive molecular assays for BEFV were published (81, 157). Stram *et al*. (157) developed a probe based real time assay similarly targeting the BEFV glycoprotein. The assay however was not evaluated against closely related ephemeroviruses and does not utilise MGB TaqMan® probe technology as described here. MGB TaqMan® probes have been demonstrated to be more specific and more sensitive than conventional dual-labelled probes (95, 102). Hsieh *et al*. (81), developed a nested PCR using a magnetic bead based probe assay also based on BEFV glycoprotein sequences. This study was limited to Taiwan BEFV strains and also did not screen related ephemeroviruses. Nevertheless the subsequent publication of the assay we developed here would require a detailed evaluation of these 2 published assays (not within the scope of our study).

4.2.5 Conclusions

- The 5' *Taq* nuclease (real time) RT-PCR developed has increased the specificity and sensitivity of bovine ephemeral fever virus detection compared with the conventional semi-nested RT-PCR assay and traditional culture virus isolation.

4.3 Leptospira

4.3.1 Introduction

The TaqMan® assay of Smythe *et al.* (155) was modified for use in diagnosing bovine leptospirosis using the Corbett RotorGene platform. This section of the report contains the modification details, development of sample handling and processing methods, and validation data for the TaqMan® assay. Validation included determinations of analytical specificity and sensitivity, comparison of the TaqMan® assay with culture using samples obtained from experimentally infected heifers and remote beef herds, and comparison of the TaqMan® assay performance in four Australian laboratories. Finally, the TaqMan® assay was applied to detailed studies of three beef herds with diminished reproductive performance.

4.3.2 Methods

4.3.1.1 ASSAY DEVELOPMENT – Stage 1

PCR

The TaqMan® assay of Smythe *et al*. (155) was modified by increasing the annealing and extension temperature from 60°C to 64°C to improve discrimination between negative and weak positive samples, and by extending the number of cycles from 40 to 50. The modified assay utilised 2 μl of template per 25 μl reaction, and used Platinum Quantitative PCR Super Mix-UDG (Invitrogen). Probe titrations showed that 50 nM was the optimum probe concentration for use on the RotorGene (results not shown). Otherwise, the assay was as described by Smythe *et al*. (2002). TaqMan® assays described in this report were performed using the modified TaqMan® assay. All samples were tested in duplicate and results were expressed as the mean threshold cycle (C_T).

Assay specificity -*Leptospira* and other organisms

L. interrogans serovar Pomona and *L. borgpetersenii* serovar Hardjo type hardjobovis were used for method development and determinations of analytical sensitivity. The *L. interrogans* serovar Pomona reference strain (Pomona) was obtained from the WHO/FAO/OIE Collaborating Centre for Reference and Research on Leptospirosis, Coopers Plains, Queensland, Australia. *L. borgpetersenii* serovar Hardjo type hardjobovis (93/94451-3) was isolated from a dairy herd in

north Queensland, Australia, and was stored in liquid nitrogen at the Biosecurity Sciences Laboratory, Yeerongpilly, Queensland, Australia.

The non-pathogenic *L. biflexa* serovar Patoc and DNA from eight pathogenic *Leptospira* were also obtained from the WHO/FAO/OIE Collaborating Centre for Reference and Research on Leptospirosis. These organisms were used in assessing the analytical specificity of the modified TaqMan® assay. Full details of these organisms are in Table 4.3.1.

Leptospira cultures were grown at 30°C in either Difco EMJH (Becton and Dickinson) supplemented with 0.9% w/v sodium pyruvate or in Bovuminar PLM-5 (Intergen Biomanufacturing Corporation).

Leptospira were counted as follows. One drop of formalin was added to 2 ml of a suitable dilution of culture in PBS (pH 7.4), and incubated for a minimum of 30 min at room temperature. The diluted culture was then loaded into a haemocytometer, and cells were counted using a dark field microscope.

Table 4.3.1. Origin of reference cultures and specificity of the *Leptospira* TaqMan® assay

Organism	Strain	Source[1]	TaqMan® Result (C_T)
Pathogenic *Leptospira*:			
L. interrogans			
Copenhageni	M20	WHO/FAO/OIE	19.6
Robinsoni	Robinson	WHO/FAO/OIE	19.8
Pomona	Pomona	WHO/FAO/OIE	18.2
Zanoni	Zanoni	WHO/FAO/OIE	19.0
L. borgpetersenii			
Hardjobovis	LT1186	WHO/FAO/OIE	18.6
	93/94451-3	BSL	N
Tarassovi	Perepelicin	WHO/FAO/OIE	19.1
Ballum	Mus 127	WHO/FAO/OIE	18.3
L. kirschneri			
Grippotyphosa	Moskva V	WHO/FAO/OIE	21.1
Saprophytic *Leptospira*:			
L. biflexa			
Patoc	Patoc 1	WHO/FAO/OIE	> 50
Other organisms:			
Tritrichomonas foetus	ATCC 30003	ATCC	> 50
	YVL-W	BSL	> 50
Campylobacter fetus subsp. *fetus*	ATCC 15296	ATCC	> 50
	98/118432	BSL	> 50
C. fetus subsp. *venerealis*	ATCC 19438	ATCC	> 50
	98/109383	BSL	> 50
Neospora caninum	ATCC 50843	ATCC	> 50
	50843	BSL	> 50
Actinomyces pyogenes	99/145243	BSL	> 50
Corynebacterium renale		BSL/IFM[2]	> 50
Escherichia coli	ATCC25922	ATCC	> 50
Streptococcus bovis	S112	BSL	> 50
Streptococcus faecalis	S161	BSL	> 50
Bovine herpesvirus type 1	IBR-1	BSL	> 50

[1]WHO/FAO/OIE, WHO/FAO/OIE Collaborating Centre for reference and Research on Leptospirosis, Coopers Plains, Queensland, Australia; BSL, Biosecurity Sciences Laboratory, Yeerongpilly, Queensland, Australia; N, not included in analytical specificity study; ATCC, American Type Culture Collection, Manassas, Virginia, USA; IFM, IFM Quality Services, Ingleburn, New South Wales, Australia
[2]This organism was isolated at BSL from material supplied by IFM as part of a veterinary bacteriology proficiency testing program.

Diagnostic samples

Fresh lung from a suspected case of leptospirosis in an alpaca was tested in the modified TaqMan® assay. Spirochaete-like objects had been observed in a silver-stained section from the lung during routine histological examination at the Toowoomba Veterinary Laboratory.

Sample preparation

Urine, vaginal mucus, preputial smegma and kidneys were used as samples for the TaqMan® assays.

Midstream urine samples were collected from the first or second void following either palpation or administration of the diuretic Frusemide (Troy Laboratories). Vaginal mucus and preputial smegma were collected into 5 ml of saline using a bull rasper as described in McMillen *et al*. (113).

Up to 5 ml of urine was centrifuged for 30 min at 2000 g. All but 0.5 ml of the supernatant was discarded. The pellet was resuspended in the remainder of the supernatant, and the suspension was centrifuged for 10 min at 4600 g in a microfuge. The pellet was resuspended in either 50 μl of sterile reverse-osmosis (RO)-purified water by vigorous pipetting or 180 μl of buffer ATL (QIAGEN).

Up to 1.5 ml of vaginal mucus or preputial smegma (in saline) was centrifuged for 10 min at 4600 g in a microfuge. The supernatant including mucus was removed and the pellet was resuspended in either 50 μl of sterile RO-purified water by vigorous pipetting or 180 μl of buffer ATL.

Suspensions in buffer ATL were processed using a QIAamp DNA Mini Kit (QIAGEN) as per the manufacturer's instructions. DNA was eluted from the QIAamp spin columns in 50 μl of QIAamp Buffer AE. Suspensions in water were processed using a heat lysis procedure (113). The suspensions were allowed to sit at room temperature for a minimum of 2 min, heated for 10 min at 95°C, then stored at -20°C. The lysates were briefly centrifuged at 15800 g in a microfuge, and the supernatants were tested in the TaqMan® assays.

Tissue samples (1-8 pieces of approximately 2 x 2 x 2 mm^3) were processed using a QIAamp DNA Mini Kit (QIAGEN). Three methods were used to prepare the tissue. For Method 1, one piece of tissue was digested for 100 min at 56° C in 180 μl of QIAamp Buffer ATL containing 2 mg/ml of proteinase K (QIAGEN). 200 μl of QIAamp Buffer AL was added, and the mixture was incubated at 70°C for 10 min. 200 μl of absolute ethanol was added, and the DNA was purified on a QIAamp DNA Mini column as per the manufacturer's instructions. DNA was eluted in 50 μl of Buffer AE.

For Method 2, eight pieces of tissue were ground in 2 ml of PBS using a mortar and pestle. 20 μl of 20 mg/ml proteinase K, then 200 μl of QIAamp Buffer AL were added to 200 μl of tissue homogenate, and the mixture was incubated for 10 min at 56°C. 200 μl of absolute ethanol was added, and the DNA purification was completed as for the first method.

Method 3 was the same as the first, except that the sample was digested overnight at 56°C in Buffer ATL plus proteinase K.

DNA was extracted from bacterial cultures and bovine herpesvirus type 1 using either a High Pure PCR Template Preparation Kit (Roche Applied Science) or a QIAamp DNA Mini Kit as per the manufacturers' instructions.

Leptospira isolation

Leptospira isolation from urine, vaginal mucus and preputial smegma was performed at the WHO/FAO/OIE Collaborating Centre for reference and Research on Leptospirosis, Coopers Plains, Qld.

Midstream urines, vaginal mucus and preputial smegma were collected as described for sample preparation. One ml of urine was immediately added to 10 ml of semisolid EMJH (EMJH broth containing 0.9% w/v sodium pyruvate, 0.01% w/v 5-fluorouracil and 0.15% w/v agar), which was chilled for transport to the laboratory. One ml of preputial smegma or vaginal mucus was immediately added to 3 ml of semisolid EMJH, which was chilled for transported to the laboratory. Cultures were incubated at 30°C for six weeks. They were examined by dark field microscopy on arrival in the laboratory and at three and six weeks. Cultures were also subcultured into fresh semisolid EMJH on arrival and at three weeks.

Urines were also cultured using a floating filter method (Myers, 1985), in an attempt to minimise overgrowth by contaminants. A 0.2 μm cellulose nitrate filter was floated on 5–6 ml of semisolid EMJH in a 45 mm diameter petri dish. Approximately 0.2 ml of urine was placed on the filter, and the petri dish was closed. The petri dish was incubated in the dark at room temperature for 6-24 hours, after which the filter and inoculum were removed, and the contents of the petri dish were added to 10 ml of semisolid EMJH. The process was continued as described above.

Pieces of kidney of approximately 1 x 1 x 1 mm^3 were placed in 10 ml of semisolid EMJH, which was cultured as described above.

Serology

Leptospiral antibodies were assayed using the microscopic agglutination test (MAT) (30).

Assay sensitivity

Ten-fold serial dilutions of actively growing *L. borgpeterseni* serovar Hardjo type hardjobovis or *L. interrogans* serovar Pomona cultures were prepared in urine and vaginal mucus from *Leptospira*-free heifers, PBS, and preputial smegma which had been submitted for purposes not related to leptospirosis. QIAamp DNA Mini extracts were prepared from 5 ml of each dilution (0.5 ml for vaginal mucus), and were tested in the modified TaqMan® assay.

DNA preparations of a *L. interrogans* serovar Pomona dilution series in first void urine from a *Leptospira*-free heifer were similarly used to evaluate the analytical sensitivity of the modified TaqMan® assay using heat lysis instead of a QIAamp DNA Mini kit.

To further assess the effect of sample extraction method on TaqMan® assay analytical sensitivity, a suspension containing 2 x 10^3 cells/ml of *L. interrogans* serovar Pomona was prepared in vaginal mucus from a *Leptospira*-free heifer. DNA was extracted from two 0.5 ml aliquots of the mucus using either the QIAamp DNA Mini Kit or heat lysis, and was tested in the modified TaqMan® assay.

Assay specificity

Eight pathogenic *Leptospira* serovars of likely veterinary relevance, one saprophytic serovar, seven bacterial, two protozoal and one viral pathogen implicated in bovine reproductive diseases were tested for reactivity in the modified TaqMan® assay (Table 4.3.1). Leptospiral DNA was tested at approximately 25-50 ng/μl. *T. foetus*, *Neospora caninum* and other bacterial DNAs were tested at approximately 1-50 ng/μl. DNA corresponding to 8 x 104 TCID50 of bovine herpesvirus type 1 was tested in the modified TaqMan® assay.

Experimental infections

Four Hereford heifers, approximately nine months old, were selected on the basis of negative MAT reactions to the following *Leptospira* serovars: Pomona, Hardjo, Tarassovi, Grippotyphosa, Celledoni, Copenhageni, Australis, Zanoni, Robinsoni, Canicola, Kremastos, Swajizak, Medanensis, Bulgarica, Cyanopteri, Ballum, Bataviae, Djasiman, Javanica, Panama and Shermani. Two heifers (designated I1 and I2) were inoculated on four consecutive days via the conjunctiva with 0.5 ml of a broth culture containing approximately 10^7 cells/ml of *L. borgpeterseni* serovar Hardjo type hardjobovis (152). On the first day only, the heifers were also inoculated by vaginal instillation with 5 ml of culture as described previously (57). The other heifers (C1 and C2) were retained as controls, and were housed in a separate building to the infected heifers. Urine and vaginal mucus samples were collected from all four heifers before inoculation commenced (day 1) and at regular intervals thereafter. First and second void urine samples and vaginal mucus were tested in the TaqMan® assay. Second void urine samples and vaginal mucus were also cultured for *Leptospira*. Cultures were received at the laboratory and processed within hours of sample collection from the heifers. *Leptospira* isolates were typed using MAT, and were compared with the inoculum isolate by random amplified polymorphic DNA fingerprinting (RAPD) (43). Blood samples were collected on day 57 for MAT.
At day 64, C1 was vaccinated with Ultravac 7 in 1 vaccine (CSL). Urine and vaginal mucus samples were collected from C1 and C2 before C1 was vaccinated, and at one and seven days after vaccination. These samples were tested by TaqMan® assay only.

At the conclusion of the experiment, all four heifers were necropsied. Urine (from the bladder after the heifers were euthanased) and kidney samples were collected for PCR and *Leptospira* isolation.

DNA extracts were prepared using a QIAamp DNA Mini Kit as described above. Initially, TaqMan® assays were performed using an annealing and extension temperature of 60ºC, and most assays incorporated probe at 200 nM as described by Smythe *et al.* (155). These TaqMan® results were used for an initial assessment of the suitability of first and second urine voids as samples for leptospirosis testing and assessing the sensitivity of the TaqMan® assay relative to culture.

To establish criteria for interpreting C_T values, selected QIAamp extracts from the experimental infection trial were retested in the modified TaqMan® assay. The extracts were from first and second void urine samples and from vaginal mucus samples from the control heifers, and culture-positive second void urine samples from the infected heifers. C_T ranges corresponding to negative, suspect and positive results were determined by relating the modified TaqMan® assay result to the infection status of the animal and the culture result for each sample.

Sample Storage Conditions

Appropriate conditions for storing samples during transport to the laboratory and at the laboratory were determined using infected material when available, and spiked samples.

First and second void urine samples collected from heifers I1 and I2 on day 31 of the experimental infections were divided into 5 ml aliquots. DNA was immediately extracted from one aliquot of each urine sample using a QIAamp DNA Mini Kit, and was stored at -20°C. The remaining aliquots were stored at -20°C, room temperature (protected from light), 4°C and 37°C. After 24 and 96 h, one aliquot from each temperature was used for DNA extraction, and the extracts were tested in the TaqMan® assay with the original extracts, using an annealing temperature of 60°C.

The effect of storage at -20°–37°C on vaginal mucus collected from a *Leptospira*-free heifer and spiked to approximately 100 cells/ml with *L. interrogans* serovar Pomona was examined using a similar experimental design to that described above for infected urine. Aliquots of 0.5 ml of vaginal mucus were processed at each time point (0 and 24 hr only) using a QIAamp DNA Mini kit. The extracts were tested in the TaqMan® assay with the original extracts, using an annealing temperature of 60°C.

To further examine the effect of freezing and thawing on infected urine, urine from two *Leptospira*-free heifers was pooled and spiked with *L. borgpeterseni* serovar Hardjo type hardjobovis to approximately 100 cells/ml, and frozen at -20°C. The urine was thawed and refrozen after one day at -20°C, and thawed again after another day at -20°C. Before freezing, and after each thaw, 5 ml was withdrawn for DNA extraction by heat lysis. The DNA extracts were tested in the modified TaqMan® assay.

4.3.1.2 FIELD SAMPLING – Stage 2

Field sampling protocols are provided in Appendix 1. Two hundred and twenty-two urine samples (first void only), 132 preputial smegma and 95 vaginal mucus samples were collected from beef cattle from 25 Queensland beef herds. The samples were submitted for testing in the modified TaqMan® assay and *Leptospira* isolation. Urine samples for TaqMan® assay were prepared using the QIAamp DNA Mini Kit and by heat lysis. Vaginal mucus and preputial smegma were prepared for TaqMan® assay using the QIAamp DNA Mini Kit. Samples generally took several days to reach the laboratory. Animals giving suspect or positive reactions in the modified TaqMan® assay were also tested by MAT for leptospiral antibodies.

4.3.1.3 MULTICENTRE EVALUATION – Stage 2

DNA extracts were prepared from 21 urine samples collected from a *Leptospira*-positive beef cattle herd. The extracts were prepared using heat lysis if the urine samples were reasonably clean, or a QIAamp DNA Mini Kit if dirty. The extracts were tested using the modified TaqMan® assay at four laboratories around Australia, three of which used Rotor-Gene 2000 or 3000 platforms, whilst the other used an iCycler (Bio-Rad). The iCycler data were analysed with the threshold adjusted manually so that the C_T value for the positive control was similar to that obtained by Laboratory 1. Correlations between the C_T values for the three laboratories using the Rotor-Gene platform were calculated, ignoring samples with C_T values > 50. The results were also classified as positive, suspect, or negative using the criteria established using samples from the experimentally infected heifers, and the degree of agreement between the laboratories was assessed using the Kappa statistic (Fleiss, 1981). A Kappa statistic ≥ 0.75 indicates excellent agreement, and a Kappa statistic between 0.40 and 0.75 indicates fair to good agreement.

4.3.1.4 CASE STUDY HERD – Stage 3

This study resulted from identification of a fertility problem in a herd in WA. Heifers, aged 14 to 16 months, were mated in late June/July 2003. In November 2003 the group was pregnancy tested and only 250 pregnant heifers were retained. These pregnant heifers were due to calve in

April-June 2004. Forty-five (18%) of these heifers failed to calve ('problem' group). This represented a much higher than normal level of loss (2-3%) between confirmed pregnancy and calving. No foetal membrane or dead foetuses were noticed by farm workers.

All 250 females were re-mated between June and August 2004. An early pregnancy diagnosis was done on 30 of the 45 from the problem group on 7/9/04 and then on the whole group on 25/10/04. Blood was also collected from 15 of the problem group on 7/9/04 and on 25/10/04 and was tested by MAT in WA. Serology for *Neospora caninum*, *Brucella abortus*, infectious bovine rhinotracheitis (IBR) and mucosal disease (MD) was also performed on the blood samples collected on 7/9/04. Bulls were vaccinated for campylobacteriosis each year.

In December 2004, blood, urine and mucus samples were collected from the majority of the females in the group. TaqMan® assays for *C. fetus* subsp. *venerealis*, *T. foetus* (83 females tested for both) and pathogenic *Leptospira* (for *Leptospira* the modified assay was used) (n=116) were conducted as well as the MAT for *Leptospira* (n=153). Vaginal mucus samples were used for *C. fetus* subsp. *venerealis* and *T. foetus* TaqMan®MGB assays (5' *Taq* nuclease) whereas urine samples were used for the *Leptospira* TaqMan® assays. All non-pregnant females were sold to slaughter in December 2004 and some of the pregnant females were relocated to another property in early 2005. The remaining females were vaccinated with Lepto Shield in March 2005 and mated again in mid 2005. Some further sampling for *Leptospira* TaqMan® assays (n=77) was conducted in March 2005 with tests being conducted in Qld. Stage of pregnancy of the remaining 101 females was subsequently assessed in November 2005.

Results from the second *Leptospira* TaqMan® assays for females (March 2005) that were not tested in December 2004 were combined with those from December 2004 to provide a more complete set of results for the group (n=170). A comparison of proportions test was used to compare the proportions pregnant in the negative vs. combined suspect and positive groups from the *Leptospira* TaqMan® assay and from the December 2004 Hardjo MAT test. Calving results were not available for the 3 matings.

Animal ethics

All work performed in Queensland involving experimental and field animals were approved by the Animal Research Institute Animal Ethics Committee. All work on the WA case study herd was performed with the owner's consent and Murdoch University animal ethics approval was not required.

4.3.3 Results

4.3.1.5 ASSAY DEVELOPMENT – Stage 1

Experimental infections

TaqMan® and culture results are shown in Table 4.3.2. TaqMan® assays were performed using an annealing and extension temperature of 60º C, and most assays incorporated probe at 200 nM as described by Smythe *et al.* (155). TaqMan® results were interpreted as positive if the C_T was ≤ 40 (155).

Overall, seven second void urine samples from the infected heifers were both culture and TaqMan®-positive, and two were culture-positive and TaqMan®-negative. *Leptospira* were not detected in vaginal mucus by TaqMan® or by culture. All cultured samples from the control heifers were culture and TaqMan®-negative except for one vaginal mucus sample, which was culture-negative but TaqMan®-positive (C_T = 34.9).

Kidney samples from the infected heifers were prepared for the TaqMan® assay using Methods 1 and 2. With Method 1, only the kidney sample from I2 was positive. When repeated using Method 2, the kidney sample from I1 was positive. Kidney samples from the control heifers were prepared using Method 1, and were negative in the TaqMan® assay. Urine and vaginal mucus samples collected from heifer C1 prevaccination and at one and seven days postvaccination were TaqMan® negative, indicating that the vaccine did not interfere with the TaqMan® assay.

Table 4.3.2. Taqman® assay and culture results for experimentally infected heifers[1]

	Infected heifer 1 (I1)						Infected heifer (I2)					
Day of trial	Urine		Vaginal mucus		Kidney		Urine		Vaginal mucus		Kidney	
	PCR	Culture	PCR	Culture	PCR	Culture	PCR	Culture	PCR	Culture	PCR	Culture
1 (Pre-infection)	-	-	-	-	ND	ND	-	-	-	-	ND	ND
10	-	-	-	-	ND	ND	-	-	-	-	ND	ND
17	-	+	-	-	ND	ND	+/-	+	-	-	ND	ND
28	+	+	-	-	ND	ND	+	+	-	-	ND	ND
38	+	+	-	-	ND	ND	+	+	-	C	ND	ND
57	+	+	-	-	ND	ND	+	+	-	-	ND	ND
74	+	+	ND	ND	+	+	ND	ND	ND	ND	+	+

[1]Urine, PCR performed on first and second void urines. Culture performed on second void only; ND, not done; +/-, first void PCR-positive, second void PCR-negative; C, contaminated.

Urine samples collected from heifer C2 with the C1 prevaccination samples were TaqMan®-positive (C_Ts for first and second void samples were 33.5 and 37.3 respectively) whilst the vaginal mucus sample was TaqMan®-negative. C1 and C2 kidney samples were culture and TaqMan®-negative. By day 57, heifers I1 and I2 had seroconverted to Hardjo (MAT titres were 1600 and 400 respectively). Heifers C1 and C2 were negative for Hardjo in the MAT. The isolates obtained from I1 and I2 most resembled serovar Hardjo in the MAT, and their RAPD fingerprints were identical to those of the inoculum culture (results not shown).

Up to 3.5 weeks were required for growth to be detected in positive cultures.

Assay optimisation

As noted above, two urine samples collected from heifer C2 during the experimental infection trial gave weak positive reactions in the TaqMan® assay. A urine sample collected from heifer I2 and a vaginal mucus sample collected from heifer I1 before the commencement of the experimental infection trial also yielded weak positive reactions in the TaqMan® assay. To improve discrimination between false positive and weak positive reactions, DNA extracts from three of these samples, a known positive urine, and from *L. borgpeterseni* serovar Hardjo type hardjobovis culture were assayed at 62ºC, 64ºC and 65ºC annealing and extension temperatures using 50 nM probe in the TaqMan® reactions. The results are shown in Table 4.3.3. Increasing the temperature to 64ºC produced higher C_Ts in the preinfection samples but not with the infected urine or *Leptospira* DNA. With an annealing and extension temperature of 65ºC, the C_Ts for the samples containing *Leptospira* DNA also started to increase. An annealing and extension temperature of 64ºC was selected to maximise discrimination between negative and weak positive samples in the TaqMan® assay.

Table 4.3.3. Comparison of original C_T values obtained using an annealing temperature of 60ºC, with C_T values obtained using annealing temperatures of 62ºC, 64º C and 65º C for known negative and positive urine and vaginal mucus samples

Sample	Original C_T	C_T 62ºC	C_T 64ºC	C_T 65ºC
I2 urine (before infection trial)	36.4	37.6	40.6	44.4
I1 mucus (before infection trial)	37.7	41.1	45.7	>50
C2 urine	33.5	36.4	39.4	43.5
I2 infected urine	24.2	24.4	25.0	26.1
Hardjobovis culture	ND	27.5	27.2	28.2

Assay interpretation

To establish criteria for interpreting C_T values, QIAamp extracts from the experimental infection trial were retested in the modified TaqMan® assay (64° C annealing and extension temperature, 50 nM probe). The extracts were from first and second void urine samples and from vaginal mucus samples from the control heifers, and culture-positive second void urine samples from the infected heifers. Two urine extracts from control heifers, and two vaginal mucus extracts from control heifers had C_T values (mean of duplicate reactions) in the range 37–40, and an additional vaginal mucus sample from a control heifer had a single reaction C_T value in this range (the duplicate reaction gave a C_T value > 50). Thirty-one urine extracts and nine vaginal mucus extracts from known negative samples had C_T values of > 40. Extracts from three culture-positive urine samples had C_T values (mean of duplicate reactions) ranging from 29 to 32, and a fourth had a C_T value of 40.8. Three additional extracts gave single reaction C_T values of up to 44.8 (the duplicate reactions had C_T values of > 50). Because of the overlap of C_T values from known positive and negative samples, the following criteria for interpreting C_T values were set: positive ≤ 37, 37 < suspect ≤ 40, negative > 40. Samples for which one reaction has C_T value of ≤ 40, and the other reaction a C_T value of > 50 are to be retested or scored as suspect. The detection limits for the assay (1-10 cells/ml) fell within suspect C_T range.

Analytical sensitivity using QIAamp Mini DNA extraction

The analytical sensitivity results are shown in Table 4.3.4. The assay detected down to 10 cells/ml in PBS, I–10 cells/ml in urine, 10 cells/ml in vaginal mucus and 1 cell/ml in preputial smegma.

Table 4.3.4.TaqMan® titration of *Leptospira* in PBS, urine, vaginal mucus and preputial smegma

Leptospira cells/ml	C_T				
	PBS	First void urine	Second void urine	Vaginal mucus	Preputial smegma
10^3	ND[1]	ND	ND	ND	29.5
10^2	33.3	ND	ND	33.3	33.1
10^1	36.8	37.3	35.0	37.6	38.2
10^0	> 50	38.4	(37.0, > 50)[2]	> 50	39.5
10^{-1}	> 50	ND	ND	ND	ND

[1]ND, not done.
[2]Only one of the duplicate reactions gave a C_T of < 50.

Using DNA prepared by heat lysis, the assay detected at least down to 1 *Leptospira* cell/ml of urine (Table 4.3.5), comparable to the analytical sensitivity obtained using the QIAamp DNA Mini Kit for DNA extraction.

Table 4.3.5. TaqMan® assay results for heat lysis extracts of urine and vaginal mucus

Leptospira cells/ml	C_T	
	First void urine	Vaginal mucus
10^2 Heat lysis	32.0	ND
10^1 Heat lysis	34.8	ND
10^0 Heat lysis	38.1	ND
2 x 10^3 QIAamp	ND	31.8
2 x 10^3 Heat lysis	ND	36.8

The results of the comparison of heat lysis and QIAamp DNA Mini extraction for *Leptospira* in vaginal mucus are shown in Table 4.3.5. Heat lysis performed poorly in comparison to the QIAamp DNA Mini Kit, with a 5 cycle increase in C_T (Table 4.3.5). Heat lysis was not tested on preputial smegma.

Analytical specificity

All eight pathogenic *Leptospira* serovars reacted in the assay whereas the other organisms did not react (C_T > 50).

TaqMan® assay diagnostic sensitivity and specificity

Data obtained from the experimental infection trial were used to assess the sensitivity and specificity of the TaqMan® assay. Using the original TaqMan® assay conditions (60°C annealing and extension temperature, and 200 nM of probe), the sensitivity compared to culture was 78%. Seven of the culture-positive urine samples was retested using the modified TaqMan® assay (64°C annealing and extension temperature, and 50 nM probe), and the C_T interpretation guidelines determined above were applied. Four of the urine samples were positive or suspect, and three were negative in the TaqMan® assay (sensitivity = 57% compared to culture). The other two samples (both of which were originally TaqMan®-positive) were not available for retesting.

The specificity of the TaqMan® assay was calculated using data from 32 urine samples collected from the control heifers (not all of which were submitted for culture). In the modified TaqMan® assay, two urine samples were scored as suspect using the revised C_T interpretation guidelines. This gives a specificity of 94%. Based on urine samples for which culture results were obtained, the specificity was 100% compared to culture.

Comparison of heat lysis and QIAamp DNA Mini Kit for sample preparation

QIAamp extracts and heat lysis extracts from 173 urine samples collected in the field trial were compared in the modified TaqMan® assay. Both types of DNA extract were negative for 147 urine samples. The C_T values for the other 26 urine samples are shown in Table 4.3.6. Overall, there was no significant difference between C_T values obtained using QIAamp and heat lysis (mean difference = 2.256; t = 1.57 with 25 degrees of freedom; P = 0.129). A two-way comparison of the classified results for QIAamp and heat lysis was performed with positive and suspect results combined to obtain a sufficiently large data set (Table 4.3.7). The results for the two extraction methods indicated a strong association between QIAamp extraction and heat lysis (χ^2 = 54.4; P < 0.001). A comparison of the proportions positive using QIA amp (12.7%) and heat lysis (8.7%) indicated no difference between the 2 methods (P=0.222).

Table 4.3.6. Comparison of C_T values obtained from positive and suspect field trial urines processed using QIAamp DNA Mini kit and heat lysis

Lab No.	QIAamp CT	Heat lysis CT	Lab No.	QIAamp C_T	Heat lysis CT
1	35.5	34.2	14	38.2	(44.0, > 50)[1]
2	35.8	35.8	15	37.6	(43.9, > 50)[1]
3	27.0	30.7	16	39.0	> 50
4	33.4	38.2	17	37.6	> 50
5	36.1	38.0	18	38.2	41.8
6	35.4	>50	19	(38.9, > 0)[1]	> 50
7	37.0	(43.0, > 50)[1]	20	(37.7, > 50)[1]	> 50
8	39.8	39.1	21	38.9	(49.1, > 50)[1]
9	37.8	37.7	22	39.0	(40.4, > 50)[1]
10	39.4	39.3	23	(47.3, > 50)[1]	36.7
11	(38.0, > 50)[1]	38.7	24	43.2	34.4
12	(39.9, > 50)[1]	38.7	25	> 50	[1]37.5
13	37.9	38.4	26	(45.4, > 50)[1]	(39.3, > 50)[1]

[1]Only one of the duplicate reactions gave a C_T of < 50.

Table 4.3.7. Comparison of TaqMan® assay results obtained from 173 urine samples prepared with either the QIAamp DNA Mini Kit or heat lysis

	Heat lysis		
QIAamp	Positive/suspect	Negative	**TOTAL**
Positive/suspect	11	11	**22**
Negative	4	147	**151**
TOTAL	**15**	**158**	**173**

Urine sampling

C_T values obtained for first and second void urine samples from the experimentally infected heifers were compared. The C_T values were similar for the first and second samples from each collection urine, with differences of < 2 cycles (Table 4.3.8).

Table 4.3.8. C_T values for first and second void urine samples collected from two heifers infected with *Leptospira*

	Heifer I1		Heifer I2	
Collection day	First void C_T	Second void C_T	First void C_T	Second void C_T
10	> 50	47.4	(41.0, > 50)[1]	(44.0, >50)[1]
17	43.7	42.5	38.5	40.6
28	33.6	31.8	32.9	31.5
38	31.3	30.3	30.5	27.3
57	38.7	38.6	26.1	26.5

[1]Only one of the duplicate reactions gave a CT of < 50.

The first and second void urine samples also gave equivalent results in the TaqMan® assay in that both voids were scored as positive in nine collections, and both as negative in four collections. In one collection, only the first void urine was positive although the C_T values differed by only approximately 2 cycles. As these assays were performed using an annealing and extension temperature of 60°C, the C_T interpretation criteria of Smythe *et al.* (155) were applied.

On this basis, either first or second void urine samples can be used for the TaqMan® assays.

Tissue processing

As an alternative to Methods 1 and 2, tissue preparation Method 3 was used on a sample of alpaca lung which was submitted for routine diagnostics, and which was shown to contain spirochaete-like objects by silver staining. The lung was positive in the modified TaqMan® assay.

Sample storage conditions

The effect of storage temperature on infected urine samples is shown in Table 4.3.9. At -20°C, C_T values peaked after 24 hr, and fell to within two cycles of their initial values. This may be due to a technical error during DNA extraction from the 96 hr samples, resulting in artificially reduced C_T values for these samples. Little change was observed in the C_T values during storage at 4°C and at room temperature, except for one urine sample for which the C_T had increased by approximately five cycles during storage at room temperature. All urine samples showed increased C_T values during storage at 37°C, indicating deterioration of the leptospiral DNA. On this basis, 4°C was selected as the optimal temperature for storing urine samples.

Table 4.3.9. C_T values for first and second void urine collected from infected heifers and stored at -20°C (A), 4°C (B), Room Temperature (C) and 37°C (D)

	I1								I2							
	First void				Second void				First void				Second void			
Time	A	B	C	D	A	B	C	D	A	B	C	D	A	B	C	D
0 hr	31.8	31.8	31.8	31.8	29.3	29.3	29.3	29.3	29.8	29.8	29.8	29.8	30.9	30.9	30.9	30.9
24 hr	37.1[1]	31.5	31.8	35.9	30.7	28.0	28.4	38.6	33.8	29.6	29.2	30.6	35.1	32.3	31.6	32.7
96 hr	33.4	30.9	31.7	35.9	29.6	27.6	33.7	36.2	31.1	31.5	30.0[1]	35.3	30.7	30.4	29.1	34.6

[1]Only one of the duplicate reactions gave a C_T of < 50.

The effect of storage temperature on infected spiked vaginal mucus samples are shown in Table 4.3.10. In contrast to the urine results, no evidence of sample deterioration (indicated by a rise in the C_T values) was observed at any temperature over the 96 hr period.

Table 4.3.10. C_T values for vaginal mucus spiked with *L. interrogans* serovar Pomona and stored at various temperatures

Time	Temperature			
	-20°C	4°C	RT[1]	37°C
0 hr	38.5	38.5	38.5	38.5
96 hr	35.9	36.2	36.1	36.0

[1]RT Room Temperature

Freezing and thawing was detrimental to spiked urine samples. After one freeze-thaw cycle, the C_T increased by approximately four cycles, but no further change was observed after the second thaw (Table 4.3.11). Freezing was therefore regarded as unsuitable for sample storage and was not tested further.

Table 4.3.11. The effect of freezing and thawing on the performance of spiked urine in the TaqMan® assay

Treatment	C_T
Before freezing	30.0
First freeze thaw	33.7
Second freeze thaw	33.7[1]

[1]Result based on single reaction only, as duplicate had dried out during the TaqMan® run.

4.3.1.6 FIELD SAMPLING – Stage 2

Training is described in Section 4.1.3.2.

In the field trial, 222 urine samples, 95 vaginal mucus samples and 132 preputial smegma samples were analysed. Twenty-seven urine samples were positive or suspect in the modified TaqMan® assay. These comprised the 26 samples which were analysed using both QIAamp DNA Mini and rapid lysis extracts (Table 4.3.6), and one sample which was analysed using a QIAamp DNA Mini extract only. Cultures from 20 positive or suspect urine samples were *Leptospira*-negative, whilst those from the other seven were overgrown by contaminants. Twenty-two of the animals from which these samples were taken had MAT titres ≥ 50 for at least one of the serovars Hardjo, Pomona and Tarassovi. One was negative for these serovars, and the other four were not tested.

Six vaginal mucus samples were suspect in the modified TaqMan® assay (C_T values were between 38 and 40), whilst all of the preputial smegma samples were negative. Two of the animals from which these samples were collected had MAT titres ≥ 50 for at least one of the serovars Hardjo, Pomona and Tarassovi. One of the animals was negative for all three serovars, and two were not tested. Urine samples from the 6 animals were negative in the TaqMan® assay. Three of the TaqMan®-positive/suspect vaginal mucus samples were overgrown by contaminants when cultured, whereas the other three were not cultured. None of the other samples grew *Leptospira* when cultured.

Of the urine samples, 12.1% were either suspect or positive, and 6.3% of the vaginal mucus samples were suspect in the modified TaqMan® assay. No TaqMan®-positive vaginal mucus or preputial smegma samples were detected. Overall, 7.3% of the samples were suspect or positive in the modified TaqMan® assay.

4.3.1.7 MULTICENTRE EVALUATION Stage 2

The C_T values obtained from the four laboratories and their classifications are shown in Table 4.3.12. There was good agreement in terms of C_T classification between the three laboratories which used the RotorGene platform (Kappa = 0.70, $P < 0.001$). Furthermore, the correlations between these laboratories in terms of C_T values were highly significant ($r = 0.898$-0.978; $P < 0.0010$).

The results from Laboratory 4 (iCycler) were not included in the statistical analyses, as the use of a different platform had an obvious effect on the C_T values. In particular, the samples classified as negative by the other laboratories had lower C_T values when tested on the iCycler at Laboratory 4.

Table 4.3.12. C_T values and classification of 21 urine samples tested by four Australian laboratories.

	Laboratory 1 (Rotor-Gene 3000)		Laboratory 2 (Rotor-Gene 3000)		Laboratory 3 (Rotor-Gene 2000)		Laboratory 4 (iCycler)
Sample Number	C_T	Classification	C_T	Classification	C_T	Classification	C_T
1	> 50	Neg	> 50	Neg	> 50	Neg	39.7
2	> 50	Neg	> 50	Neg	> 50	Neg	Not Tested
3	28.2	Pos	26.5	Pos	Not Tested	Not Tested	27.9
4	29.4	Pos	29.2	Pos	31.6	Pos	30.9
5	44.9	Neg	43.0	Neg	(44.2, > 50)[1]	Neg	32.6
6	38.4	Sus	37.1	Sus	(47.6, > 50)[1]	Neg	33.6
7	> 50	Neg	> 50	Neg	> 50	Neg	> 50
8	30.6	Pos	28.4	Pos	30.7	Pos	29
9	46.3	Neg	44.4	Neg	(49.7, > 50)[1]	Neg	37.7
10	41.9	Neg	43.4	Neg	(45.1, > 50)[1]	Neg	37.4
11	(43.7, > 50)[1]	Neg	44.7	Neg	> 50	Neg	36.5
12	> 50	Neg	> 50	Neg	43.7	Neg	> 50
13	> 50	Neg	> 50	Neg	> 50	Neg	> 50
14	> 50	Neg	> 50	Neg	> 50	Neg	> 50
15	38.6	Sus	38.1	Sus	43.1	Neg	33.9
16	38.7	Sus	37.1	Sus	44.5	Neg	33.8
17	33.1	Pos	32.5	Pos	38.2	Sus	35.5
18	34.8	Pos	34.5	Pos	36.7	Pos	34.3
19	37.6	Sus	37.1	Sus	38.6	Sus	33.4
20	37.4	Sus	35.1	Pos	37.6	Sus	32.7
21	37.6	Sus	Not Tested	Not Tested	39.8	Sus	35.4

[1]Only one of the duplicate reactions gave a CT of < 50

4.3.1.8 CASE STUDY HERD Stage 3

In the 'problem' group, by 25/10/04, 24 were pregnant, four were non-pregnant and two were not assessed (Table 4.3.13). Apparently 31 (69%) out of the problem group successfully calved in 2005 (note that we do not have identification of all of the 45 in the problem group).

Table 4.3.13. Pregnancy status of some of the problem group in the case study herd on 7/9/04 and 25/10/04

Status 7/9/04	Status 25/10/04		
	Pregnant	Non-pregnant	No pregnancy diagnosis
Pregnant	22	2	2
Non-pregnant	1	2	
No pregnancy diagnosis	1		

Of the 15 tested by MAT on 7/9/04, 12 (80%) had positive Hardjo titres ≥ 1000 and 11 of these were pregnant at the time. Hardjo titres from the 9/10/04 sampling were identical to those from 7/9/04. By the time of the second pregnancy diagnosis on 25/10/04 one of the 11 had lost its calf and the pregnancy status of one was unknown (Table 4.3.14). Of the other serological tests for which data were available, 10/10 were positive for IBR, 4/5 were positive for *N. caninum*, 4/5 were positive for MD, and 4/4 were negative for *B. abortus*.

Table 4.3.14. Pregnancy status by Hardjo titre for females in the case study herd tested on 7/9/04 and 25/10/04

	Pregnancy diagnosis 7/9/04		Pregnancy diagnosis 25/10/04		
MAT Hardjo titre	Pregnant	Non pregnant	Pregnant	Non pregnant	Unknown
0	2	1	2		1
1000	1		1		
5000	10	1	8	2	1

Overall pregnancy% of the group on 25/10/04 was 89% (n=233). Of the 170 females tested for pathogenic *Leptospira* using the modified TaqMan® assay in December 2004 and March 2005, 12 were positive and 16 suspect. Table 4.3.15 presents pregnancy% for those tested using the modified TaqMan® assay. There was no difference in pregnancy% of the negative versus combined positive and suspect group (P=0.372). Of the 25 females that were not pregnant on 25/10/04, only 4 were tested for *Leptospira* using the modified TaqMan® assay and all were negative.

There were 18 non-lactating females on 25/10/04, 16 of which were pregnant and two non-pregnant. The fact that these females are non-lactating may indicate they have aborted or lost a calf perinatally from the 2003 mating. Eleven of these 18, including the 2 non-pregnant females, were from the original problem group that were known to have lost a calf from the previous mating – 8 had positive MAT Hardjo titres, 2 negative and 1 was untested in late 2004.

Table 4.3.15. Pregnancy% on 25/10/04 by Lepto TaqMan® assay result from December 2004 or March 2005

Lepto TaqMan® assay	Number	Pregnancy%
Negative	139	97
Suspect	15	100
Positive	12	100
Unknown	67	69
Total	**233**	**89**

MAT titres for Hardjo were substantially lower for the December 2004 sampling compared with the earlier samplings (Table 4.3.16). Two samples had dropped from 5000 on 7/9/04 and 9/10/04 to 0 in December 2004.

Table 4.3.16. Comparison of Hardjo titres for samplings on 7/9/04 and December 2004

Hardjo titre 7/9/04	Hardjo titre December 2004			
	Negative (0-50)	Suspect (100)	Positive (200-800)	Not tested
Negative (0)	1		1	1
Positive (1000 or 5000)	3	2	4	2

Of the 153 females tested by MAT in December 2004, 58 were Hardjo positive (titres ≥200) and 30 were suspect (titres of 100). Table 4.3.17 presents pregnancy% for those females with Hardjo MAT results. There was no difference in pregnancy% of the negative versus combined positive and suspect group (P=0.174).

Table 4.3.17. Pregnancy% on 25/10/04 by MAT Hardjo results from December 2004

Hardjo titre	Number	Pregnancy%
Negative (≤50)	65	98
Suspect (100)	29	100
Positive (≥200)	55	91
Unknown	84	77
Total	**233**	**89**

All females tested (n=153) were negative for *T. foetus* and 3 were positive for *C. fetus* subsp. *venerealis* (2%) in December 2004. These 3 were all pregnant and one was also *Leptospir*a TaqMan® assay positive.

Pregnancy% from the third mating for the remaining 101 females assessed on 2/11/05 was 92%. Only 2 females were non-lactating on 2/11/05 indicating there was probably minimal abortion or perinatal calf loss from the 2004 mating. As there was no testing for *Leptospira* after March 2005 and females were vaccinated with Lepto Shield in March 2005 it is difficult to form any conclusions regarding these results.

4.3.4 Discussion

4.3.4.1 ASSAY DEVELOPMENT AND FIELD SAMPLING Stages 1 & 2

This report describes the optimisation and validation of the TaqMan® assay of Smythe *et al.* (155) for use in diagnosing bovine leptospirosis. Initially, the TaqMan® assay was performed as described by Smythe *et al*. (155), but reoptimisation of the probe concentration was necessary due to our use of a Corbett RotorGene instead of an ABI Prism 7700 Sequence Detector as described in the original paper. The TaqMan® assay was developed for use on specimens of human origin, which are normally collected under clean conditions. However, bovine samples (especially urine) are often contaminated with faeces and dust, which are a likely cause of the occasional weak positive reaction observed with known negative samples. Increasing the annealing and extension temperature was necessary to minimise these reactions. At 64°C annealing and extension temperature, the non-specific reactions were confined to a C_T range from 37 to over 40. Therefore samples yielding C_T values in this range are scored as suspect rather than positive.

All samples were tested in duplicate in the TaqMan® assays. It was not unusual for samples with high C_T values ($C_T \geq 37$) to have one of the duplicate reactions with a C_T value > 50. This reflects the random distribution of limiting amounts of template between the duplicate reactions. This effect is discussed by Svenstrup *et al*. (2005).

Culture performed better than the TaqMan® assays with the samples from the experimentally infected heifers (TaqMan® assay diagnostic sensitivity = 78% compared to culture). This estimate of sensitivity is very approximate due to the low sample numbers involved. The revised sensitivity of 57% for the modified TaqMan® assay is even less precise due to the reduced data set used in obtaining that value. In the field trial, culture attempts were unsuccessful, with many cultures overgrown by contaminants, whereas 27 urine samples were scored as positive or suspect in the modified TaqMan® assay. Thus culture can be very sensitive under ideal conditions such as experienced during the experimental infections, but is totally unsuitable for use as a diagnostic tool on samples from remote herds. Under these conditions the modified TaqMan® assay is a useful diagnostic tool. Even under ideal conditions, culture has the additional disadvantage of taking several weeks to obtain a result and therefore is seldom used as a veterinary diagnostic test.

A major factor in the improved performance of culture with the experimentally infected heifers is the relatively large sample volume (0.2-1 ml) used to inoculate the urine cultures. In contrast, each TaqMan® reaction receives 2 µl of DNA extract, corresponding to a maximum of 0.2 ml of the original sample. As noted above, culture failed to detect *Leptospira* under typical field conditions due to its susceptibility to contamination with extraneous bacteria, and its inherent requirement for the survival of live *Leptospira* during transport to the laboratory.

The diagnostic specificity of the modified TaqMan® assay varied from 94% to 100%, depending on the data set used. Using a restricted data set comprising urines for which a negative culture result had been obtained, the specificity was 100%. However, assuming that the control heifers were entirely *Leptospira*-free based on the fact that all culture attempts gave negative results, the specificity was recalculated as 94% for all urine samples from these heifers. This can be regarded as a more accurate estimate of sensitivity, which highlights the need for a “suspect” category for TaqMan® assay results.

Both methods of extracting DNA from urine field samples yielded similar C_T values in the modified TaqMan® assay. However, the heat lysis method is simpler to perform and requires less time than the QIAamp method, and is therefore recommended for routine use.

Leptospira were not detected in vaginal mucus from the experimentally-infected heifers by TaqMan® assay or by culture. Similarly, there was no definite evidence of the presence of *Leptospira* in field samples of vaginal mucus or preputial smegma using the modified TaqMan® assay. On this basis, urine is the sample of choice for diagnosing bovine leptospirosis using the modified TaqMan® assay.

Samples from the vaccinated heifer did not react in the modified TaqMan® assay, suggesting that vaccination does not lead to false positive results. As samples from only one heifer were used, however, further testing may be required to confirm the suitability of post-vaccination samples for testing in the modified TaqMan® assay.

Limited data were obtained for tissue samples in the TaqMan® assays. The data that were available suggest that any of the three methods could be used for DNA extraction, although only one of two infected kidneys were TaqMan®-positive when either of Methods 1 and 2 was used for template preparation. This may reflect the presence of foci of infection scattered throughout the kidneys, or inefficient extraction of DNA from the samples. Method 3 was used on a single tissue with histological evidence of *Leptospira* infection, and a positive TaqMan® result was obtained. More data is required before a firm conclusion can be reached on tissue preparation for the modified TaqMan® assay.

The modified TaqMan® assay was also trialled in three other laboratories using a standard set of samples. The laboratories used three different platforms; two different versions of the RotorGene and an iCycler. No significant difference was observed between the results from the laboratories using the RotorGene. However, the results obtained using the iCycler were noticeably different from those obtained using the RotorGenes, especially for the negative samples. Modifying the criteria for interpreting C_T values would probably be sufficient to allow the modified TaqMan® assay to be run on this platform with confidence. If not, optimising the assay for the iCycler would be necessary as the assay described in this paper was specifically optimised for the RotorGene. Spackman and Suarez (156) similarly observed that optimisation of a TaqMan® assay for avian influenza virus (AIV) for individual platforms was essential to maintain consistency across the platforms. Although the AIV TaqMan® assay is a reverse transcriptase PCR, the same appears to apply to at least some degree to the modified TaqMan® assay for *Leptospira.* Overall, the assay was sufficiently robust to allow transfer between laboratories using either version of the RotorGene, and to allow its use on other platforms with some modification.

4.3.4.2 CASE STUDY HERD Stage 3

A cohort of heifers from a beef herd in Western Australia was followed through two consecutive matings using pregnancy testing, MAT, and *Leptospira*, *C. fetus* subsp. *venerealis* and *T. foetus* TaqMan® assays. Assuming that the 15 females tested in the Hardjo MAT in September 2004 were representative of the original problem group of 45, the majority of the 45 had high Hardjo titres at this time. These titres were observed several months after they had failed to calve and while they were pregnant from the second mating. These results suggest that the failure of the 45 females to calve as a result of the first mating probably involves *Leptospira*.

There is no evidence that *Leptospira* has affected pregnancy rates from the second mating. However there is evidence from the TaqMan® assay and serology testing that Lepto is still present in the group. Without calving information the impact on *Leptospira* during the later stages of pregnancy cannot be assessed.

The data collection from this herd over the 3 matings was incomplete and we had little control over the management of the cattle. This has made it difficult to clearly identify the cause of reduced fertility in the females.

Reproductive failure in Australian beef and dairy cattle is often attributed to infection with *Leptospira*, in particular with serovars Hardjo and Pomona. Although several other serovars have been isolated from Australian cattle (23), their roles if any in bovine reproductive failure have not been established. Similarly, although antibodies to serovar Tarassovi are frequently observed in Australian beef cattle, a role in reproductive failure has not been determined (12).

Serology indicated that serovars Hardjo and, to a lesser extent, Tarassovi, were active in this herd. As noted above, Hardjo may have contributed to the failures in the first mating, but not the second mating. Serovar Hardjo exists as two genotypes, Hardjoprajitno and Hardjobovis (143, 165). Only Hardjobovis has been found in Australia (43, 147). The role of Hardjobovis in reproductive failure in Australian cattle seems very variable, with some studies reporting a close association between Hardjobovis infection and reproductive failure, and others finding no association whatsoever.

For example, Hoare and Claxton (78) reported an association between Hardjo serology and abortion and mastitis in southern NSW in 1969. Slee *et al*. (151) reported the presence of leptospirae in silver stained aborted foetal tissues, coincident with Hardjo serology in the dams in 1.9% of the abortion cases they examined. Holroyd and Smith (80) reported a significant

decrease in lactation failure among heifers in a north Queensland beef herd when vaccinated with a Hardjo vaccine.

On the other hand, Sullivan (158) failed to induce abortion in pregnant cows when inoculated at various stages of pregnancy with a Hardjo isolate of bovine origin. Carroll and Campbell (26) found no association between reproductive failure and Hardjo seroprevalence in central Queensland beef cattle. Norton *et al*. (125) found little evidence of Hardjo abortion in Atherton Tableland dairy cattle despite a high seroprevalence (49.9% among nonvaccinated breeders).

The results of this case study are consistent with the variable behaviour of Hardjobovis as a reproductive pathogen in Australian cattle.

One possibility is that Hardjo requires a synergism with some other factor(s), possibly infectious, to cause reproductive failure. The limited serology performed on the problem group in September 2004 suggested a high prevalence of IBR, MD and *N. caninum* in at least this group at the time of testing. These infections may have also contributed to the reproductive failures observed at least in the problem group in the second and maybe the first matings. Unfortunately, more data is required to further test this hypothesis.

4.3.5 Conclusions

The recommendations for diagnosing bovine leptospirosis using the modified TaqMan® assay include:

- Use urine (either first or second void) and kidney samples. Other tissues such as lung and liver may also be useful. Samples should be transported and stored refrigerated, but not frozen.
- Urine samples are to be prepared using either the QIAamp DNA Mini Kit or heat lysis. Heat lysis can be used for herd testing, but the QIAamp DNA Mini Kit is preferred for testing single animals.
- The assay should be performed on a Corbett RotorGene using 50nM probe concentration, with an annealing and extension temperature of 64° C. Samples should be tested in duplicate.
- C_T values should be interpreted as follows: positive ≤ 37, 37 < suspect ≤ 40, negative > 40. Samples for which one reaction has a C_T ≤ 40, whilst the duplicate has a C_T value > 50 should be retested or scored as suspect.

The modified TaqMan® assay is a useful test for detecting *Leptospira* infection in beef herds for the following reasons:

- Good analytical sensitivity and specificity.
- Detects *Leptospira* in urine and tissue samples.
- Ease of specimen collection and processing.
- Does not require *Leptospira* to be viable.
- No evidence of interference from vaccination.
- Rapid turnaround.
- Easily transferred to other laboratories.

5 Communication activities

5.1 Bovine venereal diseases

5.1.1 Oral presentations

- Lew A. (2006) "Bovine Reproductive Disease Research – Trials and Tribulations" Emerging Technologies Seminar Series, DPI&F (INVITED PRESENTATION)

- McMillen L, Lew A, Corney. (2004) Sensitive and specific detection of *Campylobacter fetus* subsp. *venerealis* infection in cattle by 5' *Taq* nuclease assay. National Conference for the Australian Society for Microbiology, Sydney, Australia, 29th September – 1st October 2004. (PROFFERRED PAPER)

ABSTRACT:

Sensitive and specific detection of *Campylobacter fetus* subsp. *venerealis* infection in cattle by 5' *Taq* nuclease assay

Lyle McMillen[1], Ala Lew[1], Bruce Corney[2]
[1]Agency for Food and Fibre Sciences, Department of Primary Industries and Fisheries, Brisbane, Australia
[2]Animal & Plant Health Service, Department of Primary Industries and Fisheries, Brisbane, Australia

A sensitive and specific assay for *Campylobacter fetus* subsp. *venerealis* would allow for improved diagnosis and management of this economically important bovine venereal disease. A 5' *Taq* nuclease assay, using a 3' minor groove binder-DNA probe (TaqMan® MGB), was developed from a previously identified sub-species-specific conventional PCR target. The quantitative, real time assay targets an 86 bp fragment found in *C. fetus* subsp. *venerealis* but not the morphologically, phenotypically and genetically similar *C. fetus* subsp. *fetus*, which is difficult to discriminate using current conventional diagnostic tests such as selective culture.

Assay specificity was evaluated against a range of bovine venereal microflora and related organisms, as well as field isolates of *C. fetus* subsp. *venerealis*. None of the non-target organisms assayed yielded a positive result, including *C. fetus* subsp. *fetus*. Sensitivity of the assay was evaluated and compared to that obtained with conventional diagnostic procedures, including culture and ELISA. The assay detected down to a single cell per assay, significantly improving upon the sensitivity obtained for selective culture.

Techniques for collecting, transporting and rapidly processing animal samples (smegma, vaginal mucus and urine) for *Taq* nuclease assay were adapted or developed. A sample processing method was developed that involves osmotic shock with sterile distilled water followed by heating at 95 degrees. This method may be applied directly to clinical samples with no significant loss of sensitivity or specificity, avoiding the necessity for culture or extensive commercial DNA purification systems. Sample collection, sample processing and assay procedures were evaluated with healthy and naturally infected cattle. This assay is specific, sensitive, and quantifiable, in contrast to the culture-based diagnostic tests currently in use, and is able to reliably determine the infection status of an individual animal.

This research is supported by Meat and Livestock Australia.

- Lew A, McMillen L. (2005) "Bovine Venereal Disease Diagnosis" Inaugural Meeting of the Australian Association of Veterinary Laboratory Diagnosticians, September 22-23, 2005. PIRVic, Westmeadows, Victoria. (INVITED PRESENTATION)

- Lew A. (2005). **"**Tritrichomoniasis in Australia" **In "**Protozoal Causes of Abortion WORKSHOP" - World Association for the Advancement of Veterinary Parasitology, Christchurch, New Zealand, October, 2005. (INVITED WORKSHOP PRESENTATION)

5.1.2 Poster Presentations

- McMillen L**,** Lew A, Corney B. (2004) Sensitive and specific detection of bovine *Tritrichomonas foetus* by 5' *Taq* nuclease assay. 46th Annual Scientific Meeting of the Australian Society for Parasitology Inc. Fremantle, Western Australia, September 26-30, 2004.

5.1.3 Publications (Appendix 6)

- McMillen L and Lew A. (2005) "Are your bulls carrying reproductive diseases?" March 2005 Meat & Livestock Industry Journal March 2005 p.5 (requested)

- Lew A. and McMillen L. (2005). "Use of RealMasterMix™ Probe in Bovine Venereal Disease Diagnostics" BioNews (Eppendorf, Germany) May p.5-8.

- McMillen, L., Fordyce, G., Doogan, V. J., Lew, A.E. (2005) Comparison of culture and a novel 5' *Taq* nuclease assay for the direct detection of *Campylobacter fetus* subspecies *venerealis* in clinical specimens from cattle. Journal of Clinical Microbiology 44(3): 938-945.

- McMillen, L., Lew, A.E. (2006) Sensitive and specific detection of *Tritrichomonas foetus* in cattle by 5' *Taq* nuclease assay with fluorescent 3' minor groove binder –DNA probe. Veterinary Parasitology IN PRESS.

Sensitive and specific detection of bovine *Tritrichomonas foetus* by 5' *Taq* nuclease assay

Lyle McMillen[1], Ala Lew[1]*, Bruce Corney[2]
[1]Emerging Technologies/Delivery & [2]Biosecurity, Department of Primary Industries and Fisheries, Brisbane, QLD, Australia

ABSTRACT

A sensitive and specific 5' *T aq* nuclease assay for *Tritrichomonas foetus*, an economically important bovine venereal parasite, was developed from a previously identified species-specific conventional PCR target. The quantitative, real time assay, which uses a TaqMan® MGB probe, targets the internal transcribed spacer region 1, present in multiple copies per genome. Assay specificity was evaluated against a range of bovine venereal microflora and related organisms. A single cell per assay was detected, significantly improving upon the sensitivity of selective culture using a commercial diagnostic test kit. A sample processing method was developed which may be applied directly to clinical samples with no significant loss of sensitivity or specificity, avoiding the necessity for culture or extensive commercial DNA purification systems. This research is supported by Meat and Livestock Australia.

INTRODUCTION

Trichomoniasis is a venereally transmitted reproductive disease of cattle caused by the protozoan parasite *Tritrichomonas foetus*. Infection can be in apparent in bulls but can cause early embryonic death and abortion in cows.

T. foetus is confirmed by microscopic examination of the enrichment/transport medium. However, growth of *T. foetus* can be inhibited by other microbes in the transport medium and thus fail to reach adequate detectable numbers.

Sensitive methods for the detection of pathogens in clinical samples have been simplified through the application of polymerase chain reaction (PCR) methods. One of the problems restricting the diagnostic value of PCR is the difficulty confirming specific PCR products. PCR tests developed to target *T. foetus* rRNA genes have been found to be effective, however most laboratories appear to use the PCR to detect *T.foetus* following transport in medium.

5' *Taq* nuclease assays, often also referred to as fluorogenic probe assays or real time PCR or TaqMan® assays are increasingly applied for the detection and identification of pathogens and do not require post PCR electrophoresis or processing steps. We proposed to develop real time/5' *Taq* nuclease assays to detect *T. foetus* directly in clinical samples.

METHODS

Develop Taq nuclease assay using Primer Express (ABI) based on internal transcribed spacer region, determine optimal assay conditions.

Confirm specificity of assay.

Investigate DNA processing methods: commercial DNA kits vs. heat lysis.

Compare sensitivity of InPouch culture with *Taq* nuclease assay using *T. foetus* spiked preputial and mucous samples.

Compare sampling tools during infection trial.

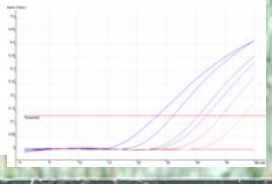
Collection of field samples.

RESULTS - SENSITIVITY

TABLE 1. Comparison of *T. foetus* detection sensitivities.

Sample type	Culture	*Taq* assay
Smegma	10000	1
Mucous	25000	1
Urine	500	1

FIGURE 1. *Taq* nuclease assay plot comparing DNA prepared from titration of *T. foetus* from spiked smegma correlating with 10e4 cells to 10e-1 cell per assay.



RESULTS - SPECIFICITY

The assay was specific for:
T. foetus strains: YVL-W* and ATCC30003 (BP-4).

The following species were negative in the *T. foetus* assay: *Tetratrichomonas gallinarum* ATCC30097, *Pentatrichomonas hominis* ATCC30000, *Trichomonas vaginalis* ATCC30001; *Campylobacter coli* NCTC11353, *C. fetus* subsp. *venerealis* 98-109383*, *C. fetus* subsp. *venerealis* ATCC19438, *C. fetus* subsp. *fetus* 98- 118432, *C. fetus* subsp. *fetus* ATCC15296, *C.jejuni* subsp. *jejuni* NCTC11168, *C. hyointestinalis* N3145*, *C. sputorum* subsp. *bubulus* Y4291-1*; *Pseudomonas aeruginosa* ATCC27853, *Proteus vulgaris* ATCC6380, *Neospora caninum* ATCC50843, *Leptospira borgpetersenii* serovar Hardjobovis 93/94451/3*, *L. interrogans* serovar PomonaPomona CCRL4*. *Field isolates DPI&F

FIGURE 2. Sampling tools investigated.

RESULTS – TOOLS & DNA prep

- Argentinean design tool found to be most suitable for VD sampling. **'TRICAMPER'**
- Higher sensitivity obtained using heat lysis method for the preparation of DNA than commercial extraction kits.

RESULTS – FIELD SAMPLING

TABLE 2. Regional field results to date

Region	Culture pos.	5' *Taq* nuclease pos.
South	0/75	0/88
Central Coast	0/54	5/85
North Coast	0/123	1/134
North West	0/8	1/8
Totals	0/260	7/315

CONCLUSIONS

- The *T. foetus* ITS *Taq* nuclease assay provided a high degree of sensitivity and specificity.
- Standard culture conditions require a high threshold of cells for a positive result.
- Preliminary data from field sampling indicate a higher prevalence of *T. foetus* in Central to North regions of Qld.

Delivery Biosecurity

Queensland Government
Department of Primary Industries and Fisheries

BEFV
Poster presented at the Australian Society for Microbiology Meeting – Canberra, Sept 2005

Real time RT-PCR detection of bovine ephemeral fever virus infections

Ala Lew[1], Bruce Corney[2], Catherine Minchin[1], Lucia Wright[2]
[1]Emerging Technologies & [2]Biosecurity, Department of Primary Industries and Fisheries, Brisbane, QLD, Australia

ABSTRACT

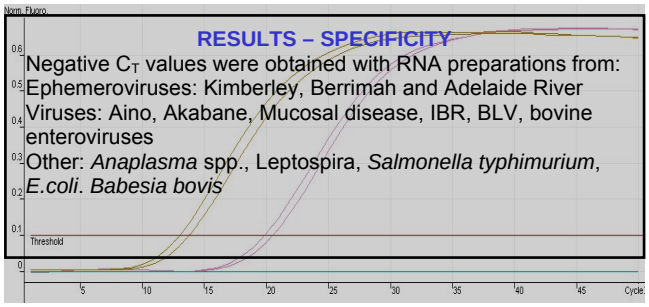
Bovine ephemeral fever virus (BEFV) is the causative agent of three-day sickness characterized by periodic summer epizootics of fever, respiratory distress, listlessness, stiffness, lameness and sometimes paralysis. BEFV is an insect-transmitted single stranded RNA virus of the genus *Ephemerovirus* in the family Rhabdoviridae. Current serological methods are not specific for BEFV and detect all *Ephemerovirus* species. As neutralizing antibodies can persist for years in an animal, these methods do not detect current infection status. Virus isolation in cell culture can confirm the presence of virus yet this approach is not always successful and can take over two weeks. A semi-nested reverse transcriptase (RT)-PCR BEFV specific test has been applied for BEFV detection. Although PCR assays can be quite sensitive, the risk of contamination and the amplification of non-specific products can become problematic. A TaqMan® MGB probe based 5' *Taq* nuclease assay was developed for the specific detection of BEFV based on conserved regions of the glycoprotein gene. RNA templates from bloods were prepared using RNeasy kits and assays were undertaken using the Invitrogen Superscript III One-step Quantitative RT-PCR System and the Corbett RotorGene 3000. The assay detected a 10-4 dilution of culture derived BEFV and approximately 0.44 ng of RNA compared with 10-3 and 4.44 ng respectively for the semi-nested RT-PCR assay. Four samples from a vaccine infectivity trial were detected by real time RT-PCR previously not positive by culture. All remaining culture positive samples correlated with real time RT-PCR results. The assay did not amplify closely related ephemeroviruses. This research was supported by MLA project AHW.036.

INTRODUCTION

- BEFV is the causative agent of three-day sickness characterized by periodic summer epizootics of fever, respiratory distress, listlessness, stiffness, lameness and sometimes paralysis
- BEFV is an insect-transmitted single stranded RNA virus of the genus *Ephemerovirus* in the family Rhabdoviridae
- Related non-pathogenic viruses present in Australia include Adelaide river, Kimberley and Berrimah
- Serological methods are not specific for BEFV and detect all non-pathogenic *Ephemerovirus* species
- Neutralizing antibodies can persist for years, serology does not detect current infection
- Virus isolation in cell culture confirms the presence of virus
- An RT-PCR BEFV specific test was developed by DPI&F and CSIRO) for BEFV detection - the risk of contamination and the amplification of non-specific products can be problematic

METHODS

- Design primers and minor groove binder (MGB) FAM-labeled probes (ABI) targeting conserved regions of nucleoprotein and glycoprotein genes for real time RT-PCR assay
- Evaluate assays using serial dilutions of BEFV and BEFV spiked blood samples using RNeasy preparations
- Determine specificity of assays using RNA preparations of related viruses and bovine pathogens
- Test 60 diagnostic trial samples submitted to DPI&F's Biosecurity section, compare real time RT-PCR to the conventional semi-nested RT-PCR assay and virus culture.



RESULTS – SPECIFICITY

Negative C_T values were obtained with RNA preparations from:
Ephemeroviruses: Kimberley, Berrimah and Adelaide River
Viruses: Aino, Akabane, Mucosal disease, IBR, BLV, bovine enteroviruses
Other: *Anaplasma* spp., Leptospira, *Salmonella typhimurium*, *E.coli*. *Babesia bovis*

RESULTS – DIAGNOSTIC TESTING COMPARISON

TABLE 1: Comparison of real time RT-PCR with conventional semi-nested RT-PCR assay (gel PCR) and virus culture isolation

Legend: C_T cycle threshold, + positive, - negative, +/-

No. of samples	C_T values	Gel PCR	Culture
40	0	-	-
1	0	+/-	-
13	24-31	+	+
5	32-37	+	-
1	37	+/-	-

RESULTS SUMMARY

METHOD: developed using SuperScript™ III Platinum® One-Step Quantitative RT-PCR System Mix using the Corbett Rotorgene 3000, following manufacturer's instructions.
GENE TARGET: Glycoprotein real time RT-PCR assay was 7 cycles more sensitive than the nucleoprotein based real time RT-PCR. The glycoprotein assay was designed based on 7 BEFV sequences to confirm specificity
SENSITIVITY: The detection limit for the real time RE-PCR was 0.44 ng RNA compared with 4.44 ng for conventional RT-PCR
SPECIFICITY: Real time RT-PCR improved specificity of conventional

CONCLUSIONS

- Glycoprotein gene proved to be the best target for the development of a sensitive BEFV assay
- Due to high sequence heterogeneity, multiple strain alignments were required to design a specific assay based on the glycoprotein gene
- The real time RT-PCR developed here is suitable for routine diagnostic application and provides significant sensitivity and specificity improvements over conventional RT-PCR and viral culture.

5.2 Leptospira

5.2.1 Oral presentations

- "Real-Time Polymerase Chain Reaction Assay For Detecting *Leptospira* infection in cattle" by B Corney et al. Australian Society for Microbiology, September 26-30, 2004. Sydney, NSW. PROFERRED PAPER

ABSTRACT:
A Real-Time Polymerase Chain Reaction Assay for Detecting *Leptospira* Infection in Cattle
Bruce Corney[1], Lee Smythe[2], Ibrahim Diallo[1], Lucia Wright[1], Glen Hewitson[1], Leonie Barnett[2], Michael Dohnt[2], Meegan Symonds[2], Lyle McMillen[3], Ala Lew[3]

[1]Department of Primary Industries and Fisheries, Animal and Plant Health Service, Brisbane, Australia
[2]WHO/FAO/OIE Collaborating Centre for Reference and Research on Leptospirosis, Queensland Health Scientific Services, Brisbane, Australia
[3]Department of Primary Industries and Fisheries, Agency for Food and Fibre Sciences, Brisbane, Australia

A *Taq* nuclease (TaqMan®) assay, which was developed for diagnosing human leptospirosis, was adapted for use in diagnosing bovine leptospirosis. The reaction mixture composition and assay running conditions were modified for use on a Corbett Research RotorGene 3000, and to eliminate occasional low-level background fluorescence observed in negative samples. The assay targets an *rrs* (16S ribosomal RNA) gene sequence which is conserved among the pathogenic *Leptospira* serovars.

The modified assay retained the ability to detect all eight pathogenic serovars against which it was tested, but did not detect the saprophytic serovar Patoc. *Tritrichomonas foetus*, *Campylobacter fetus* subsp. *fetus*, *Neospora caninum*, and bovine herpesvirus type 1 also failed to react in the assay. The detection limit of the assay was around 1 cell/ml of *Leptospira* in urine, and 1-10 cells per ml in vaginal mucus and preputial scrapings using samples spiked with *Leptospira* culture.

Leptospira isolation and the modified PCR were compared using urine samples collected from heifers which had been experimentally infected with *Leptospira* borgpetersenii serovar Hardjo. *Leptospira* isolation was performed by the standard culture method and by a filter floatation method. The PCR was more reliable than the standard culture due to overgrowth of three urine cultures by contamination. Two of the affected urine samples were positive using the filter floatation culture method, whereas only one was positive by PCR. One other urine sample was positive by the standard culture method, but negative by filter floatation and by PCR.

- "Leptospirosis" by B. Corney. Inaugural Meeting of the Australian Association of Veterinary Laboratory Diagnosticians, September 22-23, 2005. PIRVic, Westmeadows, Victoria.

5.2.2 Publications

- Corney, B., Smythe, L., Lew, A.E. McMillen, L., Diallo, I., Wright, L., Hewitson, G., Dohnt, M., Symonds, M., Barnett, L., Kelly, M., Doogan, V.J., Fenwick, S., Taylor, E., Wai'in, P., Phuetkes, P., Reid, S., Vivas-Marfisi, A., Berg, T., Rodwell, B. 2006. A 5' *Taq* nuclease assay for detecting pathogenic *Leptospira* in cattle. Vet. Microbiol. Submitted for publication. (APPPENDIX 6)

6 Success in Achieving Objectives

6.1 Evaluate and test PCR tests in laboratory samples including standardisation of methods (500 samples in total) and select herds for case studies

6.1.1 Bovine venereal diseases

Two assays were developed one each for the detection of *C. fetus* subsp. *venerealis* and *T. foetus*. The number of assays undertaken for the optimisation of each assay (temperature and titration of reagents), assay sensitivity, assay specificity, spiking of clinical material (urine, smegma, mucus), sample handling (kit extraction vs. boiled lysis), storage trials (smegma, mucus, urine) = 720 reactions per pathogen; Infection trial (32 animals, 3 sampling tools, 2 specimens per animal, 4 collections) = 768 reactions per pathogen. Thus a total number of **1488 samples** screened in 2 assays (for each pathogen) in duplicate.

6.1.2 BEFV

The preliminary evaluation of target genes for the development of a probe based real time reverse transcriptase PCR assay (*Taq* nuclease RT-PCR) confirmed that the BEFV glycoprotein gene based assay was a more sensitive assay than the nucleoprotein assay. The G protein assay detected BEFV specifically and did not amplify related ephemeroviruses and blood borne pathogens. Approximately **100 reactions** were prepared including duplicates, assay titrations and specificity and sensitivity testing for 2 different assays (N and G protein).

6.1.3 Leptospira

Total samples tested including PCR optimisation (probe titrations, annealing temperature comparisons, sampling handling and processing optimisation) = 221; Experimental infections = 164. Total = 385. These figures include positive and negative TaqMan® controls. Each sample was tested in duplicate giving a total of **770 reactions**.

6.2 Complete evaluation of field samples (400 animals in total) and multicentre laboratory evaluation of selected test samples

6.2.1 Bovine venereal diseases

Field Samples. Results from 341 animals were included in the final summaries (Table 4.1.14) with a further 99 samples collected which were either repeat collections from animals previously sampled or samples which were not included due to inconclusive results but which were not re-sampled. Thus **440 animals** were sampled in total.

Multicentre Evaluation. A multi-centre laboratory (DPI&F, DPI-VIC; DPI-NSW) evaluation using 13 *C. fetus* subsp. *venerealis* (8 positive) and 8 *T. foetus* (3 positive) field specimens was successful with good inter-laboratory correlation. An informal evaluation was undertaken with our *C. fetus* subsp. *venerealis Taq* nuclease assay at DAWA (Dept. Agriculture WA) which demonstrated higher specificity and sensitivity than the SYBR green real time PCR assay.

6.2.2 BEFV

This glycoprotein assay was re-developed and the new assay was testing using **191 diagnostic samples/animals**.

6.2.3 Leptospira

Field Samples. **222 animals** tested (on average 2 samples per animal, hence target for *Leptospira* was 200 animals instead of 400 animals).

Multi-centre Evaluation. Twenty samples were tested in each of four different laboratories for the multi-centre evaluation.

6.3 Case study herds evaluated and tests completed (one herd per pathogen) and communication material delivered

6.3.1 Bovine venereal diseases

Case study herd

Case study herds were evaluated and *Taq* nuclease tests were completed for all 3 pathogens (*C. fetus* subsp. *venerealis* and *T. foetus* screening of smegma and mucus, and *Leptospira* screening of urine). The assays successfully detected all 3 pathogens and *C. fetus* subsp. *venerealis* detection correlated with a decrease in pregnancy rates. The case study herds were not controlled trials and not all animals were pre-screened prior to mating. In addition, it would have been useful to pre-screen all female cattle using the *C. fetus* subsp. *venerealis* herd ELISA assay to confirm previous *C. fetus* subsp. *venerealis* exposure (as organisms are quickly cleared and cannot be subsequently detected using culture or PCR).

Trichomoniasis. A Station in north-west Qld agreed to participate in a case study for *T. foetus*, however *C. fetus* subsp. *venerealis* and *Leptospira* were also detected in these herds. A total number of 466 female cattle and 66 bulls were organised into 5 different mating groups with only 228 of these females screened prior to mating. This case study did demonstrate an impact of *C. fetus* subsp. *venerealis* on pregnancy rates however the impact of *T. foetus* was uncertain.

Campylobacteriosis. A south-western Qld property agreed to participate in the case study evaluation for *C. fetus* subsp. *venerealis*, however during the course of the study *T. foetus* was also detected in the herd. This study sampled 312 female cattle and 17 bulls in 3 different mating groups and similarly to above, not all animals had been pre-screened prior to mating. This case study demonstrated delayed conception rates however poor control of animals during this study precluded our ability to confirm this observation was related to campylobacteriosis. It was confirmed that *T. foetus* in this herd had not affected the overall pregnancy rate.

Communication Material. A list of formal presentations and publications are provided in Section 5 above and Appendix 6 (including a BEFV conference poster). The *T. foetus* publication, although submitted at the same time as the *C. fetus* subsp. *venerealis* manuscript, is not yet published. Considerable interest assay during the WAAVP presentation last October (New Zealand) indicated that European researchers are intending to evaluate our *T. foetus* assay. Two publications describing the *C. fetus* subsp. *venerealis* virulence gene research are currently pending preparation.

Informal communication includes a number of 'fee for service' assay requests which we undertook during the course of this project including for MLA AHW.042 ("Impact of infectious diseases on beef cattle reproduction" – neosporosis and pestivirus). Both assays have been adopted by Biosecurity DPI&F who will continue to evaluate *C. fetus* subsp. *venerealis* assay in conjunction with culture towards NATA (National Association of Testing Authorities Australia) and SCAHLS (Sub-Committee on Animal Health Laboratory Standards) approval. In addition to the multicentre evaluations, Gribbles Veterinary Pathology obtained these assays through a confidentiality agreement for use in Victoria and SA.

The VD research program included collaborators from Murdoch University's CCG (final milestone – bioinformatics resources for *C. fetus* subsp. *venerealis*) and Argentina: Universidad Nacional De San Martin (Prof. Daniel Sanchez – incomplete genome data for *C. fetus* subsp. *venerealis*) and the Instituto Nacional de Tecnología Agropecuaria (Dr Carlos Campero – *C. fetus* subsp. *venerealis* and *T. foetus* in Argentina – informal assistance with our infection trial).

The increased detection of *C. fetus* subsp. *venerealis* during the course of this research program generated considerable interest from producers, Pfizer (VibroVax producer) and collaborators. The publication of the *C. fetus* subsp. *venerealis* assay in the *Journal for Clinical Microbiology* resulted in contact from the OIE *Campylobacter* reference laboratory (Dr Marcel van Bergen, Netherlands) and the Moredun Institute (Dr Kim Willoughby, UK). Discussions are underway between our groups to develop new *C. fetus* subsp. *venerealis* research collaborations.

Communication in regard to venereal disease testing and the availability of the new assays to producers will flow through the current diagnostic services in DPI&F's Biosecurity. The publication in MLA feedback magazine did successfully lead to a number of enquiries and subsequent testing. The tricamper tool ($2.20ea, GST incl.) will be distributed with testing kits provided by the DPI&F. Instructions on how to use the tool for mucus and smegma collection has been prepared (see Appendix 1). It is hoped that these tools will be adopted and distributed with kits for VD testing provided by other state government agencies. The field sampling staff have been recommending the use of the tricamper during the field sampling and case study stages of the research undertaken here, thus the use of the tool has been actively demonstrated at various locations in Qld (2004-2006). In addition, as part of the 'Diploma in Cattle Breeding and Reproduction' (Australian Agricultural Colleges Corporation, Emerald Campus), students are taught to use the tricamper sampling tool and the benefits in the diagnosis of venereal diseases is promoted in bull fertility examination. The tricampers have been provided to DPI&F extension staff to include in their producer communication forums and for inclusion in activities as described here. Updated information will also soon be available on DPI&F government web-sites.

6.3.2 Leptospira

Case study herd

This case study demonstrates firstly the difficulty in determining the cause(s) of bovine reproductive failure, and secondly the value of the TaqMan® assays for *Leptospira, C. fetus* subsp. *venerealis* and *T. foetus* in combination with serology in studying reproductive failure. The TaqMan® assays demonstrated the continuing presence of *Leptospira* in the heifers, and serological tests for bovine herpesvirus type 1 and *Neospora caninum* also demonstrated widespread exposure to these organisms among the heifer cohort, suggesting a multifactorial cause of reproductive failure in the cohort. There was no evidence that *Leptospira* was acting as the sole cause of reproductive failure, but the possibility of a synergism between *Leptospira* and the other organisms should be considered.

The presence of *Leptospira* in the heifer cohort as demonstrated using the modified TaqMan® assay is important from a workplace health and safety perspective.
233 animals were tested in the modified *Leptospira* TaqMan® assay.

153 animals tested in each of the *C. fetus* subsp. *venerealis* and *T. foetus* TaqMan® assays.

Communication Material. Formal presentations are listed in Section 5 above and provided in Appendix 6. Informal conversations by telephone and email were held with private veterinarians and property owners concerning the availability and application of the TaqMan® assays for Leptospira, C. fetus subsp. venerealis and T. foetus. Dr Bruce Corney is also responsible for the

molecular diagnostic applications in Biosecurity DPI&F and for the delivery of these new protocols for DPI&F (Qld).

6.4 Preliminary screen of virulence genes within Australian isolates of *Campylobacter fetus* subsp. *venerealis*

Twenty-seven field extracts (positive using the *C. fetus* subsp. *venerealis Taq* nuclease assay) and 3 Australian *C. fetus* subsp. *venerealis* reference strains were screened using 32 assays developed to detect putative virulence genes.

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

7.1 Bovine Venereal Diseases – campylobacteriosis and trichomoniasis

The primary feature of these diseases is the failure to conceive and embryo loss. Normal fertility resumes after initial exposure to *C. fetus* subsp. *venerealis* whereas *T. foetus* infections in cows can recur. Bulls are carriers and are asymptomatic. Reproductive wastage in infected herds represents are large economic loss for producers, where gross margins can be reduced by as much as 36-66% and 50% respectively for *C. fetus* subsp. *venerealis* and *T. foetus*. Campylobacteriosis can be managed through the use of a commercial vaccine, whereas for trichomoniasis there is no treatment or vaccine available in Australia. Both diseases are OIE notifiable diseases and are significant in the international trade of animals and animal products.

The OIE recommended protocols for the detection of these pathogens have long been suspected as poorly sensitive. The isolation of *C. fetus* subsp. *venerealis* depends on direct isolation of the organism by selective culture from semen, preputial smegma or vaginal mucus, or through the detection of previous exposure using a cervico-vaginal ELISA method. Both methods are prone to false negative and false positive results with a high percentage of *C. fetus* subsp. *venerealis* susceptible to the antibiotics used in the selective media. Microscopic examination of smegma following transport/enrichment is recommended for the recognition of *T. foetus* without the need for further selective isolation. This method itself is not sensitive and can deliver false positive results through the contamination of morphologically similar intestinal or coprophilic trichomonads. The *Taq* nuclease assays demonstrated a 100 and a 1000 fold increase in the sensitivity of detection for *C. fetus* subsp. *venerealis* and *T. foetus* in smegma samples respectively.

Improved detection of both of these diseases '**now**', has provided tools for future epidemiological studies to enable veterinarians and clinicians to determine the impact of the diseases on herd health. Both pathogens were detected during the course of this research program however, it is not known how the pathogens survive in a herd and under what conditions reproductive losses are induced. The assays are very sensitive and can provide a quantitative estimate which can be correlated to cells/ml for each pathogen. This will assist to answer some of the epidemiological questions which resulted from the case studies undertaken here. In addition, widespread adoption and evaluation of the methods both in Australia and overseas will assist to gather further epidemiological data under a variety of production systems.

In **5 years time**, Meat and Livestock industries may recommend that a trichomoniasis vaccine become available in Australia (Trichguard®, Fort Dodge). Currently there is no treatment and treating bulls prior to mating would seem the best option for the management of this disease. This will confirm the disease free status of our export cattle ($9.6B industry for Australia) and minimise disease impacts within herds. An additional factor requiring investigation is the impact of *T. foetus* reservoirs such as feral pigs (*T. foetus* is an intestinal commensal) and management of pigs may simply decrease the spread of this pathogen.

The research described here undertaken with the detection of *C. fetus* subsp. *venerealis* was convincing, however, research in the northern hemisphere has cast doubts upon the use of the gene used here for the identification of this subspecies. This however, may not be the case in Australia, but in a global sense, the test may not be applicable. Our final milestone research indicated that it is feasible that less pathogenic organisms occupy the bull prepuce and further research is required to determine the impact of this new knowledge. Genome sequencing had made the advance in this knowledge possible. Thus for campylobacteriosis, in **5 years**, we may

be able to confirm which subspecies and which genes are best targeted for the identification of pathogenic *C. fetus* subsp. *venerealis* implicated in campylobacteriosis.

*Thus in summary, the impact on meat and livestock industries **now***:

- the availability of 2 new sensitive assays for the detection of *C. fetus* subsp. *venerealis* and *T. foetus* available for herd studies
- the sensitive and direct detection of bovine venereal diseases from bull smegma without the need for transport medium

*And, the impact on the meat and livestock industries in **5 years***:

- decreased venereal disease and improvement in productivity due to the ability to detect *T. foetus* both in bulls and in other reservoirs
- improved management of trichomoniasis world-wide through the wide spread adoption of new molecular diagnostic tools
- improved understanding of *C. fetus* subsp. *venerealis* differences at both the subspecies and biovar level
- new molecular diagnostic tools to enable the detection of virulent bovine campylobacteriosis pathogens, thus enabling improved management of delayed conceptions within affected herds
- decreased economic loss attributed to bovine venereal diseases (up to 66% of gross margins)
- improved trade and market access
- ability to ensure disease free status of semen and breeding stock

7.2 Ephemeral Fever

Bovine ephemeral fever is an insect vectored disease of cattle and water buffalo occurring in tropical, subtropical and temperate climate zones. In Australia it is controlled by vaccination but can cause heavy economic losses due to reduced milk production and lowered male fertility. The impact on Meat and Livestock industries **now** from this research is the availability of a sensitive test for this virus. In **5 years** the assay would have provided improved service to industry by ensuring sensitive detection of the pathogen and thus providing advice for the implementation of vaccination programs. Together with the vaccines available for BEF, a new sensitive diagnostic tool will enable early detection and thus assist to prevent future production losses in the event of an outbreak.

7.3 Leptospira

This is a report on the development and partial validation of a diagnostic test for bovine leptospirosis. The test '**now**' has the following advantages over traditional methods such as serology and culture:

- It will detect low levels of *Leptospira* in urine and kidney.
- It does not require the *Leptospira* to be viable when the samples arrive at the laboratory.
- It can be used to detect all pathogenic serovars.
- Results can be available within 1-2 days of the samples arriving at the laboratory.

It therefore reveals the infection status of animals in "real-time" and can be used on poor quality samples or when transportation to the laboratory takes several days, as is often the case with extensively-managed beef herds.

The test is currently being offered as a routine diagnostic test at BSL and results are reported with the following disclaimer:

"The PCR test for *Leptospira* is experimental and is not NATA-accredited. This test is undergoing final validation for use in disease diagnosis. A diagnosis made using this test is only presumptive."

This disclaimer will be removed once test validation is completed, and the test has been NATA accredited.

Uptake by other laboratories is expected to follow once the test is submitted to and approved by SCAHLS as an Australian Standard Diagnostic Test.

The main impact of this test on the Australian beef industry will be in terms of prevention and treatment of reproductive failure, and reduced risk of infection to farm and abattoir workers. To illustrate, economic modelling for tropical Queensland predicted that, in this region alone, if only 20% of producers implement leptospirosis control measures yielding a 3% increase in weaning rates, the accrued benefit to the local beef industry would be in the vicinity of $2.5 million. Improved diagnosis and control of bovine leptospirosis would yield similar benefits to the beef industry in other regions of tropical Australia. Furthermore, 25% of the 177 human notifications Australia-wide in 2004 for which occupation data are available were meatworkers/inspectors/butchers, graziers, or station hands (161). Thus leptospirosis has a significant financial impact on the industry through absence from work due to illness, lost productivity and possible workcover claims. Improved management of leptospirosis resulting from improved disease diagnosis would enable reduced risks to human health associated with handling and slaughtering infected cattle. This, in turn, will yield significant savings for the beef industry nationally and the public health system. We expect these savings to gradually increase as the test is promoted and uptake of the test by field veterinarians increases.
Other expected impacts on the Australian beef industry from the use of this test are:

- Improved ability to ensure disease free status of semen and breeding stock
- Improved fertility through the implementation of appropriate management programs
- Improved trade and market access
- Improved disease regulation through improved tools for diagnosis.

These impacts will be experienced progressively as uptake of the test increases but it may take 1-2 years (and thus by **5 years**) for the full impact to be realised.

8 Conclusions and Recommendations

8.1 Bovine Venereal Diseases

8.1.1 Trichomoniasis

Conclusions

- The 5' *Taq* nuclease assay (based on the internal transcribed spacer region 1) developed for the detection of *T. foetus* improved the detection of this pathogen ~1000 fold compared with the gold standard methods.
- Direct detection of *T. foetus* in diagnostic specimens simplifies laboratory transport.
- Crude lysis provided a higher sensitivity than kit extracts for *T. foetus* detection in clinical specimens.
- The sequence used as the target for this assay is highly conserved and specific for *T. foetus.*
- Bull smegma, vaginal mucus and bull urine can be used for detection of *T. foetus*, however, smegma is the most reliable diagnostic specimen due to female cattle pathogen clearance soon after infection and the inability to detect *T. foetus* in urine samples from infected bulls.
- A low prevalence of *T. foetus* was detected in Qld during the course of this study.
- The detection of *T. foetus* in female cattle did not have a definitive impact on reproductive failure in the herds studied.

Recommendations

- The 5' *Taq* nuclease assay (probe-based real time assay) for *T. foetus* is best conducted by screening smegma collected using the tricamper sampling tool and sent to the laboratory in saline. Specimens can be sent at ambient temperatures without subsequent losses in assay sensitivity or specificity.
- NATA and SCAHLS approval should be sought through DPI&F Biosecurity laboratories.
- International evaluation of the method should be undertaken.
- Investigation of herd disease epidemiology which includes frequent sampling of animals pre- and post-mating will assist to determine the impact of the quantified infective load of the pathogen in a herd on delayed conceptions and embryonic loss.
- Determine the economic benefit of *T. foetus* vaccination and determine whether Australian beef industries would benefit from the availability of a trichomoniasis vaccine.

8.1.2 Campylobacteriosis

Conclusions

- The 5' *Taq* nuclease assay (based on the Hum PCR target shown to identify Australian *C. fetus* subsp. *venerealis*) developed for the detection of *C. fetus* subsp. *venerealis* improved the detection of this pathogen ~100 fold compared with the gold standard methods.
- Direct detection of *C. fetus* subsp. *venerealis* in diagnostic specimens simplifies laboratory transport.
- Crude lysis provided a higher sensitivity than kit extracts for *C. fetus* subsp. *venerealis* detection in clinical specimens.
- Recent literature suggests that the gene target for this assay may not be suitable for the identification of *C. fetus* subsp. *venerealis* isolates in England, however, phenotypic methods used to identify the subspecies are also questionable.

- Bull smegma, vaginal mucus and bull urine can be used for detection of *C. fetus* subsp. *venerealis*, however, smegma is the most reliable diagnostic specimen due to female cattle pathogen clearance soon after infection and the poor success in detecting *C. fetus* subsp. *venerealis* in urine samples from smegma positive bulls.
- A high prevalence of *C. fetus* subsp. *venerealis* was detected in Qld during the course of this study.
- The detection of *C. fetus* subsp. *venerealis* (using the assay developed in this study) in bulls pre- and post- mating correlated with a decrease in conception rates in the corresponding female cattle.
- Following the preliminary screening of 3 Australian reference strains and 27 field preparations of *C. fetus* subsp. *venerealis* in 32 assays detecting 32 putative *C. fetus* subsp. *venerealis* virulence genes, it was concluded that it is feasible that the bull prepuce harbours *C. fetus* subsp. *venerealis*–like organisms with lowered virulence.

Recommendations

- The 5' *Taq* nuclease assay (probe-based real time assay) for *C. fetus* subsp. *venerealis* is best conducted by screening smegma collected using the tricamper sampling tool and sent to the laboratory in saline. Specimens can be sent at ambient temperatures without subsequent losses in assay sensitivity or specificity.
- The availability of the tricamper sampling tool should be promoted in a future MLA Feedback Article - to enable wider awareness of the benefits of using the tool for VD screening and how to obtain the tool currently.
- NATA and SCAHLS approval should be sought through DPI&F Biosecurity laboratories.
- Investigation of herd disease epidemiology which includes frequent sampling of animals pre- and post-mating will assist to confirm the impact of the quantified infective load of pathogen in a herd on delayed conceptions and embryonic loss.
- Campylobacteriosis vaccine efficacy needs to be re-determined under control trial conditions, in collaboration with the vaccine manufacturer.
- International collaboration will assist to confirm the identification of subspecies and biovars and their impact on campylobacteriosis disease outcome.
- Completed genome sequencing of *C. fetus* subsp. *venerealis* should be undertaken (only 72% of Argentina strain completed) to allow comprehensive genomic comparison with *C. fetus* subsp. *fetus* and other related *Campylobacter* species.
- Genome sequencing of *C. fetus* subsp. *venerealis* biovar *intermedius* will assist to confirm similarity to *C. fetus* subsp. *venerealis* and to enable further research to determine whether this pathogen is clinically implicated in bovine campylobacteriosis.
- The evaluation of the 5 new *C. fetus* subsp. *venerealis* 5' *Taq* nuclease assays developed here is required (not within the scope of this study).
- Further screening of the virulence genes using characterised strains of *C. fetus* subsp. *venerealis* and *C. fetus* subsp. *fetus* needs to be undertaken (include collections from collaborators and other countries).
- The presence of less pathogenic *C. fetus* subsp. *venerealis* –like organisms needs to be confirmed and their capacity to cause venereal disease needs to be determined.
- Publication of the bioinformatics and virulence gene analyses undertaken in the final milestone research – these analyses are a world first.

8.1.3 Bovine Ephemeral Fever

Conclusions

The 5' *Taq* nuclease RT-PCR assay described here improved upon the sensitivity and specificity of current bovine ephemeral fever virus detection methods and with a high degree of correlation with current methods was demonstrated.

Recommendations

The BEFV assay should be fully adopted by veterinary diagnostic laboratories for the investigation of BEFV outbreaks where applicable.

8.1.4 Leptospira

Conclusions

Use of the modified TaqMan® assay. The data demonstrate that the modified TaqMan® assay of Smythe *et al.* (2002) is useful for diagnosing bovine leptospirosis, especially as a herd test, and is a significant improvement on traditional tests. Urine (first or second void after administering a diuretic) and tissue samples are suitable for the modified TaqMan® assay. Urine samples may be prepared using either the QIAamp DNA Mini kit, or heat lysis, although the latter is preferred due to its speed and simplicity. The assay was sufficiently robust and easily transferred to other laboratories.

Recommendations

Use of the modified TaqMan® assay. Because of the often intermittent nature of *Leptospira* shedding in urine (Faine, 1982) and the low cell numbers often present (as reflected in the C_T values observed in the field trial), the assay is best used as a herd test when investigating poor reproductive performance. A reasonable sample size would be about 15-20 animals per group, based on the proportion of positive or suspect urine samples observed in the field study.

The modified TaqMan® assay may also be used on tissues from aborted foetuses and tissues from post mortems when investigating abortion cases or cases of overt leptospirosis in individual animals.

Recommendations for future work

- The modified TaqMan® assay is validated to a standard sufficient for use as a diagnostic test for bovine leptospirosis. However, further validation is required for NATA-accreditation and SCAHLS approval as a standard test for leptospirosis. This would include confirmation that vaccines do not interfere with the modified TaqMan® assay, further validation of the use of the modified TaqMan® assay on tissues, and collecting more data comparing the modified TaqMan® assay to culture using urine.
- A statistically designed trial needs to be conducted to demonstrate that common Leptospirosis vaccines do not interfere with the modified TaqMan® assay.
- A statistically designed trial comparing the three methods of tissue extraction with each other and with culture or silver staining is required. This would involve experimentally infecting a number of animals, and collecting tissue samples once the excretion of *Leptospira* in urine is demonstrated.
- Although a comparison of the modified TaqMan® assay and culture was obtained using samples from experimentally infected heifers, the data obtained from this was limited. Culture failed completely when used on samples from remote field herds. Samples need to be collected from beef or dairy cattle in relative proximity to Brisbane, to maximise the chance of successfully isolating *Leptospira*. Also, the culture method used in AHW.036 was developed

for isolating *Leptospira* from human samples, which are collected under cleaner conditions than bovine samples and are thus less susceptible to contamination with other bacteria. Culture methods that were previously used at ARI (111) with reasonable success might be more suitable.

- The *Leptospira* case study raised the possibility of synergistic effects between *L. borgpeterseni* serovar Hardjo and other pathogens having a role in bovine reproductive failure. Although a project to test this hypothesis would be expensive, it could provide invaluable insight into the causes and prevention of reproductive failure in cattle.
- Promotion of the test to field veterinarians has been limited due to the need for further validation. Promotion will be resumed as more validation data is acquired.

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10 Appendices

10.1 Appendix 1 - Stage 2 field Sampling Instructions

Materials required in each sampling exercise:

Component	Heifer (A*)	Bull (B*)	Slaughtered heifer (C*)	Slaughtered bull (D*)
Vacutainer	1	1	2	2
Vacutainer needle and collar	1	1	2	2
2 mL syringe + 19G needle	1	1	1	1
5 mL syringe + 21G needle		1		
Tricamper	1	1	1	1
Transfer pipettes	4	4	4	4
5 mL saline (tube **5**)	1	1	1	1
5 mL Mod. Weybridge (tube **2**)	1	1	1	1
InPouch TF test kit	1	1	1	1
3 mL lepto media (tube **4**)	1	1	1	1
25 mL specimen container (tube **7**)	1	2	1	1
10 mL lepto media (tube **6**)	1	1		
Frusemide		1		
Sharps bin	Yes	Yes	Yes	Yes
Biopsy tools			Yes	Yes
Labels	Yes	Yes	Yes	Yes
Zip-lock bags	Yes	Yes	Yes	Yes

* Detailed procedures in sections A, B, C and D.

A. Bovine venereal disease diagnostics (Heifer)

This is a guide to for field staff in sample collection and handling from live heifers to achieve best laboratory results. The target organisms are:

Campylobacter fetus subsp. *Venerealis*	referred to hereafter as “Camp”
Tritrichomonas foetus	referred to hereafter as “Trich”
pathogenic *Leptospira*	referred to hereafter as “Lepto”.

The assays these samples are used for are extremely sensitive and specific, and are capable of detecting a single target organism. Avoiding cross-contamination is critical, so there are a couple of basic procedures that are strongly recommended.

- Wash hands with disinfectant and rinse well with water between animals. *C. fetus* subsp. *venerealis*, *T. foetus*, and pathogenic *Leptospira* should be readily susceptible to common disinfectants.
- Do not re-use any sampling equipment between animals.


All three pathogens are capable of infecting a wide range of mammals (including humans), and can cause serious illness or even death. Reasonable precautions must be taken against possible infection – major infectious routes are ingestion, inhalation, cuts and abrasions, and eyes, as well as venereal. A mask and safety glasses are strongly recommended, as is keeping any injuries covered.

Materials
(per heifer)

Vacutainer tube (plain) with 18 G vacutainer needle & collar (primary blood sample)
Sampling tool (tricamper)
5 mL saline in yellow-capped sample tube (primary mucus sample) (tube **5**)
2 mL syringe with 19G needle (to rinse tricamper)
5 mL modified Weybridge media in yellow-capped sample tube (tube **2**, Black media)
InPouch TF test kit
3 mL Leptospira media (tube **4**)
25 mL specimen container for urine (primary urine sample) (tube **7**)
10 mL Leptospira media in yellow-capped bottle (tube **6**)
2 mLTransfer pipettes (4) (sub-sampling primary samples)

Additional materials:
- Tissues
- Needle notcher for sharps disposal
- Sheets of labels (33 labels/sheet)
- Ice brick
- Packing material (shredded paper/cotton wool)
- Diagnostic specimen transport pack

General method
Collect the 3 primary samples (blood, mucus, and urine) crush-side from each animal into labelled tubes. It is very important to have an assistant for this procedure. Remember the advice concerning cross-contamination and potential for infection above and act accordingly.
Avoid samples being exposed to elevated temperatures or directly to the sun; best practice is to place samples into an esky with a freezer pack until sub-sampling is carried out.
Transfer to a suitable place for sub-sampling each sample into the required labelled containers and media.
Following sub-sampling, package samples in a chilled esky according to instructions and ship to lab.

1 Sample collection

1.1 Sample from behind a vet gate without head bailing.
1.2 Blood: Blood is collected from heifers using a 6 mL plain vacutainer, with an 18 G vacutainer needle. Blood may be collected by jugular venipuncture or tail stab.
1.3 Vaginal mucus: The vulva is opened with one hand, and a tricamper inserted in a dorso-cranial direction with the leading edge of the instrument in contact with the dorsal vagina. Once there is no risk of the instrument entering the urethra, entry progresses to a cranial movement as for artificial breeding so that the anterior end reaches the cervix. The tricamper is moved gently backwards and forwards. The end of the tricamper is blocked (typically by a finger) to prevent any of the collected material being suctioned out, and then removed. The instrument is held just off horizontal, the tip inserted into the tube of saline (tube **5**) and the block removed from the end of the instrument. While an assistant holds the sample bottle, the mucus is rinsed off with 5 mL physiological saline, using a syringe with attached needle to physically rinse off mucus. Alternatively, the black head of the tricamper may be cut off with side-cutters into the saline.
1.4 Urine: Vertical stroking of the perineum towards the ventral commissure of the vulva is used to induce urination, and 20 mL mid-stream first void urine is collected in tube **7**.

2 Sample preparation

2.1 Fill out the provided labels with animal ID, location, date, operator, and sample type (see table below), and label each of the 7 samples to be sent to the lab.

2.2 Using transfer pipettes, 0.5 mL of the mucus in saline (tube **5**) is transferred into modified Weybridge media (tube **2**), and a further 0.5 mL used to inoculate InPouch TF test kits. 1 mL of the mucus is used to inoculate 3 mL of Leptospira transport medium (tube **4)**. The approximately 3 mL remaining is retained for transport to the lab. 1 mL of the urine (tube **7**) is used to inoculate 10 mL of the Leptospira transport medium (tube **6**), and the remaining urine retained for transport to the lab.

Sample number	Primary sample	Volume inoculated into	Assays in lab
1	Blood	Transported as is	Lepto ELISA
2	Mucus in saline (**5**)	0.5 mL into Modified Weybridge (**2**)	Camp culture
3	Mucus in saline (**5**)	0.5 mL into InPouch TF test	Trich culture
4	Mucus in saline (**5**)	1 mL into *Leptospira* media (3 mL) (**4**)	Lepto culture
5	Mucus in saline	Transported as is	*Taq* assays, ELISA
6	Urine (**7**)	1 mL into *Leptospira* media (9 mL) (**6)**	Lepto culture
7	Urine	Transported as is	*Taq* assays

3 Sample transport

3.1 Samples must be packed in accordance with IATA packing instruction 650.

3.2 Ensure sample tubes are correctly labelled and lids are on tight.

3.3 Pack all tubes from a given animal into the zip-lock bag they came in.

3.4 All samples are placed into a Bio-bottle, along with an ice brick and as much absorbent material (eg shredded paper, cotton wool) as can be fitted. The bottle is sealed, and placed inside the insulated container.

3.5 An itemized list of contents (ie: X veterinary diagnostic samples in PBS, Y diagnostic samples in modified Weybridge media, etc) must be included between the insulated container and the cardboard box. Place the insulated in the cardboard box, and seal it.

3.6 Add labels for shipping. The package and consignment notice must include the text: ”DIAGNOSTIC SPECIMEN PACKED IN COMPLIANCE WITH IATA PACKING INSTRUCTION 650”, and the “Dangerous Goods” box on the consignment notice must be ticked.

3.7 Ship to the ARI via courier.

B. Bovine venereal disease diagnostics (Bull)

This is a guide to for field staff in sample collection and handling from live bulls to achieve best laboratory results. The target organisms are:

Campylobacter fetus subsp. *Venerealis*	referred to hereafter as "Camp"
Tritrichomonas foetus	referred to hereafter as "Trich"
pathogenic *Leptospira*	referred to hereafter as "Lepto".

The assays these samples are used for are extremely sensitive and specific, and are capable of detecting a single target organism. Avoiding cross-contamination is critical, so there are a couple of basic procedures that are strongly recommended.

- Wash hands with disinfectant and rinse well with water between animals. *C. fetus* subsp. *venerealis*, *T. foetus*, and pathogenic *Leptospira* should be readily susceptible to common disinfectants.
- Do not re-use any sampling equipment between animals.

All three pathogens are capable of infecting a wide range of mammals (including humans), and can cause serious illness or even death. Reasonable precautions must be taken against possible infection – major infectious routes are ingestion, inhalation, cuts and abrasions, and eyes, as well as venereal. A mask and safety glasses are strongly recommended, as is keeping any injuries covered.

Materials

Field test kit (per bull):

Vacutainer tube (plain) with 18 G vacutainer needle & collar (primary blood sample)
Sampling tool (tricamper)
2 mL syringe with 19G needle (to rinse tricamper)
5 mL saline in yellow-capped sample tube (primary smegma sample) (tube **5**)
5 mL modified Weybridge media in yellow-capped sample tube (Tube **2**, Black media)
InPouch TF test kit
3 mL Leptospira media (Tube **4**)
Frusemide with 5 mL syringe & 21G needle
2 × 25 mL specimen containers for urine (primary urine sample) (Tubes **7** & **7.1**)
10 mL Leptospira media in large yellow-capped tube (tube **6**)
2 mLTransfer pipettes (4) (sub-sampling primary sample)

Additional materials:

Tissues
Needle notcher for sharps disposal
Sheets of labels (33 labels/sheet)
Ice brick
Packing material (shredded paper/cotton wool)
Diagnostic specimen transport pack

General method

Collect the 3 primary samples (blood, smegma, urine) crush-side from each animal into labelled tubes. It is very important to have an assistant for this procedure. Remember the advice concerning cross-contamination and potential for infection above and act accordingly.
Avoid samples being exposed to elevated temperatures or directly to the sun; best practice is to place samples into an esky with a freezer pack until sub-sampling is carried out.
Transfer to a suitable place for sub-sampling each sample into the required labelled containers and media.

Following sub-sampling, package samples in a chilled esky according to instructions and ship to lab.

1 Sample collection

1.1 A head bail and back bar in a crush with a lower-half side gate is recommended to ensure safe sampling from the prepuce.

1.2 Blood: Blood is collected from bulls using a 6 mL plain vacutainer, with an 18 G vacutainer needle. Blood may be collected by jugular venipuncture or tail stab.

1.3 Preputial smegma: Whilst holding the anterior aspect on the sheath with one hand, insert the sampling tool into the prepuce, with the end adjacent to the penis. Move the tool back and forward, so that it scrapes across the preputial mucosa and surface of the penis. The end of the tricamper is blocked (typically by a finger) to prevent any of the collected material being suctioned out, and then removed. The instrument is held just off horizontal, the tip inserted into the tube of saline (tube **5**) and the block removed from the end of the instrument. While an assistant holds the sample bottle, the smegma is rinsed off with 5 mL saline, using a syringe with attached needle to physically rinse off smegma. Alternatively, the black head of the tricamper may be cut off with side-cutters into the saline.

1.4 Urine: Bulls often dribble, so urine may be collected this way. If the bull isn't urinating, inject 5-10 mL frusemide subcutaneously in the neck area. Use a 21G needle, and introduce the needle as slowly as possible to minimise reaction from the bull. Two bottles of urine (First void in tube **7**, mid-stream in tube **7.1**) can then be collected using a sample container in a holder.

2 Sample preparation

1.5 Fill out the provided labels with animal ID, location, date, operator, and sample type (see table below), and label each of the 8 samples to be sent to the lab.

1.6 Using transfer pipettes, 0.5 mL of the smegma in saline (tube **5**) is transferred into modified Weybridge media (tube **2**), and a further 0.5 mL used to inoculate InPouch TF test kits. 1 mL of the smegma is used to inoculate 3 mL of Leptospira transport medium (tube **4**). The approximately 3 mL remaining is retained for transport to the lab. 1 mL of the mid-stream urine (Tube **7.1**) is used to inoculate 10 mL of the Leptospira transport medium (tube **6**), and the remaining urine retained for transport to the lab.

Sample number	Primary sample	Volume inoculated into	Assays in lab
1	Blood	Transported as is	Lepto ELISA
2	Smegma in saline (**5**)	0.5 mL into Modified Weybridge (**2**)	Camp culture
3	Smegma in saline (**5**)	0.5 mL into InPouch TF test	Trich culture
4	Smegma in saline (**5**)	1 mL into *Leptospira* media (3 mL) (**4**)	Lepto culture
5	Smegma in saline	Transported as is	*Taq* assays, ELISA
6	Urine (**7.1**)	1 mL into *Leptospira* media (9 mL) (**6**)	Lepto culture
7 & 7.1	Urine	Transported as is	*Taq* assays

3 Sample transport

3.1 Samples must be packed in accordance with IATA packing instruction 650.

3.2 Ensure sample tubes are correctly labelled and lids are on tight.

3.3 Pack all tubes from a given animal into the zip-lock bag they came in.

3.4 All samples are placed into a Bio-bottle, along with an ice brick and as much absorbent material (eg shredded paper, cotton wool) as can be fitted. The bottle is sealed, and placed inside the insulated container.
3.5 An itemized list of contents (ie: X veterinary diagnostic samples in PBS, Y diagnostic samples in modified Weybridge media, etc) must be included between the insulated container and the cardboard box. Place the insulated in the cardboard box, and seal it.
3.6 Add labels for shipping. The package and consignment notice must include the text: ”DIAGNOSTIC SPECIMEN PACKED IN COMPLIANCE WITH IATA PACKING INSTRUCTION 650”, and the “Dangerous Goods” box on the consignment notice must be ticked.
3.7 Ship to the ARI via courier.

C. Bovine venereal disease diagnostics (Slaughtered Heifer)
This is a guide to for field staff in sample collection and handling from slaughtered heifers to achieve best laboratory results. The target organisms are:

Campylobacter fetus subsp. *Venerealis*	referred to hereafter as “Camp”
Tritrichomonas foetus	referred to hereafter as “Trich”
pathogenic *Leptospira*	referred to hereafter as “Lepto”.

The assays these samples are used for are extremely sensitive and specific, and are capable of detecting a single target organism. Avoiding cross-contamination is critical, so there are a couple of basic procedures that are strongly recommended.

- Where possible, wash hands with disinfectant and rinse well with water between animals. *C. fetus* subsp. *venerealis*, *T. foetus*, and pathogenic *Leptospira* should be readily susceptible to common disinfectants.
- Do not re-use any sampling equipment between animals.


All three pathogens are capable of infecting a wide range of mammals (including humans), and can cause serious illness or even death. Reasonable precautions must be taken against possible infection – major infectious routes are ingestion, inhalation, cuts and abrasions, and eyes, as well as venereal. A mask and safety glasses are strongly recommended, as is keeping any injuries covered.

Materials
(per heifer carcase)
2 Vacutainer tubes (plain) with 18 G vacutainer needles & collar (primary blood sample, & primary urine sample from bladder)
Sampling tool (tricamper)
2 mL syringe with 19G needle (to rinse tricamper)
5 mL saline in yellow-capped sample tube (primary mucus sample) (tube **5**)
5 mL modified Weybridge media in yellow-capped sample tube (Black media) (Tube **2**)
InPouch TF test kit
3 mL Leptospira media (Tube **4**)
25 mL specimen container for kidney biopsy (primary biopsy sample) (Tube **7**)
2 mLTransfer pipettes (3) (sub-sampling primary sample)

Additional materials:
Tissues
Needle notcher for sharps disposal
Sheets of labels (33 labels/sheet)
Ice brick
Packing material (shredded paper/cotton wool)
Diagnostic specimen transport pack

General method
Collect the 4 primary samples (blood, mucus, urine, & kidney) from each animal into labelled tubes. It will probably be most practical to collect the required organs from the carcase (vagina, kidney, bladder) and a blood sample into a separate container on the slaughterhouse floor for sampling as soon as possible in a more convenient location. Remember the advice concerning cross-contamination and potential for infection above and act accordingly.
Avoid samples being exposed to elevated temperatures; best practice is to place samples into an esky with a freezer pack until sub-sampling is carried out.
Following sub-sampling, package samples in a chilled esky according to instructions and ship to lab.

1 Sample collection

1.1 Blood: If feasible, blood is collected from the carcase using a 6 mL plain vacutainer, with an 18 G vacutainer needle.
1.2 Vaginal mucus: The vulva is opened with one hand, and a tricamper inserted so that the anterior end reaches the cervix. The tricamper is moved backwards and forwards, and then removed. The collected mucus is rinsed off into approximately 5 mL saline (Tube **5**) using a syringe with attached needle to physically rinse off mucus. Alternatively, the black head of the tricamper may be cut off with side-cutters into the saline.
1.3 Urine: If feasible, urine is collected from the carcase by puncturing the bladder with an 18G needle, attached to a 6 mL vacutainer.
1.4 Kidney biopsy: A kidney biopsy, approximately 2 cm by 2 cm by 2 cm, is taken from the carcase.

2 Sample preparation

2.1 Fill out the provided labels with animal ID, location, date, operator, and sample type (see table below), and label each of the 7 samples to be sent to the lab.
2.2 Using transfer pipettes, 0.5 mL of the mucus in saline (tube **5**) is transferred into modified Weybridge media (tube **2**), and a further 0.5 mL used to inoculate InPouch TF test kits. 1 mL of the mucus is used to inoculate 3 mL of Leptospira transport medium (tube **4**). The approximately 3 mL remaining is retained for transport to the lab.

Sample number	Primary sample	Volume inoculated into	Assays in lab
1	Blood	Transported as is	Lepto ELISA
2	Mucus in saline (**5**)	0.5 mL into Modified Weybridge (**2**)	Camp culture
3	Mucus in saline (**5**)	0.5 mL into InPouch TF test	Trich culture
4	Mucus in saline (**5**)	1 mL into *Leptospira* media (3 mL) (**4**)	Lepto culture
5	Mucus in saline	Transported as is	*Taq* assays, ELISA
6	Urine	Transported as is	*Taq* assays
7	Kidney Biopsy	Transported as is	Lepto culture

3 Sample transport

3.1 Samples must be packed in accordance with IATA packing instruction 650.
3.2 Ensure sample tubes are correctly labelled and lids are on tight.
3.3 Pack all tubes from a given animal into the zip-lock bag they came in.
3.4 All samples are placed into a Bio-bottle, along with an ice brick and as much absorbent material (eg shredded paper, cotton wool) as can be fitted. The bottle is sealed, and placed inside the insulated container.

3.5 An itemized list of contents (ie: X veterinary diagnostic samples in PBS, Y diagnostic samples in modified Weybridge media, etc) must be included between the insulated container and the cardboard box. Place the insulated in the cardboard box, and seal it.
3.6 Add labels for shipping. The package and consignment notice must include the text: ”DIAGNOSTIC SPECIMEN PACKED IN COMPLIANCE WITH IATA PACKING INSTRUCTION 650”, and the “Dangerous Goods” box on the consignment notice must be ticked.
3.7 Ship to the ARI via courier.

D. Bovine venereal disease diagnostics (Slaughtered Bull)

This is a guide to for field staff in sample collection and handling from slaughtered bulls to achieve best laboratory results. The target organisms are:

Campylobacter fetus subsp. *Venerealis*	referred to hereafter as "Camp"
Tritrichomonas foetus	referred to hereafter as "Trich"
pathogenic *Leptospira*	referred to hereafter as "Lepto".

The assays these samples are used for are extremely sensitive and specific, and are capable of detecting a single target organism. Avoiding cross-contamination is critical, so there are a couple of basic procedures that are strongly recommended.

- Where possible, wash hands with disinfectant and rinse well with water between animals. *C. fetus* subsp. *venerealis*, *T. foetus*, and pathogenic *Leptospira* should be readily susceptible to common disinfectants.
- Do not re-use any sampling equipment between animals.


All three pathogens are capable of infecting a wide range of mammals (including humans), and can cause serious illness or even death. Reasonable precautions must be taken against possible infection – major infectious routes are ingestion, inhalation, cuts and abrasions, and eyes, as well as venereal. A mask and safety glasses are strongly recommended, as is keeping any injuries covered.

Materials

(per bull carcase)

2 Vacutainer tubes (plain) with 18 G vacutainer needles & collar (primary blood sample, & primary urine sample from bladder)
Sampling tool (tricamper)
2 mL syringe with 19G needle (to rinse tricamper)
5 mL saline in yellow-capped sample tube (primary smegma sample) (tube **5**)
5 mL modified Weybridge media in yellow-capped sample tube (Black media) (tube **2**)
InPouch TF test kit
3 mL Leptospira media (Tube **4**)
25 mL specimen container for kidney biopsy (primary biopsy sample) (tube **7**)
2 mLTransfer pipettes (3) (sub-sampling primary samples)

Additional materials:

Tissues
Needle notcher for sharps disposal
Sheets of labels (33 labels/sheet)
Ice brick
Packing material (shredded paper/cotton wool)
Diagnostic specimen transport pack

General method

Collect the 4 primary samples (blood, smegma, urine, & kidney) from each animal into labelled tubes. It will probably be most practical to collect the required organs from the carcase (preputial sheath, kidney, bladder) and a blood sample into a separate container on the slaughterhouse floor for sampling as soon as possible in a more convenient location. Remember the advice concerning cross-contamination and potential for infection above and act accordingly.
Avoid samples being exposed to elevated temperatures; best practice is to place samples into an esky with a freezer pack until sub-sampling is carried out.
Following sub-sampling, package samples in a chilled esky according to instructions and ship to lab.

1 Sample collection

1.1 Blood: If feasible, blood is collected from the carcase using a 6 mL plain vacutainer, with an 18 G vacutainer needle (if necessary).

1.2 Preputial smegma: The prepuce is pulled back, exposing the penis. Preputial smegma is collected by scraping a tricamper along the surface of the penis and internal prepuce near the fornix. The collected smegma is rinsed off into approximately 5 mL saline (tube **5**) using a syringe with attached needle to physically rinse off smegma. Alternatively, the black head of the tricamper may be cut off with side-cutters into the saline.

1.3 Urine: If feasible, urine is collected from the carcase by puncturing the bladder with an 18G needle, attached to a 6 mL vacutainer.

1.4 Kidney biopsy: A kidney biopsy, approximately 2 cm by 2 cm by 2 cm, is taken from the carcase.

2 Sample preparation

1.5 Fill out the provided labels with animal ID, location, date, operator, and sample type (see table below), and label each of the 7 samples to be sent to the lab.

1.6 Using transfer pipettes, 0.5 mL of the smegma in saline (tube **5**) is transferred into modified Weybridge media (tube **2**), and a further 0.5 mL used to inoculate InPouch TF test kits. 1 mL of the smegma is used to inoculate 3 mL of Leptospira transport medium (tube **4**). The approximately 3 mL remaining is retained for transport to the lab.

Sample number	Primary sample	Volume inoculated into	Assays in lab
1	Blood	Transported as is	Lepto ELISA
2	Smegma in saline (**5**)	0.5 mL into Modified Weybridge (**2**)	Camp culture
3	Smegma in saline (**5**)	0.5 mL into InPouch TF test	Trich culture
4	Smegma in saline (**5**)	1 mL into *Leptospira* media (3 mL) (**4**)	Lepto culture
5	Smegma in saline	Transported as is	*Taq* assays, ELISA
6	Urine	Transported as is	*Taq* assays
7	Kidney Biopsy	Transported as is	Lepto culture

3 Sample transport

3.1 Samples must be packed in accordance with IATA packing instruction 650.

3.2 Ensure sample tubes are correctly labelled and lids are on tight.

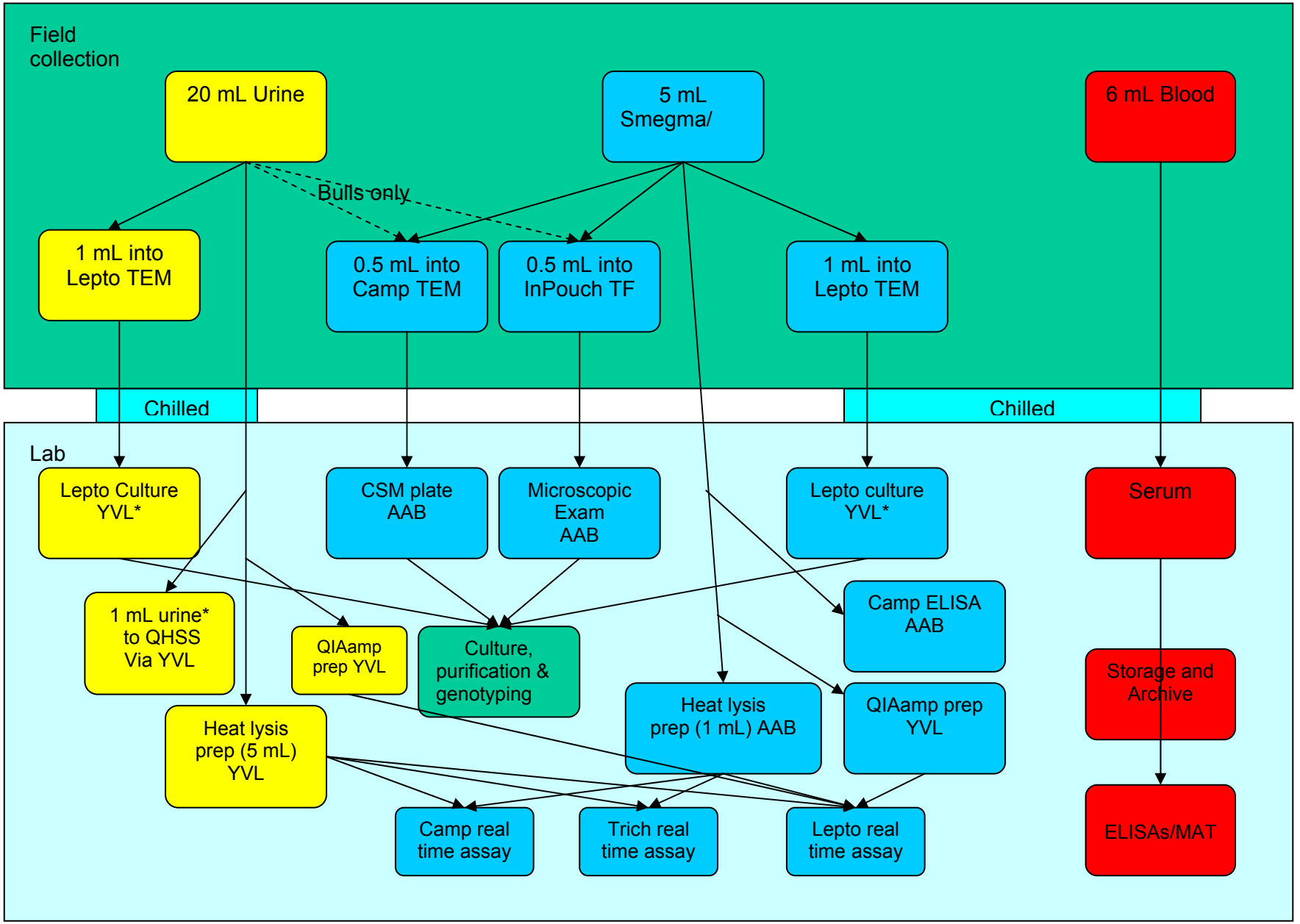
3.3 Pack all tubes from a given animal into the zip-lock bag they came in.

3.4 All samples are placed into a Bio-bottle, along with an ice brick and as much absorbent material (eg shredded paper, cotton wool) as can be fitted. The bottle is sealed, and placed inside the insulated container.

3.5 An itemized list of contents (ie: X veterinary diagnostic samples in PBS, Y diagnostic samples in modified Weybridge media, etc) must be included between the insulated container and the cardboard box. Place the insulated in the cardboard box, and seal it.

3.6 Add labels for shipping. The package and consignment notice must include the text: ”DIAGNOSTIC SPECIMEN PACKED IN COMPLIANCE WITH IATA PACKING INSTRUCTION 650”, and the “Dangerous Goods” box on the consignment notice must be ticked.

3.7 Ship to the ARI via courier.


Field collection
20 mL Urine
5 mL Smegma/
6 mL Blood
Bulls only
1 mL into Lepto TEM
0.5 mL into Camp TEM
0.5 mL into InPouch TF
1 mL into Lepto TEM
Chilled
Chilled
Lab
Lepto Culture YVL*
CSM plate AAB
Microscopic Exam AAB
Lepto culture YVL*
Serum
1 mL urine* to QHSS Via YVL
QIAamp prep YVL
Culture, purification & genotyping
Camp ELISA AAB
Storage and Archive
Heat lysis prep (5 mL) YVL
Heat lysis prep (1 mL) AAB
QIAamp prep YVL
Camp real time assay
Trich real time assay
Lepto real time assay
ELISAs/MAT

10.2 Appendix 2 – Centre for Comparative Genomics - bioinformatics analysis

The bioinformatics analyses were prepared by Paula Moolhuijzen (Senior Bioinformatics Officer) of WA's CCG (Murdoch University). Additional data is provided here by the CCG to outline the detailed methodologies applied to undertake the sequence analyses and searches, and to describe the database resources established at CCG to compare the available *C. fetus* subsp. *venerealis* genome data (UNSAM) to *C. fetus* subsp. *fetus* genome (NCBI) data.

The CCG received 273 FASTA format *C. fetus* subsp. *venerealis* contig sequences greater than 2 b of the 1187 assembled contigs from Prof. Daniel Sanchez (UNSAM, Argentina). Gene prediction was conducted using Glimmer3 (52, 144) for gene lengths greater than 100 nucleotide bases resulting in 1370 putative genes.

The 273 *C. fetus* subsp. *venerealis* contigs and 1370 putative genes were subsequently screened against public NCBI protein, patent databases and 1700+ *C. fetus* subsp. *fetus* protein sub-data set using BLASTX 2.2.13 (3). These results were then parsed using BIOPERL scripts into five categories below:

1. No hits to any proteins
2. *C. fetus* subsp. *fetus* with low significance alignments, expectation values greater than 1e-05
3. *C. fetus* subsp. *fetus* with expectation values lower than 1e-05
4. Low significance alignments, expectation values greater than 1e-05 to other species than *C. fetus* subsp. *fetus*
5. Significant alignments, expectation values less than 1e-05 to other species than *C. fetus* subsp. *fetus*.

Bioperl scripts were written to discern significant alignments in the contig sequences outside of the predicted gene regions.

The CCGs SOE is a web-based interactive database and visualisation tool used to store and display multiple sequence analyses. Multiple sequence alignment results were stored and viewed in SOE under password protection for *C. fetus* (thus accessible by QDPI&F). Figure 1 displays the alignments of *C. fetus* subsp. *venerealis* contigs, genes, and global proteins to *C. fetus* subsp. *fetus* genome sequences. Figure 2 displays the gene and protein alignments of flagellin B (motility) for *C. fetus* subsp. *fetus* Contig 3 nucleotide region 20000 to 30000 with *C. fetus* subsp. *venerealis*. These figures are based on computer screen images as seen when accessing SOE to provide a visual example of this resource provided specifically for this MLA project.

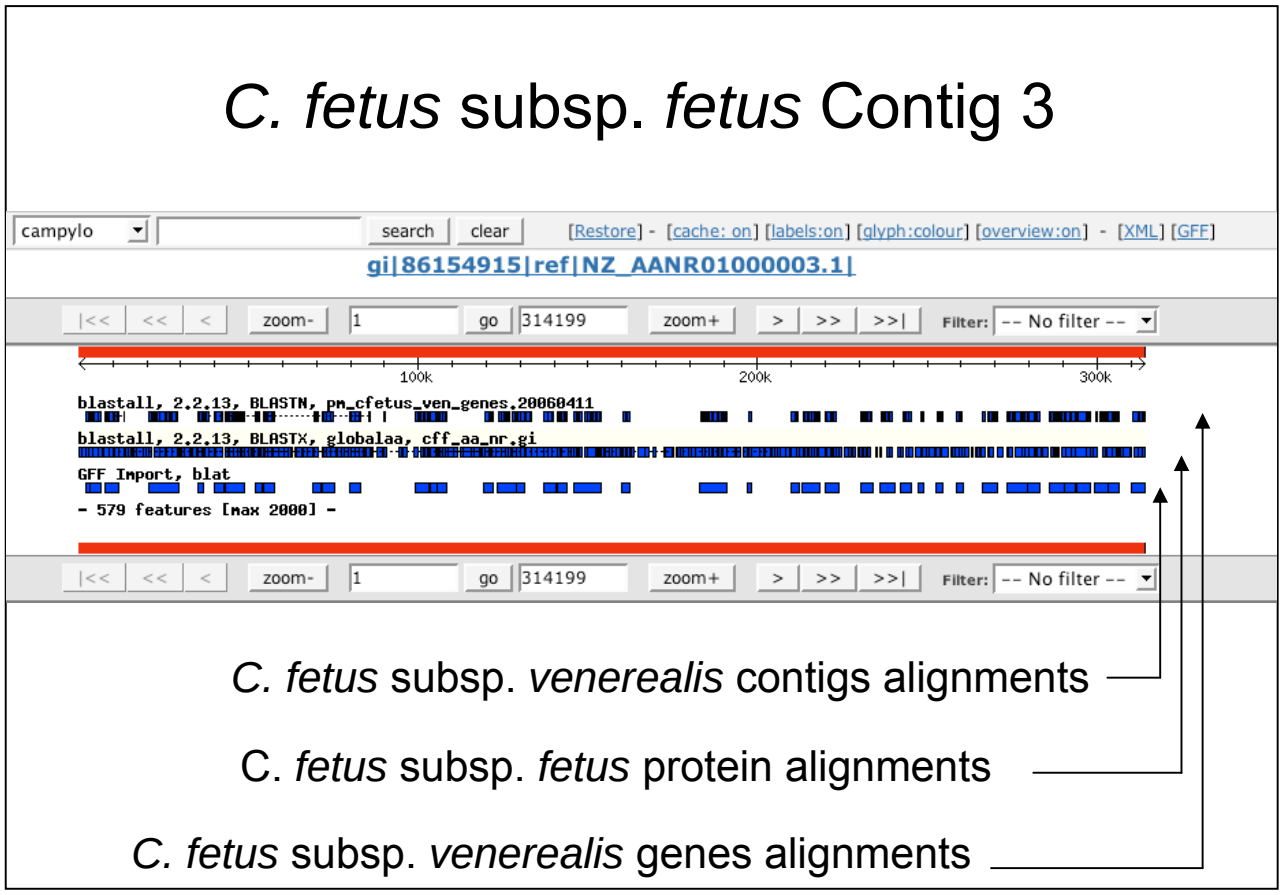


Figure 1. Representation of *C. fetus* subsp. *venerealis* contig and gene alignments against the *C. fetus* subsp. *fetus* genome sequence for contig 3

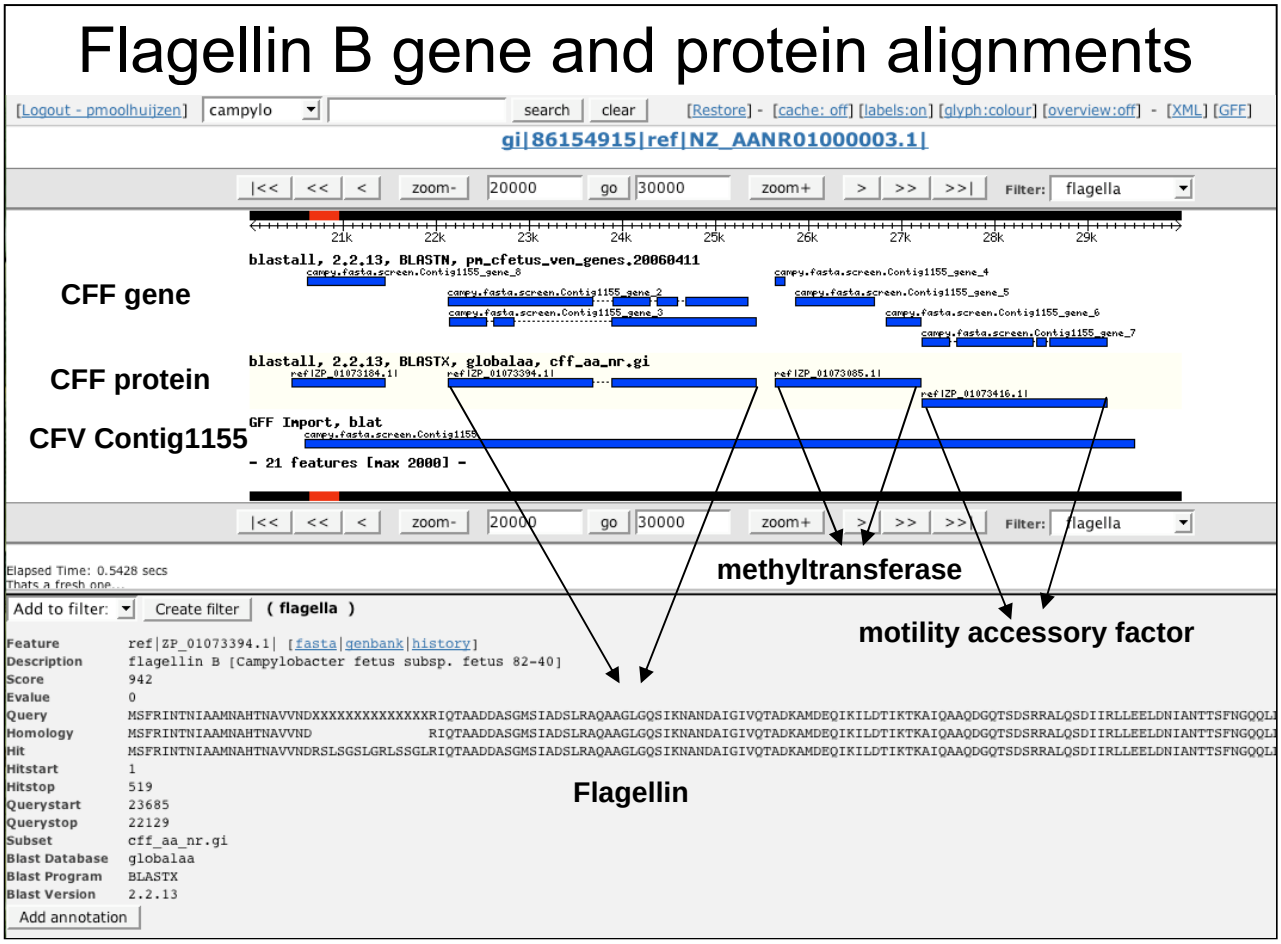


Figure 2. This figure displays the flagellin B (motility) gene and protein alignments for *C. fetus* subsp. *fetus* contig 3 nucleotide region 20000 to 30000 with corresponding *C. fetus* subsp. *venerealis* regions and sequence

10.3 Appendix 5 –BEFV *Taq* RT-PCR assay diagnostic sample screening

Table 1. Comparison of realtime (5' *Taq* nuclease) BEFV RT-PCR results with conventional gel-based RT-PCR results from diagnostic samples

RNeasy sample	Realtime Ct	Gel PCR	RNeasy sample	Realtime Ct	Gel PCR
20975	0.00	-	3/101751	21.02	+
156384	0.00	-	2/194846	22.81	+
155976	0.00	-	2/189064	21.27	+
156001	0.00	-	3/109545	17.80	+
04/19836	0.00	-	3/109700	21.21	+
4/155036-2	0.00	-	3/125270/2	23.38	+
4/150615	0.00	-	3/124622	24.13	+
4/18141	0.00	-	2/128635	0.00	-
4/151820	0.00	-	2/08982	29.34	+
4/151696	0.00	-	2/08773	22.02	+
4/151135	0.00	-	2/123681	21.97	+
17675	0.00	-	2/123402	22.46	+
50355	0.00	-	2/114202	21.89	+
150855	0.00	-	2/114055	20.06	+
144000	0.00	-	2/05040	24.33	+
16602	0.00	-	2/04810	20.19	+
142160-4	0.00	-	2/108430	23.08	+
142160-3	0.00	-	1/108051	23.16	+
142160-2	0.00	-	2/107570	22.38	+
142160-1	0.00	-	2/107531	30.92	+
4/140733-1	24.06	+	2/04060	28.72	+
4/138042-1	22.86	+	2/03996	27.75	+
4/138541	20.95	+	2/105772	25.29	+
4/137584	21.13	+	2/03586	29.55	+
4/137456	19.52	+	2/106156	23.15	+
4/135280	20.97	+	2/104583	20.05	+
4/134922	20.65	+	2/104432	19.93	+
4/134713	19.65	+	2/104424	26.93	+
4/09513	24.69	+	2/02366	22.29	+
4/09351	22.23	+	2/02223	26.02	+
4/09196	28.56	+	2/02173	23.67	+
4/128225	19.35	+	2/01630	25.68	+
4/127930	24.25	+	2/01541	27.14	+
4/08750	22.80	+	2/01162	24.37	+
		+			+
4/07592	22.46		2/00696	25.14	
4/126780	30.16	+	1/56626	24.23	+
4/126474	23.90	+	2/128241	21.52	+
4/125993	21.20	+	2/09111	20.25	+
4/124065	19.64	+	128635	0.00	-
4/124050	21.70	+	2/100353	25.88	+

RNeasy sample	Realtime Ct	Gel PCR	RNeasy sample	Realtime Ct	Gel PCR
4/7441-2	21.66	+	1/222810	26.22	+
4/07112	23.28	+	1/214884	26.51	+
4/112300	24.44	+	1/213254	30.24	+
4/05152	17.63	+	1/212146	19.04	+
1/04984	22.85	+	1/210751	32.04	+
4744	21.07	+	1/210380/2	24.29	+
3/157116	0.00	+/-	1/210380/1	20.69	+
3/11454	28.08	+	1/185074	23.20	+
3/10660	24.62	+	1/159371	23.17	+
3/131274	24.47	+	1/44714	18.69	+
3/09455	22.38	+	1/142716	18.69	+
3/130151	33.28	+	1/412585	25.23	+
3/129693	26.27	+	1/136380	21.63	+
3/129693/1	23.10	+	1/43491	23.07	+
3/129600/2	22.62	+	1/134454/2	22.51	+
3/129600/1	24.37	+	1/72076	25.60	+
3/128585	29.12	+	1/107941	22.27	+
3/128554	18.85	+	1/40651	0.00	-
3/125900	22.32	+	1/142651	22.43	+
3/124854	24.69	+	1/08966	27.09	+
03/125270/1	21.32	+	1/155125	31.38	+
03/116143	29.80	+	1/157936	31.05	+
03/115894/2	22.65	+	1/07561	27.70	+
3/40554	22.79	+	1/160202	27.70	+
3/105540	27.55	+	2/04825	20.92	+
3/104641	26.70	+			

Table 2. Comparison of realtime BEFV RT-PCR test results from the AQIS Viremia Trial with the conventional gel-based RT-PCR and traditional culture isolation

SAMPLE animal no./date	Realtime C_T	Gel PCR	Virus Isolation
859 27/2	0.00	-	-
859/1 1/3	0.00	-	-
859/1 2/3	0.00	-	-
859/1 3/3	0.00	-	-
859/1 4/3	0.00	-	-
859/1 5/3	0.00	-	-
859/1 6/3	0.00	-	-
859/1 7/3	0.00	-	-
859/1 8/3	0.00	-	-
859/1 9/3	0.00	-	-
861P 27/2	0.00	-	-
861/1 1/3	0.00	-	-
861/1 2/3	0.00	-	-
861/1 3/3	35.50	+	-

SAMPLE animal no./date	Realtime C_T	Gel PCR	Virus Isolation
861/1 4/3	28.28	+	+
861/1 5/3	29.65	+	+
861/1 6/3	29.51	+	+
861/1 7/3	0.00	+/-, -	-
861/1 8/3	0.00	-	-
861/1 8/3	0.00	-	-
861/1 9/3	0.00	-	-
861/2 1/3	0.00	-	-
861/2 2/3	0.00	-	-
861/2 3/3	33.85	+	-
861/2 4/3	26.57	+	+
861/2 5/3	24.14	+	+
861/2 6/3	31.61	+	+
861/2 7/3	0.00	-	-
861/2 8/3	0.00	-	-
861/3 1/3	0.00	-	-
861/3 3/3	30.30	+	+
861/3 4/3	31.07	+	+
861/3 5/3	26.79	+	+
861/3 6/3	37.49	+	-
861/3 7/3	0.00	-	-
861/3 8/3	0.00	-	-
862P 27/2	0.00	-	-
862/1 1/3	0.00	-	-
862/1 2/3	0.00	-	-
862/1 3/3	0.00	-	-
862/1 4/3	0.00	-	-
862/1 5/3	0.00	-	-
862/1 6/3	0.00	-	-
862/1 7/3	0.00	-	-
862/1 8/3	0.00	-	-
862/1 9/3	0.00	-	-
863P 27/2	0.00	-	-
863/1 1/3	0.00	-	-
863/1 3/3	26.48	+	+
863/1 4/3	25.77	+	+
863/1 5/3	33.19	+	-
863/1 6/3	0.00	-	-
863/1 7/3	0.00	-	-
863/2 1/3	0.00	-	-
863/2 2/3	32.96	+	-
863/2 3/3	26.01	+	+
863/2 4/3	28.03	+	+
863/2 5/3	37.38	+/-,-	-
863/2 6/3	0.00	-	-
863/3 1/3	0.00	-	-

10.4 PUBLICATIONS

Eppendorf BioNews Application Notes

The use of the Eppendorf RealMasterMix Probe in the detection of bovine venereal diseases

Ala Lew & Lyle McMillen,
Emerging Technologies, Department of Primary Industries and Fisheries, Queensland, Australia

Abstract

A Campylobacter fetus subsp. venerealis-specific Taq nuclease assay displayed significant improvements in C_t, ΔF and sensitivity when performed using Eppendorf's RealMasterMix Probe, compared to a leading competitor's qPCR mix. Using the RealMasterMix Probe, the C_t was reduced by 3-4 cycles when purified DNA was assayed and by up to 8 cycles when crudely processed clinical specimens were assayed. Normalised ΔF was also higher in both cases. The significant improvements in amplification efficiency observed when assaying crudely processed clinical specimens in RealMasterMix Probe mix are most likely due to lower inhibition in the presence of PCR inhibitors found in many clinical specimens. This improved performance allows reliable and sensitive detection of target organisms from minimally processed clinical specimens.

Introduction

The use of *Taq* nuclease assays either on minimally processed clinical specimens, or directly on the clinical specimen itself without the need for extensive and expensive nucleic acid purification, would significantly improve the cost effectiveness and speed of diagnostic *Taq* nuclease assays. PCR-based diagnostic assays confront two significant challenges when applied directly to clinical specimens – releasing nucleic acids from the target organism, and overcoming the activity of PCR inhibitors present in a range of clinical specimens.

A specific 5' *Taq* nuclease assay for *Campylobacter fetus* subsp. *venerealis* has been developed that may be applied to clinical specimens (preputial smegma or cervico-vaginal mucus from cattle) with minimal or no processing. The assay was developed using a TaqMan® MGB probe, a leading quantitative real-time PCR mix (qPCR Mix "X"), and a Corbett Research RotorGene RG-3000™ real-time PCR platform (Corbett Research, Sydney, Australia). Distinct improvements in sensitivity, C_t and ΔF were observed when performing the assay using RealMasterMix Probe, compared to qPCR Mix X.

Clinical specimens regularly contain faeces, blood, semen, pus, or epithelial cells as well as smegma or mucus. As a consequence, significant quantities of PCR inhibitors may be present, which can be removed by purification of the specimen using a commercial DNA isolation kit. Improved assay performance in the presence of any PCR inhibitors will allow specimen preparation to be less exhaustive, faster and cheaper while retaining sensitivity and specificity.

The *Taq* nuclease assay uses a three-step thermal cycle, as opposed to the conventional two-step thermal cycle, as the assay performs poorly under higher annealing conditions. The assays were duplicated using RealMasterMix Probe and qPCR Mix X, with extension temperatures of either 68 °C (the optimum for RealMasterMix Probe) or 72 °C (optimum for qPCR Mix X), and the C_t, ΔF, and sensitivities compared.

Materials and Methods

Clinical specimens
Collection and transport: Preputial smegma was collected from 2-5 year old breeding bulls (*Bos taurus* x *Bos indicus*) by scraping the penis and internal prepuce near the fornix with a plastic pipette (10 mm diameter), and rinsing off the smegma with 5 ml PBS. The smegma was transported to the laboratory within 48 h in a chilled, insulated container.

Processing via heat lysis
200 µl of the diluted smegma was centrifuged at maximum speed in a microcentrifuge for 3 min, and the supernatant discarded. The pellet was resuspended in 100 µl sterile distilled water, and the suspension heated at 95 °C for 10 min to lyse bacterial cells. The suspension was briefly centrifuged to pellet any large debris, and 5 µl assayed for *C. fetus* subsp. *venerealis* by *Taq* nuclease assay.

Processing via commercial DNA isolation kit
Genomic DNA was isolated from clinical specimens using a commercial DNA isolation kit (QIAamp® DNA Minikit, Valencia, CA, USA), with an initial sample volume of 200 µl and eluting in 100 µl.

***Taq* nuclease assay**

Sub-species specific primers and MGB probe had been designed using PrimerExpress 2.0 (Applied Biosystems, Foster City, CA, USA), and the assay optimised using a leading competitor's quantitative real-time PCR mix. Table 1 describes the reaction conditions and set-up for each of the experiments presented.

Taq Nuclease Assay Reaction Conditions		Temp (°C)	Time (s)
Initial denaturation		95	120
Denaturation		95	20
Annealing		50	20
Extension (Experiment 1, qPCR Mix X optimum)		72	20
Extension (Experiment 2, RealMasterMix Probe optimum)		68	20
No. of cycles	45		
Hold		25	

Table 1

Application Hotline Tel.: +49-180-3666 789 · E-Mail: application-hotline@eppendorf.com
USA: Tel.: 800-645-3050 Ext. 2258 · E-Mail: apps@eppendorf.com

The use of the Eppendorf RealMasterMix Probe in the detection of bovine venereal diseases

Experiment 1:
Comparison at qPCR Mix X optimum extension temperature (72 °C)

Quantitative controls of a well-characterised *C. fetus* subsp. *venerealis* field isolate (98-109383, Animal Research Institute, Department of Primary Industries and Fisheries, Queensland, Australia) and 3 clinical specimens (preputial scrapes) were prepared via heat lysis, and a complete set of reactions (controls, specimens, and NTCs) was prepared using each real-time PCR mix (see Table 2).

The assay was conducted with the optimal conditions of qPCR Mix X (72 °C), the threshold set to a normalised fluorescence of 0.1 and C_t values recorded.

Experiment 1: *Taq* Nuclease Assay Reaction		
Reaction Component	Final Amount	Source
RealMasterMix Probe	1×	Eppendorf
QR qPCR Mix X	1×	Leading competitor
Forward Primer	900 nM	Custom synthesis
ReversePrimer	900 nM	Custom synthesis
Fluorescent 3' MGB probe (TaqMan®)	170 nM	Custom synthesis
Template	5 µl	Clinical specimen
Molecular Biology Grade Water	to 25 µl	Eppendorf

Table 2

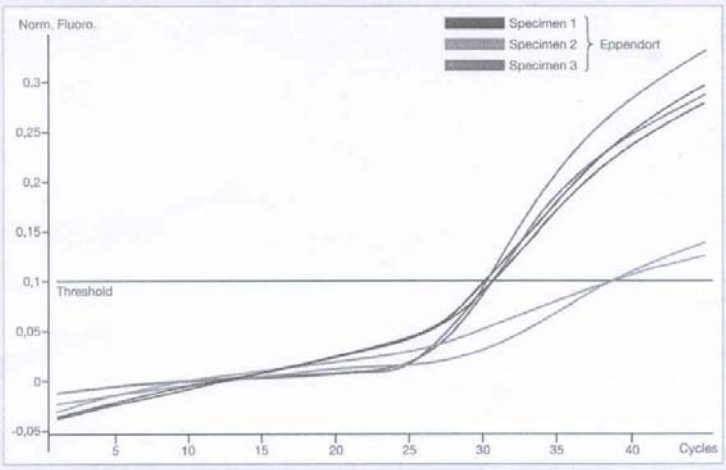


Fig. 1a: Assay of heat lysed clinical specimens, with a 72 °C extension step, specimen 1 to 3 RealMasterMix Probe (Eppendorf)

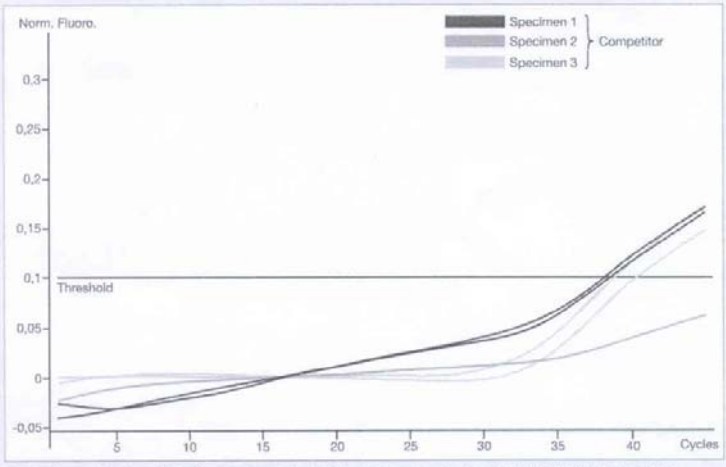


Fig. 1b: Assay of heat lysed clinical specimens, with a 72 °C extension step, specimen 1 to 3 qPCR Mix X (Competitor)

The use of the Eppendorf RealMasterMix Probe in the detection of bovine venereal diseases

Experiment 2:
Comparison at RealMasterMix optimum extension temperature (68 °C)

Quantitative controls of a well-characterised *C. fetus* subsp. *venerealis* field isolate (98-109383) were prepared via heat lysis, and 3 clinical specimens (preputial scrapes) were prepared using a commercial DNA isolation kit.

A complete set of reactions (controls, specimens, and NTCs) was prepared using each real-time PCR mix (see Table 3).

The assay was conducted with the optimal conditions of RealMasterMix Probe (68 °C), the threshold set to a normalised fluorescence of 0.1 and C_t values recorded.

Experiment 2: *Taq* Nuclease Assay Reaction		
Reaction Component	Final Amount	Source
RealMasterMix Probe	1×	Eppendorf
QR qPCR Mix X	1×	Leading competitor
Forward Primer	900 nM	Custom synthesis
ReversePrimer	900 nM	Custom synthesis
Fluorescent 3' MGB probe (TaqMan®)	170 nM	Custom synthesis
Template	5 µl	gDNA
Molecular Biology Grade Water	to 25 µl	Eppendorf

Table 3

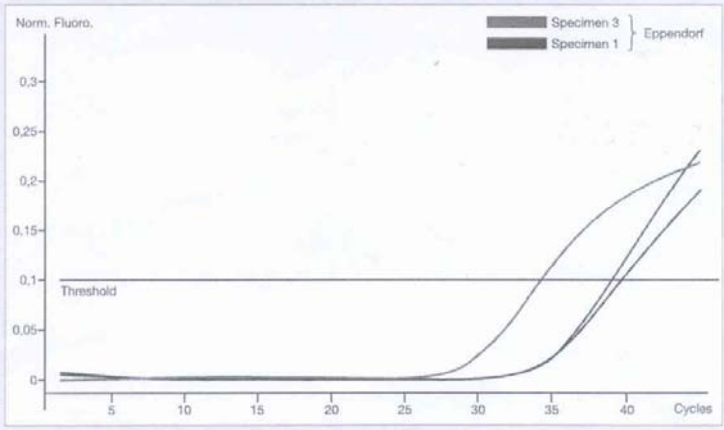


Fig. 2a: Assay of purified gDNA from clinical specimens, with a 68 °C extension step, specimen 1 and 3 RealMasterMix Probe (Eppendorf)

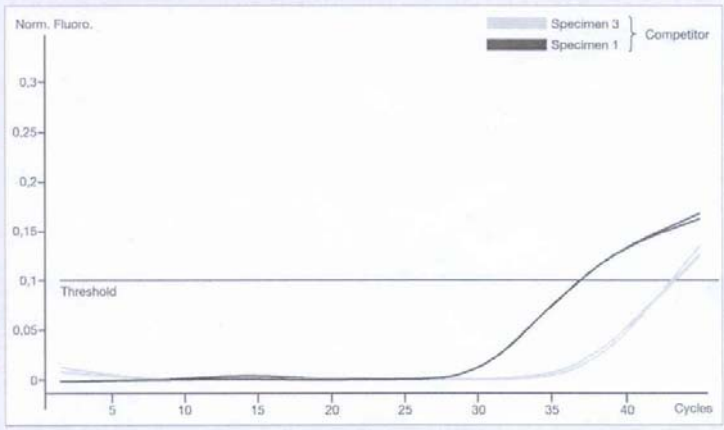


Fig. 2b: Assay of purified gDNA from clinical specimens, with a 68 °C extension step, specimen 1 and 3 qPCR Mix X (Competitor)

The use of the Eppendorf RealMasterMix Probe in the detection of bovine venereal diseases

Results:

Comparison of specimens assayed at an extension temperature of 72 °C in both real-time PCR mixes (Experiment 1; Figure 1 a+b) indicated a significant difference in C_t and ΔF between the two qPCR mixes. Specimens assayed in RealMasterMix Probe consistently displayed a lower C_t, with reductions of 3 to 8 cycles commonly displayed (Fig. 1a; Table 4). This difference was most pronounced in heat lysed clinical specimens, with differences of approximately 8 cycles regularly evident. An increase in normalised ΔF was also noticed when com-paring RealMasterMix Probe with qPCR Mix X. In addition, some positive specimens (specimen 2) were consistently detected using RealMasterMix Probe, but not qPCR Mix X (Table 4).

Similar differences were observed for heat lysed specimens assayed with a 68 °C extension step (data not shown).

Assay of the same specimens processed with a commercial DNA isolation kit in order to remove any PCR inhibitors reduced the differences in C_t values obtained using RealMasterMix Probe and qPCR Mix X (Figure 2a; Table 5).

Discussion

Reduced C_t and ΔF values for *Taq*-nuclease assay of bovine vibriosis using both purified gDNA and specimens prepared using a crude heat lysis protocol indicated a significant improvement in reaction efficiency using RealMasterMix Probe, compared to a leading competitor's quantitative real-time PCR mix. The observed differences in C_t may be attributed to two principal factors:

The RealMasterMix Probe is more efficient than qPCR Mix X at both 68 °C (RealMasterMix Probe optimal extension temperature) and 72 °C (leading competitor's mix optimal extension temperature), leading to a reduction in C_t of approximately 3 to 4 when assaying purified gDNA templates containing no significant potential PCR inhibitors.

The RealMasterMix Probe is more tolerant of the PCR inhibitors associated with preputial smegma specimens than qPCR Mix X. Significant differences in C_t were observed between crude lysis preparations of clinical specimens assayed using RealMasterMix Probe and qPCR Mix X, in addition to the overall improved efficiency described above.

Crudely prepared clinical specimens are likely to have a higher yield of target sequence than specimens that have undergone an extensive DNA purification process. A robust assay able to function efficiently in the presence of crudely prepared clinical specimens and any associated PCR inhibitors will be able to reliably detect positive clinical specimens with low numbers of target organisms.

RealMasterMix Probe provides an efficient, rugged quantitative real-time PCR, with improved sensitivity compared to a qPCR Mix X, and suitability for assay of minimally processed clinical specimens.

Template	Av. C_t (RealMasterMix Probe)	Av. C_t (qPCR Mix X)	C_t reduction
10^6 cells/ml control	24.65	28.1	3.45
10^5 cells/ml control	28.52	31.68	3.16
10^4 cells/ml control	31.13	34.44	3.31
10^3 cells/ml control	40.52	40.56	0.04
Specimen 1	30.59	38.3	7.71
Specimen 2	38.88	-	-
Specimen 3	30.44	39.69	9.25

Table 4: Comparison of average C_t values obtained with RealMasterMix Probe and qPCR Mix X at 72 °C extension. Control templates are heat lysed quantitated axenic cultures of C. fetus subsp. venerealis, specimens are heat lysed bovine preputial scrapings in PBS.

Template	Av. C_t (RealMasterMix Probe)	Av. C_t (qPCR Mix X)	C_t reduction
Specimen 1	36.77	43.08	6.31
Specimen 2	-	-	-
Specimen 3	34.14	38.31	4.17

Table 5: Comparison of average C_t values obtained with RealMasterMix Probe Mix and qPCR Mix X at 68 °C extension. Specimens are bovine preputial scrapings in PBS, isolated using a commercially available kit.

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ON-FARM

Are your bulls carrying reproductive diseases?

By Dr Lyle McMillen and Dr Ala Lew

Venereal diseases of cattle cause significantly reduced calving rates through reduced fertility in females, delayed conception and early pregnancy abortion. Two common venereal diseases in Australia are trichomoniasis and campylobacteriosis (or vibriosis).

The NSW Department of Agriculture estimates economic losses through reduced calf yield can reach 65.5% and a reduction in gross margins of 35.7% compared with non-infected herds.

While uncommon in southern Australian states, both diseases remain endemic in the extensive cattle areas of northern Australia, and are widely distributed in areas with extensive management and natural breeding.

The NSW Department of Agriculture estimates economic losses through reduced calf yield can reach 65.5% and a reduction in gross margins of 35.7% compared with non-infected herds.

While a commercial vaccine is available to control campylobacteriosis (Vibrovax, Pfizer Animal Health), no vaccine is available for trichomoniasis.

However, detection of infection and management of breeding are the most effective means of controlling the impact of these diseases on calf production.

And now a new and improved test for detecting these diseases is available. Queensland Department of Primary Industries and Fisheries scientists, in collaboration with MLA, have developed sensitive DNA-based diagnostic tests for trichomoniasis and campylobacteriosis that provide significant improvements on the current tests.

Detection of infection and management of breeding are the most effective means of controlling the impact of these diseases on calf production.

The DNA-based assays for both diseases are significantly more sensitive than the previous tests, and are much more reliable and specific. These assays are applied to genital specimens, but do not share the limitations of the older diagnostic tests. A purpose built sampling tool has also been developed which has improved both sample collection practises and the reliability of the tests.

This technology will provide a number of benefits to producers:

- **Definitive identification of infected animals**: These new tests are over 1000-fold more sensitive than the currently used culture-based tests, and are not prone to many of the difficulties associated with current diagnostic techniques.
- **Improved profitability through better management**: Improved diagnosis of these diseases should allow more effective management of calf losses through poor reproductive performance.
- **Improved export access through world-leading technology**: Both diseases are list B notifiable diseases under OIE regulations. Infection status of cattle and semen exports will be more confidently determined using these tests.

SYMPTOMS AND SPREAD

Both trichomoniasis and campylobacteriosis present very similar symptoms – low calving rates caused by dry females, delayed cycling and abortions. Carrier bulls spread the diseases during mating or through contaminated semen, and show no sign of infection. However, infected females are likely to clear infection within three months and develop a temporary immunity to reinfection. Damage to the reproductive tract can render a cow permanently barren, although they will continue to cycle regularly.

The assays are now available through the Queensland Department of Primary Industries Biosecurity division. For more information and instructions for sampling, contact:

QLD: Dr Bruce Corney, ARI, Brisbane
Phone: 07 3362 9578
Email: Bruce.Corney@dpi.qld.gov.au.

NSW: Dr Michael Hornitzky, EMAI, Menangle
Phone: 02 4640 6311
Email: Michael.Hornitzky@agric.nsw.gov.au

In addition, DPI&F fact sheets on both diseases are available by phoning the DPI&F call centre on 13 25 23.

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Comparison of Culture and a Novel 5′ *Taq* Nuclease Assay for Direct Detection of *Campylobacter fetus* subsp. *venerealis* in Clinical Specimens from Cattle

Lyle McMillen, Geoffry Fordyce, Vivienne J. Doogan, and Ala E. Lew*

Queensland Department of Primary Industries and Fisheries, QLD, Australia

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A *Campylobacter fetus* subsp. *venerealis*-specific 5′ *Taq* nuclease PCR assay using a 3′ minor groove binder-DNA probe (TaqMan MGB) was developed based on a subspecies-specific fragment of unknown identity (S. Hum, K. Quinn, J. Brunner, and S. L. On, Aust. Vet. J. 75:827–831, 1997). The assay specifically detected four *C. fetus* subsp. *venerealis* strains with no observed cross-reaction with *C. fetus* subsp. *fetus*-related *Campylobacter* species or other bovine venereal microflora. The 5′ *Taq* nuclease assay detected approximately one single cell compared to 100 and 10 cells in the conventional PCR assay and 2,500 and 25,000 cells from selective culture from inoculated smegma and mucus, respectively. The respective detection limits following the enrichments from smegma and mucus were 5,000 and 50 cells/inoculum for the conventional PCR compared to 500 and 50 cells/inoculum for the 5′ *Taq* nuclease assay. Field sampling confirmed the sensitivity and the specificity of the 5′ *Taq* nuclease assay by detecting an additional 40 bulls that were not detected by culture. Urine-inoculated samples demonstrated comparable detection of *C. fetus* subsp. *venerealis* by both culture and the 5′ *Taq* nuclease assay; however, urine was found to be less effective than smegma for bull sampling. Three infected bulls were tested repetitively to compare sampling tools, and the bull rasper proved to be the most suitable, as evidenced by the improved ease of specimen collection and the consistent detection of higher levels of *C. fetus* subsp. *venerealis*. The 5′ *Taq* nuclease assay demonstrates a statistically significant association with culture (χ^2 = 29.8; $P < 0.001$) and significant improvements for the detection of *C. fetus* subsp. *venerealis*-infected animals from crude clinical extracts following prolonged transport.

Bovine venereal campylobacteriosis or vibriosis is a major cause of abortion and infertility in cattle and is one of the most important bovine venereal diseases in Australia (6). The disease is caused by *Campylobacter fetus* subsp. *venerealis* and is spread by infected bulls during servicing, by contaminated semen, or between bulls (41). Campylobacteriosis is an Office International des Epizooties list B notifiable disease that is considered to have socioeconomic and/or public health implications and is thus significant in the international trade of animals and animal products. In addition, international semen export guidelines require that bulls be *C. fetus* subsp. *venerealis* negative. Symptoms in female cattle include irregular estrus cycles, infertility through uterine infection, and early embryonic death, while bulls are asymptomatic carriers (6). The symptoms of campylobacteriosis are very similar to those of trichomoniasis (caused by the protozoan *Tritrichomonas fetus*), and these venereal diseases tend to occur in areas with extensive cattle management and natural breeding, such as western North America, Australia, Africa, and Latin America (11). In Australia, it has been estimated that vibriosis causes significant reproductive wastage in infected beef and dairy herds and represents a large economic loss for producers, particularly in the first year of infection, where gross margins can be reduced by as much as 66% (19). When the disease becomes established, gross margins may be 36% below those of noninfected herds (19). Similarly, in Argentina, bovine venereal diseases are considered to be causes of low reproductive efficiency with severe economic losses (5). Several killed bacterial campylobacteriosis vaccines are available, e.g., Vibrovax (Pfizer Animal Health, Australia); Vibrio-Lepto-5 (Boehringer Ingelheim Vetmedica, Inc.); Bioabortogen-H, Biogenesis, and Repropolivac (San Jorge Bago, Argentina); and *Campylobacter (Vibrio) fetus* vaccine (Onderstepoort Biological Products Ltd., South Africa) (9, 10), and such vaccines are considered the most effective means of managing the disease.

The diagnosis of infection is by the direct isolation of the causative agent by selective culture from semen, preputial smegma, or vaginal mucus (20, 31) or through the detection of an immune response in cervico-vaginal mucus by using an enzyme linked immunosorbent assay (ELISA) (23). Several methods for the collection of preputial smegma and vaginal mucus have been investigated in order to improve the reliability of selective culture-based diagnostic procedures or ELISA. These procedures have included preputial washes, scrapes (55), mucus swabs, blotting (23), and the commonly used scrape/aspiration methods with a pipette (26, 41, 55). The use of swabs and blotting has been limited to female cattle. A comparison of three collection methods for preputial smegma (scraping, aspiration, and washing) demonstrated that scraping with a specialized tool that was developed for the collection of preputial smegma for *Tritrichomonas fetus* culture reduced contaminant levels and improved isolation rates compared to those for aspiration and washing (55). Both aspiration and washing require manipulation of a syringe or bulb as well as the

* Corresponding author. Mailing address: Queensland Department of Primary Industries and Fisheries, c/o Animal Research Institute, Locked Mail Bag No. 4, Moorooka, 4105 QLD, Australia. Phone: 61 7 3362 9502. Fax: 61 7 3362 9429. E-mail: ala.lew@dpi.qld.gov.au.

pipette, requiring at least two people during the collection of diagnostic specimens.

The traditional culture and ELISA diagnostic procedures present with sensitivity and specificity limitations. The ELISA is known to produce false-positive and false-negative results, and a high percentage of *C. fetus* subsp. *venerealis* strains are susceptible to polymyxin B, an antibiotic used in all *Campylobacter* selective media and transport enrichment media (TEM) (20, 26). *Campylobacter* colonies from preputial scrapes and vaginal mucus are visible within 48 h in a microaerobic environment, and the slow-growing *C. fetus* subsp. *venerealis* is readily overgrown by a range of microbes, leading to inaccurate diagnoses (31). These methods are not very sensitive or specific, and discrimination between *C. fetus* subsp. *venerealis* and the morphologically, phenotypically, and genetically similar *C. fetus* subsp. *fetus* is not reliable (57). A direct immunofluorescence test (DIFT) has been developed and applied to the detection of *C. fetus*, and although not widely evaluated, it may present with false-positive results due to nonspecific fluorescence and the inability to differentiate *C. fetus* subspecies (38). *C. fetus* subsp. *fetus* occurs mainly in the intestinal tracts of cattle and sheep and causes only sporadic abortion in these animals (50). Conversely, *C. fetus* subsp. *venerealis* is highly adapted to the genital tract of cattle and cannot survive in the bovine intestine (4). It is thus essential to identify *C. fetus* subspecies in the diagnosis of bovine venereal diseases. Molecular methods such as PCR (22), amplified fragment length polymorphism (57), and pulsed-field gel electrophoresis (17, 44) have been used to discriminate between the two *Campylobacter fetus* subspecies. However, PCR has not been routinely applied for the diagnosis of bovine venereal campylobacteriosis and field studies continue to rely upon either selective culture (20), ELISA (21), or DIFT (38).

Although the isolation and identification of *C. fetus* subsp. *venerealis* appears to be difficult, the adaptation of sensitive molecular methods for direct detection in clinical samples has not been forthcoming. In comparison to conventional PCR techniques, 5′ *Taq* nuclease assays are highly sensitive and specific and the amount of target DNA in the assay can also be accurately quantified (37). The implementation of 5′ *Taq* nuclease assays has improved the detection of a wide range of pathogenic organisms, including *Salmonella enterica* (18), pathogenic *Leptospira* spp. (51), *Campylobacter jejuni* (43), *Actinobacillus pleuropneumoniae* (3), and *Mycobacterium avium* subsp. *paratuberculosis* (28). Minor groove binder (MGB) probes demonstrate higher specificities and sensitivities than non-MGB probes in 5′ *Taq* nuclease assays (30) and thus are highly suited for routine diagnostic applications as demonstrated for the detection of bovine retroviruses (34, 35). This study describes the optimization of sampling, transport, and processing protocols for the diagnosis of bovine venereal campylobacteriosis by using a novel 5′ *Taq* nuclease PCR assay utilizing a 3′ TaqMan MGB probe.

MATERIALS AND METHODS

Bacterial and protozoan culture. Isolates of several *Campylobacter* species were obtained from the Animal Research Institute, Department of Primary Industries and Fisheries (DPI&F), from the American Type Culture Collection, and from the National Collection of Type Cultures (Table 1). *Campylobacter* strains were grown at 37°C in brain-heart infusion broth (Oxoid), 0.2% yeast extract, 0.07% Bacto agar for between 1 and 3 days. *Tritrichomonas fetus* was grown at 37°C in 1.25% neutralized liver digest, 0.5% tryptose, 0.15% Bacto agar, 50% sterile heat-inactivated bovine serum, 0.1% P/S solution (0.75% penicillin and 0.082% streptomycin). *Pseudomonas aeruginosa* and *Proteus vulgaris* were grown at 37°C on blood agar plates for 24 h. *Neospora caninum* tachyzoites were cultured in Vero cells as previously described (14).

TABLE 1. Reference species and isolates used in this study

Species	Strain	Source
C. fetus subsp. *venerealis*	98-109383	Field isolate (DPI&F)
C. fetus subsp. *venerealis*	19438	ATCC
C. fetus subsp. *venerealis*	Biovar venerealis	Pfizer Animal Health, Australia
C. fetus subsp. *venerealis*	Biovar intermedius	Pfizer Animal Health, Australia
C. fetus subsp. *fetus*	98-118432	Field isolate (DPI&F)
C. fetus subsp. *fetus*	15296	ATCC
C. jejuni subsp. *jejuni*	11168	NCTC
Campylobacter hyointestinalis	N3145	Field isolate (DPI&F)
Campylobacter sputorum subsp. *bubulus*	Y4291-1	Field isolate (DPI&F)
Campylobacter coli	11353	NCTC
Tritrichomonas foetus	YVL-W	Field isolate (DPI&F)
T. foetus	30003	ATCC
Tetratrichomonas gallinarum	30097	ATCC
Pentatrichomonas hominis	30000	ATCC
Trichomonas vaginalis	30001	ATCC
Pseudomonas aeruginosa	27853	ATCC
Proteus vulgaris	6380	ATCC
Neospora caninum	50843	ATCC
Leptospira borgpetersenii serovar Hardjobovis	93/94451/3	Field isolate (DPI&F)
Leptospira interrogans serovar Pomona	Pomona	CCRL[a]

[a] CCRL, WHO/FAO/OIE Collaborating Centre for Reference and Research on Leptospirosis, Australia and Western Pacific Region.

Animal sampling. Three techniques were evaluated for the collection of smegma from bulls and the collection of vaginal mucus from female cattle. Collection techniques were evaluated on the basis of ease of use for the veterinarians, lack of adverse impact upon the animals, and suitability of the material gained for assay and culture.

Preputial smegma samples were collected from eight bulls by using sterile pipettes, swabs, or bull rasperts. The bulls were restrained in a veterinary crush during the collection procedures. A sterile pipette (10-mm internal diameter, with a beveled edge) was gently scraped along the surface of the penis and internal prepuce near the fornix, with gentle aspiration being applied with an attached bulb or syringe. The collected smegma was rinsed into approximately 5 ml sterile phosphate-buffered saline (PBS) or physiological saline. A sterile McCullough uterine mare swab (Minitube Australia Pty Ltd.) was gently scraped along the surface of the penis and internal prepuce near the fornix. The collected smegma was expressed into approximately 5 ml sterile PBS or physiological saline. A bull rasper (polyethylene, 60 cm long with a 75-mm-long, 8-mm-diameter corrugated scraper head with a 1.5-mm collection bore attached to 6-mm-diameter tubing with a 1.5-mm internal diameter, similar to those produced by Elastecnica, Argentina; based on the original design that was described previously [52]) was gently scraped along the surface of the penis and internal prepuce near the fornix. No aspiration was necessary. The collected smegma was rinsed into approximately 5 ml sterile PBS or physiological saline.

Vaginal mucus samples were collected from eight cows by using artificial insemination pipettes, swabs, or bull rasperts during restraint in a veterinary crush. A sterile artificial insemination infusion pipette was inserted so that the anterior end reached the cervix. Gentle suction was applied by using a rubber bulb while moving the pipette gently backwards and forwards. The pipette was removed, and the collected mucus was rinsed into approximately 5 ml sterile physiological saline. A sterile 15-cm swab, held by sterile forceps, was inserted so that the anterior end reached the cervix. The swab was gently moved backwards and forwards while being rotated to saturate the head with mucus. The swab was removed, and the collected mucus was expressed into approximately 5 ml sterile physiological saline. A bull rasper was inserted so that the anterior end reached the cervix. The rasper was moved gently backwards and forwards. No aspiration was necessary. The rasper was removed, and the collected mucus was rinsed into approximately 5 ml sterile physiological saline.

TABLE 2. Primers and fluorescent 3' MGB-DNA probe used in this study

Primer	Sequence (5' to 3')
VenSF	CTTAGCAGTTTGCGATATTGCCATT
VenSR	GCTTTTGAGATAACAATAAGAGCTT
Mg3F	GGTAGCCGCAGCTGCTAAGAT
Mg4R	TAGCTACAATAACCACAACT
CFVF	CCCAGTTATCCCAAGCGATCT
CFVR	CGGTTGGATTATAAATTTTAGCTTGGT
CFVP1	6-FAM-CATGTTATTTAATACCGCAA[a]

[a] The probe was labeled with 6-carboxyfluorescein (6-FAM) phosphoramidite as the 5' reporter dye and included a nonfluorescent quencher attached to the 3' MGB moiety.

Urine was investigated to determine its feasibility as an alternative clinical specimen for the diagnosis of venereal *C. fetus* subsp. *venerealis* infection in bulls. Urine from two consecutive voids was collected in a series of sterile collection containers following the subcutaneous administration of a diuretic (Frusemide; Ilium Veterinary Products, Australia). The first container collected was discarded as being the most likely to be heavily contaminated with fecal material, hair, and other debris.

Diagnostic culture. Culture-based diagnosis for *C. fetus* subsp. *venerealis* was conducted by the inoculation of 5 ml modified Weybridge medium (32) with 0.5 ml of preputial smegma in PBS or vaginal mucus in saline, followed by transport at ambient temperatures for up to 48 h. Upon arrival at the laboratory, 100 μl of the inoculated modified Weybridge medium was plated onto *Campylobacter fetus* selective medium (Skirrow's) (49) and incubated at 37°C in a microaerobic environment that was generated by using an anaerobic jar and a Campygen sachet (Oxoid). The presence of *C. fetus* subsp. *venerealis* was indicated by the presence of small (about 0.5 mm in diameter), smooth, translucent colonies arising after 48 to 72 h, followed by microscopic confirmation of *Campylobacter*-like morphology.

PCR template preparation. DNA was extracted from liquid culture, resuspended colonies, preputial smegma, vaginal mucus, and urine samples by using a commercial kit (QIAamp DNA mini kit; QIAGEN) as per the manufacturer's protocol, except for elution of the final product in 50 μl rather than 400 μl.

Crude cell lysates were prepared for the 5' *Taq* nuclease assay by a heat lysis method. One milliliter of the sample (preputial smegma in PBS; vaginal mucus in saline or urine) was centrifuged for 5 min at 12,000 × *g*, and the supernatant was discarded. The pellet or compressed mucus was resuspended in 500 μl sterile distilled water and heated at 95°C for 10 min. The suspension was centrifuged for 30 s at 2,000 × *g*, and the supernatant was assayed by 5' *Taq* nuclease assay.

PCR amplification, cloning, and conventional PCR assay. All primers used in this study were synthesized by Proligo Australia Pty Ltd. The 142-bp *C. fetus* subsp. *venerealis*-specific product was amplified in a 15-μl reaction mixture volume by using 500 nM VenSF and VenSR primers (Table 2) (22), 1× PCR buffer with $MgCl_2$ (Roche Diagnostics), 200 μM dNTPs, 1 U *Taq* DNA polymerase (Roche Diagnostics), and 1 ng of target *C. fetus* subsp. *venerealis* DNA. The reactions were cycled in a GeneAmp PCR system model 2700 (Applied Biosystems Inc.) using the following conditions: initial denaturation at 95°C for 10 min, 30 cycles at 95°C for 20 s, annealing at 50°C for 20 s, and extension at 72°C for 2 min, with a final extension at 72°C for 10 min.

PCR assays were conducted under the same conditions using both the VenSF-and-VenSR primer pair and MG3F-and-MG4R primer pair (Table 2), which amplify a 960-bp *C. fetus*-specific product (22). Two microliters of QIAGEN kit-purified genomic DNA (gDNA) prepared from smegma, mucus, and urine extracts was added as template for conventional PCR assays. The presence of *C. fetus* subsp. *venerealis* is indicated by the presence of both the 960-bp *C. fetus*-specific amplicon and the 142-bp *C. fetus* subsp. *venerealis*-specific amplicon.

Amplification products were separated in 2% TBE (89 mM Tris borate, 2 mM EDTA [pH 8]) agarose gels by using size markers (Marker XIV; Roche Molecular Biochemicals, Germany) and were visualized under UV illumination by ethidium bromide staining.

Sequencing. The 142-bp *C. fetus* subsp. *venerealis*-specific amplicon from strain 98-118432 was ligated into a cloning vector (pCR2.1, TOPO-TA cloning kit; Invitrogen Corporation) as described in the manufacturer's protocol. Plasmids with inserts were sequenced using the T7 and M13 reverse primers and the BigDye Terminator mix (Applied Biosystems, Inc.), following the manufacturer's protocols. Sequences were analyzed by the Griffith University DNA Sequencing Facility (School of Biomolecular and Biomedical Science, Griffith University, Nathan, QLD 4111, Australia) on an ABI 377 DNA sequencer.

5' *Taq* nuclease assay. Primer and probe combinations for 5' *Taq* nuclease assay using fluorescent 3' MGB-DNA probes (synthesized by Applied Biosystems, Inc.) were designed for *C. fetus* subsp. *venerealis* by using Primer Express, version 2 (Applied Biosystems, Inc.), and BLASTn searches (http://www.ncbi.nlm.nih.gov/BLAST/) were conducted to confirm sequence specificity. Sequences of primers and the probe are provided in Table 2. The 5' *Taq* nuclease assay for *C. fetus* subsp. *venerealis* was conducted in a 25-μl volume by using either Platinum Quantitative PCR SuperMix-UDG (Invitrogen Life Technologies) or RealMasterMix probe mix (Eppendorf) with 900-nM CFVF and CFVR primers, 170 nM CFVP1 fluorescent 3' MGB-DNA probe, and 5 μl of either heat-lysed cells or kit-purified DNA templates in a Corbett Rotor-Gene RG-3000 (Corbett Research, Australia). The thermal profile was 50°C for 2 min, 95°C for 2 min, and 45 cycles of denaturation at 95°C for 20 s, annealing at 50°C for 20 s, and extension at 72°C for 20 s. The acquisition of fluorescence occurred at the end of each extension step. A positive result was indicated by the fluorescence (normalized to a no-template control) passing a threshold of 0.1. Assay conditions were optimized by using serial dilutions of *C. fetus* subsp. *venerealis*-purified DNA. Quantitative estimates of target cells/ml were calculated by using a standard curve generated with either kit DNA extracts or crude cell extracts prepared from serial dilutions of known numbers of *C. fetus* subsp. *venerealis* isolates. All 5' *Taq* nuclease assays were prepared in duplicate, with cycle threshold or quantitative cell estimates averaged for each sample. Inconsistently positive samples (one of two repeats) were repeated.

Sensitivity evaluation and effects of transport. *C. fetus* subsp. *venerealis* strain 98-109383 cells from 2-day-old fresh cultures were treated 1:1 with methanol to reduce cell motility and were counted by using a hemocytometer. Serial log dilutions of the 2-day-old *C. fetus* subsp. *venerealis* cultures were prepared at 10^5 cells/ml and were diluted to 1 cell/ml. These dilutions were inoculated into smegma, mucus, and urine that were obtained from healthy animals which had previously tested negative for both *C. fetus* subsp. *venerealis* (by selective culture and 5' *Taq* nuclease assay) and *T. fetus* (microscopic examination of InPouchTF cultures) (Biomed Diagnostics). The viability of the cells that were used for these evaluations was determined by spreading 100 μl of each dilution onto prepoured Columbia sheep blood agar plates (Oxoid), and colonies were counted following 2 days of incubation at 37°C in a microaerobic environment (as described above). Genomic DNA was extracted from the spiked specimens by using a commercial DNA extraction kit (QIAamp DNA mini kit; QIAGEN), which was assayed by both conventional PCR and 5' *Taq* nuclease assays. Inoculated specimens were also prepared for 5' *Taq* nuclease assay by heat lysis. Modified Weybridge TEM were inoculated with these laboratory-spiked specimens, as was done for diagnostic culture. DNA was also extracted from aliquots of inoculated TEM by using a commercial kit (QIAamp; QIAGEN) and assayed by both 5' *Taq* nuclease assay and conventional PCR assay. Selective media were inoculated from the TEM as was done for diagnostic culture. Estimates of cell equivalents/assay or cells/inoculum were calculated from the enumerated spiked specimens by determining equivalent cell numbers contained in the final volume used either as template for PCR or as inoculum in cultures.

5' *Taq* nuclease assays were scored positive if the fluorescence (normalized to a no-template control) passed a threshold of 0.1. A positive conventional PCR assay required the detection of both the 960-bp, species-specific and the subspecies-specific, 142-bp amplicons. Results from cultured samples were scored positive for the presence of *Campylobacter*-like colonies, followed by microscopic confirmation of *Campylobacter*-like morphology and motility.

Sample transport was simulated by storing the inoculated TEM and animal samples at ambient temperatures for smegma and mucus and at 4°C for urine for up to 5 days. Samples for 5' *Taq* nuclease assays, conventional PCR assays, and selective culture were processed as described above at time zero and after 2 and 5 days of storage. To examine changes in cell numbers under these storage conditions, quantified cell estimates were compared by using 5' *Taq* nuclease assay results of the 10^6 cells/ml-spiked samples.

Field sampling evaluation. Preputial smegma ($n = 249$) and cervico-vaginal mucus ($n = 120$) specimens were collected from 369 animals originating from 38 properties throughout northeastern Australia (Queensland) by using the bull rasper and the collection protocols described above. Specimens were assayed for *C. fetus* subsp. *venerealis* by both diagnostic culture and 5' *Taq* nuclease assay. Urine was collected (as described above) from 16 bulls whose samples were positive by smegma 5' *Taq* nuclease assay and processed for 5' *Taq* nuclease assay testing. A chi-square test was used to compare the distribution of positive and negative results for the two methods of testing, culture and 5' *Taq* nuclease assay. The proportions that were detected as positive by the two methods were also compared by using a normal approximation. Mucus samples from female cattle were also tested by using the *C. fetus* subsp. *venerealis* ELISA as described previously (23).

TABLE 3. Colony counts of dilution series prepared from fresh 2-day cultures of *C. fetus* subsp. *venerealis* subsequently used for sensitivity and transport inoculation experiments

Cells/inoculum[a]	Avg colony count	SD
2×10^3	>1,000[b]	NA[c]
2×10^2	210	28.28427
2×10^1	22	4.242641
2×10^0	2	1.414214
2×10^{-1}	0	0

[a] Cells/inoculum are based on an initial direct count using a hemocytometer followed by serial dilution. Inoculum volume was 100 μl/plate.
[b] The plate contained too many colonies to accurately count, with many colonies in physical contact.
[c] NA, not applicable.

RESULTS

Assay specificity and sensitivity. The genomic sequence of the species-specific amplicon from *C. fetus* subsp. *venerealis* strain 98-118432 is described in GenBank under accession no. AY903214. 5′ *Taq* nuclease primer and probe sequences are described in Table 2. BLASTn searches identified sequence identity between the primers, the probe, and the *C. fetus* subsp. *venerealis* plasmid ParA-like protein gene (GenBank accession no. AY750964). A single base difference was observed between the ParA-like protein gene sequence and the species-specific *C. fetus* subsp. *venerealis* amplicon that is described here.

By using serial dilutions of *C. fetus* subsp. *venerealis* genomic DNA (gDNA), optimal amplification and fluorescence were obtained using a three-step thermal profile (described in Materials and Methods) as opposed to the probe manufacturer's two-step preferred protocol (results not shown). 5′ *Taq* nuclease assay of genomic DNA from a range of related organisms and bovine venereal microflora (Table 1) did not produce any nonspecific amplification. Notably, the morphologically, phenotypically, and genetically similar *C. fetus* subsp. *fetus* did not produce a positive assay result in the *C. fetus* subsp. *venerealis* assay.

The viability counts of *C. fetus* subsp. *venerealis* dilutions correlated well with the hemocytometer counts as indicated in Table 3. The 5′ *Taq* nuclease assay ably detected approximately a single cell equivalent per assay from heat-lysed spiked preputial smegma and vaginal mucus preparations and 100 cell equivalents per assay from urine (Table 4). Culture-based detection was less sensitive (Table 4), and the *C. fetus* subsp. *venerealis* selective media suffered from significant levels of overgrowth by non-*Campylobacter*-like organisms, particularly from vaginal mucus. Conventional PCR assays generally improved upon the sensitivity of culture-based diagnosis (Table 4), with the greatest improvements being observed when vaginal mucus specimens were assayed. The 5′ *Taq* nuclease assay provided at least a 10-fold increase in sensitivity compared to those of the other methods that were evaluated and an improvement of 250-fold or higher compared to that for selective culture (Table 4).

Sample processing. A number of the samples collected from healthy animals contained visible contamination with feces, semen, and blood. Despite repeated attempts, the conventional PCR did not yield detectable products from crude cell extracts, and kit-purified gDNA had to be used as a template for these assays. The sensitivity limits and quantitative estimates that were observed by using the 5′ *Taq* nuclease assay did not change in the presence of potentially inhibitory substances in crude extracts; in fact, the sensitivity of detection improved slightly compared to that of gDNA extracts (Tables 4 and 5). Smegma specimens that were prepared for conventional PCR assay by a commercial DNA

TABLE 4. Detection limits for *Campylobacter fetus* subsp. *venerealis* from stored samples determined by selective culture, conventional PCR assay, and 5′ *Taq* nuclease assay[a]

Sample type	Storage time (days)[b]	Sensitivity limit[c]					
		Selective culture	Assay postenrichment culture[d]		Conventional PCR	5′ *Taq* nuclease assay	
			5′ *Taq* nuclease assay	Conventional PCR	QIAamp	QIAamp	Heat lysis
Smegma	0	>25,000	500	5,000[e]	>1,000	10	1
	2	25,000	500	5,000[e]	100	10	1
	5	2,500	5,000	>5,000	100	1	10
Mucus	0	>25,000	50	50	100	10	10[e]
	2	>25,000	500	500[e]	10	10[e]	1
	5	>25,000	500	5,000	10	10	1
Urine	0	500	ND[f]	ND	ND	10	100[e]
	2	>25,000	ND	ND	ND	10	1
	5	>25,000	ND	ND	ND	10	1

[a] See Table 3 for viability counts of each dilution.
[b] Smegma and mucus samples for 5′ *Taq* nuclease or conventional PCR assay were stored at ambient temperatures in either PBS or physiological saline. Urine samples were stored at 4°C. Samples for culture were stored in modified TEM at ambient temperatures.
[c] Sensitivity limits for both conventional PCR and 5′ *Taq* nuclease assay are given in cell equivalents/assay. Selective culture sensitivity limits are indicated as the minimum number of cells/inoculum in TEM required to produce *Campylobacter*-like colonies on subsequently inoculated selective media. Standard deviations on viable cell counts for each dilution are presented in Table 3.
[d] DNA was extracted from TEM for 5′ *Taq* nuclease and conventional PCR assay using a commercial kit (QIAamp).
[e] Positive assay results were possible for the log dilutions below this concentration but were not reliably obtained.
[f] ND, not done.

TABLE 5. Comparison of quantitative estimates for *Campylobacter fetus* subsp. *venerealis* by 5′ *Taq* nuclease assay of stored samples inoculated with 10^4 cells

Sample type	Storage time (days)[a]	Avg no. of cells/ml		
		Assay postenrichment culture[b,c]	5′ *Taq* nuclease assay	
			QIAamp	Heat lysis
Smegma	0	15,510	379	1,259
	2	3,380	94	1,836
	5	0	47	183
Mucus	0	7,905	996	5,282
	2	5,948	253	1,838
	5	2,764	206	810
Urine	0	ND[d]	976	693
	2	ND	414	426
	5	ND	393	776

[a] Smegma and mucus samples for 5′ *Taq* nuclease or conventional PCR assay were stored at ambient temperatures in either PBS or physiological saline. Urine samples were stored at 4°C. Samples for culture were stored in modified TEM at ambient temperatures.
[b] DNA was extracted from TEM for 5′ *Taq* nuclease assay using a commercial kit (QIAamp).
[c] Quantitative 5′ *Taq* nuclease assay estimates are given in cells/ml.
[d] ND, not done.

purification kit appeared to be inhibited relative to vaginal mucus specimens prepared the same way (Table 4).

Sample storage/transport. Selective culture of vaginal mucus samples for *C. fetus* subsp. *venerealis* led to significant levels of overgrowth by other venereal microflora, limiting identification of the slow-growing *Campylobacter* colonies following prolonged storage. Urine that had been stored at 4°C for 2 or more days also proved to have significant levels of contaminating organisms that were capable of growing on the *Campylobacter* selective media. Both the 5′ *Taq* nuclease and conventional PCR assays generally improved upon the detection sensitivity of selective culture following enrichment in TEM. But detection limits for both PCR-based techniques rose over the course of 5 days while detection limits for selective culture either improved or were unaltered (Table 4).

The direct detection of *C. fetus* subsp. *venerealis* from clinical specimens that were not stored in culture medium exhibited higher sensitivity by both conventional PCR and 5′ *Taq* nuclease assay compared with that of PCR detection postenrichment culture. In fact, estimates of *C. fetus* subsp. *venerealis* numbers in the TEM obtained from 5′ *Taq* nuclease assay quantitation were shown to drop steadily over the course of storage (Table 5). The sensitivity of the conventional PCR improved slightly when specimens were tested at days 2 and 5 (Table 4). A similar trend was generally observed for the 5′ *Taq* nuclease assay results following sample storage. Overall, 5′ *Taq* nuclease assay detection of crude cell lysates prepared from uncultured clinical material provided the highest level of *C. fetus* subsp. *venerealis* detection despite prolonged storage or transport at ambient temperatures (for mucus and smegma). Thus, this protocol was applied for detection of *C. fetus* subsp. *venerealis* for all subsequent animal testing.

Sample collection. The three techniques that were assessed for the collection of genital mucus specimens from cattle caused minimal adverse impact and no obvious discomfort upon the animals. The 10-mm pipette and artificial insemination infusion pipette both required the application of suction via a bulb or syringe, often requiring two operators in order to obtain a suitable sample. The bull rasper was less cumbersome to use and could be effectively manipulated with one hand. Three of the eight bulls tested were identified as having natural *C. fetus* subsp. *venerealis* infections, based upon at least one positive result with selective culture. Selective culture and 5′ *Taq* nuclease assay results for these three animals are presented in Table 6. Specimens that were collected by using the bull rasper obtained positive selective culture results for all three bulls, while those that were collected by using either a pipette or swab provided selective culture positive results from only one of the three bulls. The 5′ *Taq* nuclease assay provided positive results for all three bulls by using each collection technique, and estimates of cells/ml in the original specimens were highest in the specimens that were collected with the bull rasper (Table 6).

TABLE 6. Diagnostic assay results from successive testing of naturally infected bulls via different specimen collection tools

Bull	Collection tool	Selective culture[a]	Quantitative 5′ *Taq* nuclease assay[b]
1	Pipette	−	1,430
	Rasper	+	2,258
	Swab	−	85
2	Pipette	+	72
	Rasper	+	1,501
	Swab	−	148
3	Pipette	−	2,876
	Rasper	+	8,118
	Swab	+	2,910

[a] +, positive culture result; −, negative culture result.
[b] Quantitative 5′ *Taq* nuclease assay estimates are given in cells/ml, which is an average value calculated from duplicate results.

Field sampling evaluation. Results of the diagnostic culture and 5′ *Taq* nuclease assays for bull testing (smegma) are presented in Table 7. All mucus samples were negative by both culture and 5′ *Taq* nuclease assay; however, 14 were positive by ELISA. In two herds with four ELISA-positive results, bulls associated with these herds were identified as positive by the 5′ *Taq* nuclease assay. However, bulls associated with properties of herds for the remaining 10 ELISA positives were not tested. Of the 249 bull smegma samples, 13 were positive by culture, with 9 of these correlating to samples that were positive by the 5′ *Taq* nuclease assay. The four culture-positive specimens which were 5′ *Taq* nuclease assay negative were confirmed as false culture positives following 16S rRNA gene sequencing

TABLE 7. Comparison of diagnostic culture and 5′ *Taq* nuclease assay results from smegma specimens collected from northern Australian properties

Culture result	No. of 5′ *Taq* nuclease assay results that were:		Total
	Positive	Negative	
Positive	9	4[a]	13
Negative	30	206	236
Total	39	210	249

[a] Confirmed that the four 5′ *Taq* nuclease assay negative-culture positive isolates were nonspecific positives.

and conventional subspecies-specific PCR. A further 30 bulls were negative by culture yet were positive by 5′ *Taq* nuclease. Of the 16 urine samples collected from 16 smegma-positive bulls, only 2 urine samples tested positive with the 5′ *Taq* nuclease assay (results not shown). Chi-square analysis of the data indicated a strong association between results for the 5′ *Taq* nuclease assay and selective culture ($\chi^2 = 29.8$; $P < 0.001$). Comparison of the proportions that were positive for the 5′ *Taq* nuclease assay (16%) and selective culture (5%) indicated the 5′ *Taq* nuclease assay is detecting significantly more infected animals ($P < 0.001$).

DISCUSSION

Real-time PCR-based techniques, such as 5′ *Taq* nuclease assays, have been applied to the clinical diagnosis of a wide range of pathogens from various sources, including *C. jejuni* from human stools (24, 43) and *Trichomonas vaginalis* from female genital secretions (27). These assays provide improvements in sensitivity and specificity relative to selective culture and direct microscopic examination, and diagnoses can be obtained in significantly less time. Real-time PCR techniques are less labor intensive than conventional PCR-based assays, as there is no need for electrophoretic analysis, and the use of specific probes offers improved assay sensitivity and specificity (58). These assays can also provide quantitative measures of target organisms, providing useful tools to clinicians and diagnosticians (13, 37, 53). The assay described here is the first real-time probe-based PCR assay that was developed for the specific detection and quantification of *C. fetus* subsp. *venerealis* to improve the identification of campylobacteriosis in bull carriers.

The assay designed here was based on a subspecies-specific PCR target that was shown to previously identify *C. fetus* subsp. *venerealis* successfully, differentiating among 99 strains of *C. fetus* subspecies (22). Subsequently, this PCR has demonstrated suitability for subspecies-specific diagnostic and research identification of *C. fetus* subsp. *venerealis* (42, 56, 57) and was therefore considered a suitable target for the development of the 5′ *Taq* nuclease assay that is described here. The sensitivity of the 5′ *Taq* nuclease assay was higher than that of the conventional PCR assay, as demonstrated during testing of laboratory-spiked clinical specimens or DNA prepared from similarly inoculated transport medium. In addition, the conventional PCR did not consistently amplify product from crude extracts, requiring pure DNA as a template from both clinical specimens and culture medium. The specificity of this target fragment for the identification of *C. fetus* subsp. *venerealis* was further confirmed by specific detection by 5′ *Taq* nuclease assay of all strains of this subspecies that were tested in this study while also providing considerable improvement on the conventional PCR assay based on this same target.

The 5′ *Taq* nuclease assay provides several significant improvements over conventional culture diagnostic methods. Approximately one single target cell was sufficient for a positive result from smegma or cervico-vaginal mucus, whereas a culture-based diagnosis to isolate *Campylobacter*-like colonies required a minimum of 1,000 cells. A method which can withstand prolonged transport conditions is critical for the sampling of animals from extensively grazed cattle regions. Prolonged transport results in poor culture isolation of *C. fetus* subsp. *venerealis*, and it is recommended that for successful culture, samples be transported for up to only 48 h prior to subculture onto selective medium (7). The slow growth and fastidious nutritional requirements of *C. fetus* subsp. *venerealis* allow rapid overgrowth by more vigorously multiplying contaminating organisms. The organism also maintains limited viability under normal levels of atmospheric oxygen, limiting its survival during transport (8). In addition, antimicrobial susceptibility differs between isolates of *C. fetus* subsp. *venerealis*, with a majority of isolates showing susceptibility to polymyxin B, which is used in most *Campylobacter fetus* selective media (26). The decline in quantitative 5′ *Taq* nuclease assay estimates of *C. fetus* subsp. *venerealis* numbers during 5 days of simulated transport in modified Weybridge media illustrates the impact of these factors on the subsequent isolation of the pathogen. Therefore, these factors reduce the effectiveness of culture-based diagnosis, leading to false-negative results for infected animals as confirmed by our field investigation in this study.

ELISA-based diagnosis of campylobacteriosis has several significant limitations as a diagnostic tool. It is an indirect diagnostic method, detecting, rather than the organism itself, immunoglobulin A antibodies that are specific for the organism. This immune response can persist for up to 10 months after infection, long after the infection has been eliminated (23). As such, it is not an indicator of current infection status, but rather of exposure within the previous 10 months. The ELISA is unsuitable for use in diagnosis of bulls, due to a lack of sufficient titers of antibody in preputial fluids (59). All results for female cattle tested in this study were negative by culture and 5′ *Taq* nuclease assays despite the demonstrated high sensitivity of 5′ *Taq* nuclease assay in spiked mucus samples. We did, however, identify previously infected females by using the ELISA, demonstrating the effectiveness of the ELISA to detect previous exposure. It is feasible that the seasonal timing of our sample collections did not coincide with current or recent infection of the female cattle that were sampled for this study. We did not have access to the DIFT used in Argentina to determine whether this assay is suitable as an alternative confirmatory diagnostic tool (38).

The direct PCR detection of pathogens in clinical specimens without culture enrichment is increasingly being applied for disease diagnosis (1, 12, 16, 39, 46, 47, 54, 60). Although PCR methods have been developed to differentiate the *C. fetus* subspecies following enrichment culture (22, 44), very few studies describe the direct amplification from clinical specimens (15). Preputial and cervico-vaginal mucus specimens may contain a range of contaminating materials, including blood, urine, feces, pus, and semen. These potentially inhibitory materials can limit the effectiveness of PCR as a reliable diagnostic tool unless adequate DNA purification steps are undertaken (2, 25). The 5′ *Taq* nuclease assay described here suffers only minor inhibition in the presence of urine following crude cell lysis and no significant loss of sensitivity or specificity in the presence of smegma or mucus, including specimens contaminated with the blood, feces, or semen as observed during this study. Urine is commonly used for the diagnosis of human venereal diseases in males but is less suitable for similar diagnoses in females (33, 48). Laboratory-spiked urine specimens were suitable for 5′ *Taq* nuclease assay, but the suitability of

urine as a clinical specimen proved less satisfactory than smegma in our study. Preputial smegma is recommended as the most reliable clinical sample for the specific diagnosis of *C. fetus* subsp. *venerealis* from bulls. Mucus samples can also be tested by using the protocols described here, but success is dependent upon recent colonization of the bacteria in infected female cattle as described above.

Heat lysis techniques have been successfully applied for the isolation of template DNA from diagnostic specimens and thus offer considerable time and labor savings for the routine application of DNA-based diagnostics (29, 36, 40, 45, 61). Although crude sample processing does not remove all potential PCR-inhibitory substances, 5′ *Taq* nuclease assays appear to be more robust, enabling successful amplification of target material as demonstrated in this study. This also simplifies the requirements for transport from the field to the laboratory, without the need for complex transport enrichment media. Although, with prolonged storage of some samples, the sensitivity of detection following heat lysis diminished slightly, it was determined that amplification following this crude preparation method proved more sensitive than that of 5′ *Taq* nuclease assay by using kit-purified templates. Further transport studies examining the statistical differences of each processing method and *C. fetus* subsp. *venerealis* viability are required to confirm the preliminary outcomes that were identified in this study. Nevertheless, the heat lysis processing of clinical samples, followed by 5′ *Taq* nuclease assay, provided the most sensitive and practical protocol for the reliable detection of *C. fetus* subsp. *venerealis* for future routine application in diagnostic laboratories.

Previous studies have demonstrated that the bull rasper could be an effective tool for the collection of venereal samples for diagnosis (55). By comparing quantitative 5′ *Taq* nuclease assay results, we were able to confirm that specimens collected from infected bulls by using the bull rasper yielded higher estimates of *C. fetus* subsp. *venerealis* cells than did other collection tools. This was also confirmed by a higher success rate of positive culture from some specimens. In addition, the bull rasper led to marked improvements in the ease of specimen collection from both male and female cattle. Ease of use for the veterinarian, combined with improved specimen quality and no notable adverse impact upon the animal, makes the bull rasper a superior tool for the collection of genital specimens from cattle for the diagnosis of campylobacteriosis by either selective culture or 5′ *Taq* nuclease assay. A bull rasper may be an effective tool for the collection of diagnostic specimens for other venereally localized organisms, such as *Trinichomonas fetus*.

In summary, the 5′ *Taq* nuclease assay described here is a reliable, sensitive, and specific detection method for *C. fetus* subsp. *venerealis* in bovine venereal diagnostic specimens, providing reliable detection of as few as approximately one cell equivalent per assay, and is able to readily discriminate between the target organism and the phenotypically and genotypically similar *C. fetus* subsp. *fetus*. Specimen collection from male and female cattle by using a bull rasper has been found to be simple and efficient, and specimens that are suspended in physiological saline have proven to be stable during transport at ambient temperatures. Diagnostic specimens can be processed by a simple and rapid heat lysis technique rather than DNA extraction, with no loss of sensitivity. Significant improvements in sensitivity and specificity over those obtained with selective culture-based and conventional PCR-based techniques was observed, with bull testing proving to be the most reliable specimen for screening herds for this pathogen. The assay should be suitable for routine use within diagnostic laboratories with continued use of "gold standard" culture methods. A multicenter evaluation of the specimen collection, transport, processing, and assay procedures should prove valuable. As the detection of *C. fetus* subsp. *venerealis* is significant for trade restrictions, it will be crucial to develop standardized and robust internal positive and negative control protocols, to develop an alternative sensitive assay to confirm positive results, and to obtain sequential samples from animals to confirm test results.

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ADDENDUM IN PROOF

The data summarized in Table 7 were subjected to reanalysis after the paper was submitted. Some samples were removed from the analysis, with slight consequent changes to the statistical results. The changes are reflected in the text and do not affect the interpretation of the results or any subsequent conclusions.

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Improved detection of *Tritrichomonas foetus* in bovine diagnostic specimens using a novel probe-based real time PCR assay

Lyle McMillen, Ala E. Lew *

Department of Primary Industries and Fisheries, Locked Mail Bag No. 4, Moorooka, 4105 Qld, Australia

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Abstract

A *Tritrichomonas foetus*-specific 5′ *Taq* nuclease assay using a 3′ minor groove binder-DNA probe (TaqMan® MGB) targeting conserved regions of the internal transcribed spacer-1 (ITS-1) was developed and compared to established diagnostic procedures. Specificity of the assay was evaluated using bovine venereal microflora and a range of related trichomonad species. Assay sensitivity was evaluated with $\log_{10}$ dilutions of known numbers of cells, and compared to that for microscopy following culture (InPouch™ TF test kit) and the conventional TFR3-TFR4 PCR assay. The 5′ *Taq* nuclease assay detected a single cell per assay from smegma or mucus which was 2500-fold or 250-fold more sensitive than microscopy following selective culture from smegma or mucus respectively, and 500-fold more sensitive than culture followed by conventional PCR assay. The sensitivity of the conventional PCR assay was comparable to the 5′ *Taq* nuclease assay when testing purified DNA extracted from clinical specimens, whereas the 5′ *Taq* nuclease assay sensitivity improved using crude cell lysates, which were not suitable as template for the conventional PCR assay. Urine was evaluated as a diagnostic specimen providing improved and equivalent levels of *T. foetus* detection in spiked urine by both microscopy following culture and direct 5′ *Taq* nuclease detection, respectively, compared with smegma and mucus, however inconclusive results were obtained with urine samples from the field study. Diagnostic specimens ($n = 159$) were collected from herds with culture positive animals and of the 14 animals positive by 5′ *Taq* nuclease assay, 3 were confirmed by selective culture/microscopy detection (Fisher's exact test $P < 0.001$). The 5′ *Taq* nuclease assay described here demonstrated superior sensitivity to traditional culture/microscopy and offers advantages over the application of conventional PCR for the detection of *T. foetus* in clinical samples.

Keywords: *Tritrichomonas foetus*; Bovine venereal disease; 5′ *Taq* nuclease; Minor groove binder; TaqMan®

1. Introduction

Trichomoniasis is a venereally transmitted reproductive disease of cattle caused by the protozoan parasite *Tritrichomonas foetus* (Levine, 1973), and is widely distributed in regions relying on extensive herd management and natural breeding, including areas of the USA, Canada, Spain, Africa, Asia, Australia and Latin America (Behera et al., 1987; BonDurant et al., 1990; Copeland et al., 1994; Erasmus et al., 1989; Griffiths et al., 1984; Kvasnicka et al., 1989; Martin-Gomez et al., 1998; McCool et al., 1988; Perez et al., 1992; Rae et al., 2004; Takizawa and Ito, 1977). Infection can be inapparent in bulls, but can cause early pregnancy abortion in cows. Bulls (particularly over 5 years old) are the primary carrier, and may reinfect previously infected cows during service, while cows clear the infection after a few months (Riley et al., 1995; Yule et al., 1989). Trichomoniasis is similar to

* Corresponding author. Tel.: +61 7 3362 9502; fax: +61 7 3362 9429.
E-mail address: ala.lew@dpi.qld.gov.au (A.E. Lew).

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campylobacteriosis caused by the bacterium *Campylobacter fetus* subsp. *venerealis* and thus diagnosis is crucial for the development of disease specific control measures (reviewed by BonDurant, 2005). Management practises include a commercially available *T. foetus* vaccine (Trichguard®, Fort Dodge), the culling of positive bulls, the use of artificial insemination over natural breeding, separating females into pregnant >5 months/pregnant <5 months/cull opens, or restricting breeding to bulls less than 5 years old (BonDurant, 2005; Clark et al., 1974; Felleisen et al., 1998; Yule et al., 1989).

Diagnostic specimens (preputial smegma or cervicovaginal mucus) are typically collected by washing or scraping with an aspirated AI infusion pipette, however a unique tool with a scraper head or 'metal brush' can improve the collection of secretions by reducing contamination levels and improving isolation rates compared to aspiration or washing (BonDurant et al., 2003; Irons et al., 2002; Kittel et al., 1998; Mancebo et al., 1995; McMillen et al., 2006; Mukhufhi et al., 2003; Parker et al., 1999; Tedesco et al., 1977). Diagnosis of infection is typically based on microscopic examination of an inoculated enrichment/transport medium (e.g. InPouch™, BioMed Diagnostics) to identify spindle-shaped trichomonads with characteristic darting motility (Kittel et al., 1998). *T. foetus* is considered easily recognised within 7 days without the need for further selective isolation, but increasing evidence indicates that microscopic examination may be insufficient to confirm the taxa of trichomonads isolated from the prepuce of bulls due to contamination with intestinal or coprophilic trichomonads (Campero et al., 2003; Kittel et al., 1998; Taylor et al., 1994). A 100% diagnostic sensitivity of culture has been demonstrated, however rates of 81–90% of naturally infected bulls and only 58–78% of infected female cattle are more commonly reported (Bryan et al., 1999; Clark et al., 1971; Fitzgerald et al., 1954; Lun et al., 2000; Parker et al., 1999; Reece et al., 1983; Skirrow, 1987; Skirrow et al., 1985). Success of culture in these studies appears to vary with the sampling technique used, the temperature during sample transport, the time in transit, use of different media, the number of repetitive samples taken from bulls (thereby increasing the likelihood of isolation), different isolate growth characteristics and the number of *T. foetus* organisms inoculated (Bryan et al., 1999; Parker et al., 2003). As field samples are often subjected to suboptimal conditions, there has long been a need for the development of a more robust diagnostic test for trichomoniasis (BonDurant, 2005; Bryan et al., 1999).

Diagnostic culture followed by microscopic examination to detect the presence of *T. foetus* organisms is considered the 'gold standard' protocol and researchers have developed polymerase chain reaction or DNA based assays to enhance the culture diagnosis success rate (Felleisen et al., 1998; Ho et al., 1994; Nickel et al., 2002). The internal transcribed spacer-1 (ITS-1) based PCR method developed by Felleisen et al. (1998) has found wide acceptance and has been applied to detect *T. foetus* in culture to improve upon microscopic examination and to differentiate *T. foetus* from morphologically similar trichomonads (Campero et al., 2003; Cobo et al., 2003; Hoevers et al., 2003; Parker et al., 2001, 2003). A new ITS-1 PCR using a fluorescently labeled primer has been developed and differentiates *T. foetus* from other trichomonads based on amplicon size, however the diagnostic sensitivity or applicability of this assay has not been demonstrated (Grahn et al., 2005). Results using the Felleisen et al. PCR directly on clinical samples or cultured clinical material have shown either equivalent or improved detection by PCR when compared with conventional culture and microscopy (BonDurant et al., 2003; Felleisen et al., 1998; Mukhufhi et al., 2003; Parker et al., 2001). Some researchers have suggested that the culture or clinical specimen DNA preparations contain inhibitory substances and novel DNA processing procedures have subsequently improved the sensitivity of the PCR test (Chen and Li, 2001; Mukhufhi et al., 2003). The aim of this study was to develop a robust 5′ *Taq* nuclease (probe-based real time) PCR assay targeting the ITS-1 region of *T. foetus* to circumvent the specificity and processing issues observed using conventional PCR for the improved detection of this pathogen.

5′ *Taq* nuclease assays using fluorescent probes are highly sensitive and specific, and also allow the quantification of target DNA within the sample (Mackay, 2004). 5′ *Taq* nuclease assays have improved the detection of a wide range of protozoan parasites, including *Trichomonas vaginalis* (Hardick et al., 2003; Jordan et al., 2001), *Plasmodium chabaudi* (Cheesman et al., 2003), *Cryptosporidium parvum* and *Giardia lamblia* (Fontaine and Guillot, 2002; Guy et al., 2003). Minor groove binder (MGB) probes demonstrate higher specificity and sensitivity than non-MGB probes in 5′ *Taq* nuclease assays (Kutyavin et al., 2000), and thus are highly suited for routine diagnostic application as demonstrated for the detection of bovine retroviruses and *C. fetus* subsp. *venerealis* from clinical material (Lew et al., 2004a,b; McMillen et al., 2006). This study describes firstly the development of a *T. foetus*-specific 5′ *Taq* nuclease assay, followed by the optimisation of

sampling, transport, and specimen processing protocols for the sensitive and specific diagnosis of bovine venereal trichomoniasis.

2. Materials and methods

2.1. Bacterial and protozoan culture

Isolates of *T. foetus* were obtained from the Animal Research Institute (DPI&F), and the American Type Culture Collection (Table 1). *T. foetus* strains were grown at 37 °C in 1.25% neutralised liver digest, 0.5% tryptose, 0.15% bacto agar, 50% sterile inactivated bovine serum, 0.1% P + S solution (0.75% penicillin, 0.082% streptomycin). *Campylobacter* species were grown at 37 °C in 1× Brain-Heart Infusion (Oxoid), 0.2% yeast extract, 0.07% Bacto agar for between 1 and 3 days. *Pseudomonas aeruginosa* and *Proteus vulgaris* were grown at 37 °C on blood agar plates for 24 h. *Neospora caninum* tachyzoites were cultured in vero cells as previously described (De Meerschman et al., 2002).

2.2. Animal samples

Preputial smegma samples were collected from bulls (while restrained in a veterinary crush) using either a bull rasper or aspirated using an artificial insemination (AI) infusion pipette. A bull rasper (polyethylene, 60 cm long with a 75 mm long, 8 mm diameter corrugated scraper head with a 1.5 mm collection bore attached to 6 mm diameter tubing with a 1.5 mm internal diameter, similar to those produced by Elastecnica, Argentina based on Sutka and Katai, 1969) was gently scraped along the surface of the penis and internal prepuce near the fornix. No aspiration was necessary. The collected smegma was rinsed into approximately 5 ml physiological saline. A sterile AI infusion pipette was gently scraped along the surface of the penis and internal prepuce near the fornix, applying gentle suction with a syringe or rubber bulb. The collected smegma was rinsed into approximately 5 ml sterile physiological saline.

Vaginal mucus samples were collected from heifers with a bull rasper during restraint in a veterinary crush. A bull rasper was inserted so that the anterior end reached the vulva. The rasper was moved gently backwards and forwards. No aspiration was necessary. The rasper was removed and the collected mucus rinsed into approximately 5 ml sterile physiological saline.

Urine was investigated to determine its feasibility as an alternative clinical specimen for diagnosis of venereal *T. foetus* infection. Urine from two consecutive voids was collected in a series of sterile collection containers following either palpation of the perineum to induce urination in heifers or subcutaneous or intramuscular administration of a diuretic (Frusemide, Ilium Veterinary Products, Australia).

Table 1
Reference species and strains used in this study

Species	Strain	Source
Tritrichomonas foetus	YVL-W	Field isolate (DPI&F, Qld)[a]
T. foetus	BP-4 (30003)	ATCC[b]
Tetratrichomonas gallinarum	TP-79 (30097)	ATCC
Pentatrichomonas hominis	hs-3 (30000)	ATCC
Trichomonas vaginalis	C-1 (30001)	ATCC
Campylobacter coli	11353	NCTC[c]
C. fetus subsp. *venerealis*	98-109383	Field isolate (DPI&F, Qld)
C. fetus subsp. *venerealis*	19438	ATCC
C. fetus subsp. *fetus*	98-118432	Field isolate (DPI&F, Qld)
C. fetus subsp. *fetus*	15296	ATCC
C. jejuni subsp. *jejuni*	11168	NCTC
C. hyointestinalis	N3145	Field isolate (DPI&F, Qld)
C. sputorum subsp. *bubulus*	Y4291-1	Field isolate (DPI&F, Qld)
Pseudomonas aeruginosa	27853	ATCC
Proteus vulgaris	6380	ATCC
Neospora caninum	50843	ATCC
Leptospira borgpetersenii serovar Hardjobovis	93/94451/3	Field isolate (DPI&F, Qld)
L. interrogans serovar Pomona	Pomona	CCRL[d]

[a] Animal Research Institute, Department of Primary Industries and Fisheries, Qld, Australia.
[b] American Type Culture Collection, Virginia, USA.
[c] National Collection of Type Cultures, London, United Kingdom.
[d] WHO/FAO/OIE Collaborating Centre for Reference and Research on Leptospirosis, Australia and Western Pacific Region.

The first container collected was discarded as being the most likely to be heavily contaminated with fecal material, hair, and other debris.

2.3. Diagnostic culture/microscopy

Culture-based diagnosis for *T. foetus* was conducted by inoculating InPouch™ TF media (Biomed Diagnostics) with 0.5 ml of preputial smegma or vaginal mucus in saline followed by transport to the laboratory at ambient temperatures (up to 48 h) and incubated at 37 °C. The media was examined microscopically every 24 h for 5 days for the presence of motile trichomonads.

2.4. PCR template preparation

DNA was extracted from liquid culture, preputial smegma, vaginal mucus or urine samples using a commercial kit (QIAamp DNA mini kit, Qiagen) as per the manufacturer's protocol, except using a final elution volume of 50 μl rather than 400 μl. DNA concentration was determined following spectrophotometric measurement at A_{260} using a biophotometer (Eppendorf).

Crude cell lysates were prepared for the 5′ *Taq* nuclease assay by a heat lysis method. One millilitre of the sample (preputial smegma or vaginal mucus in saline, urine) was centrifuged for 5 min at 12,000 × *g*, and the supernatant discarded. The pellet or compressed mucus was resuspended in 500 μl sterile dH_2O, and heated at 95 °C for 10 min. The suspension was centrifuged for 30 s at 2000 × *g*, and 5 μl supernatant was assayed by 5′ *Taq* nuclease assay.

2.5. PCR amplification and conventional PCR assay

All primers used in this study were synthesised by Proligo Australia Pty Ltd. The 347 bp *T. foetus*-specific product was amplified in a 15 μl reaction volume, using 500 nM TFR3 and TFR4 primers (Felleisen et al., 1998), 1× PCR reaction buffer with $MgCl_2$ (Roche Diagnostics), 200 μM dNTPs, 1 U *Taq* DNA polymerase (Roche Diagnostics) and 1 ng of *T. foetus* DNA. For smegma, mucus and urine extracts, 2 μl of kit purified DNA was added as template for conventional PCR assays. The reactions were cycled in a GeneAmp PCR system model 2700 (Applied Biosystems Inc.), using the following temperature profile: an initial denaturation at 94 °C for 90 s, followed by 40 cycles of denaturation at 94 °C for 30 s, annealing at 67 °C for 30 s, and extension at 72 °C for 90 s, including a final single extension for 15 min at the end of the profile. Amplification products were separated in 2% TBE (89 mM Tris borate, 2 mM EDTA, pH 8) agarose gels using size markers (Marker XIV, Roche Molecular Biochemicals, Germany) and were visualized under UV illumination by ethidium bromide staining. The presence of *T. foetus* was indicated by the presence of the 347 bp *T. foetus*-specific amplicon.

2.6. Sequencing

The 347 bp *T. foetus*-specific amplicon from strain YVL-W was ligated into a cloning vector (pCR2.1, TOPO-TA cloning kit, Invitrogen Corporation) as described in the manufacturer's protocol. Plasmids with inserts were sequenced using the T7 and M13 Reverse primers, and BigDye Terminator Mix (Applied Biosystems Inc.), following the manufacturer's protocols. Sequences were analysed by the Griffith University DNA Sequencing Facility (School of Biomolecular and Biomedical Science, Griffith University, Nathan, Qld 4111, Australia) using an ABI 377 DNA Sequencer.

2.7. 5′ Taq nuclease assay

Primer and probe combinations for 5′ *Taq* nuclease assay using fluorescent 3' minor groove binder-DNA probes were designed for *T. foetus* ITS-1 using Primer Express Version 2 (Applied Biosystems Inc.), and BLASTn searches (http://www.ncbi.nlm.nih.gov/BLAST/) were conducted to confirm sequence specificity. Primer and probe sequences are: TFF2: GCGGCTGGATTAGCTTTCTTT; TFR2: GGCGCG-CAATGTGCAT; TRICHP2: 6-FAM-ACAAGTTC-GATCTTTG-MGB-BHQ. 5′ *Taq* nuclease assay for *T. foetus* was conducted in a 25 μl volume, using either Platinum Quantitative PCR SuperMix-UDG (Invitrogen Life Technologies) or RealMasterMix probe mix (Eppendorf), with 900 nM TFF2 and TFR2 primers, and 80 nM TRICHP2 fluorescent 3′ minor groove binder-DNA probe in a Corbett Rotor-Gene RG-3000 (Corbett Research, Australia). The thermal profile was 50 °C for 2 min, 95 °C for 2 min, and 40 cycles of denaturation at 95 °C for 20 s and annealing/extension at 60 °C for 45 s. Fluorescence acquisition occurred at the end of each annealing/extension cycle. A positive result was indicated by the fluorescence (normalised to a no-template control) passing a threshold of 0.1. Assay conditions were optimized using serial dilutions of *T. foetus* purified DNA. Quantitative estimates of target cells/ml were calculated using a standard curve generated with either gDNA or crude cell extracts prepared from serial dilutions of known numbers of

T. foetus from 10^5 to 1 cell(s)/ml. All 5′ *Taq* nuclease assays were prepared in duplicate with cycle threshold (C_T) or quantitative cell estimates averaged for each sample. Inconsistently positive samples (one of two repeats) were repeated.

2.8. Sensitivity evaluation and effects of transport

T. foetus strain YVL-W cells were counted using a hemocytometer and inoculated into smegma, mucus and urine collected from healthy animals which previously tested negative by both selective culture/microscopy and 5′ *Taq* nuclease assay. Serial log dilutions started at 10^5 cells/ml and were diluted to 1 cell/ml. Genomic DNA was extracted from the spiked specimen using a commercial DNA extraction kit (QIAamp DNA minikit, Qiagen), and assayed by both conventional PCR assay and 5′ *Taq* nuclease assay. Inoculated specimens were also prepared for 5′ *Taq* nuclease assay by heat lysis. InPouch™ TF test kits were inoculated with laboratory-spiked specimens, as for diagnostic culture. DNA was also extracted from aliquots of inoculated InPouch™ TF media using a commercial kit (QIAamp, Qiagen) and assayed by both 5′ *Taq* nuclease assay and conventional PCR assay. Estimates of cell equivalents/assay or cells/inoculum were calculated from the enumerated spiked specimen by determining equivalent cell numbers contained in the final volume used as PCR assay template or for the culture inoculum.

5′ *Taq* nuclease assays were scored positive if the fluorescence (normalised against a no-template control) passed a threshold of 0.1. A positive conventional PCR assay required detection of a 347 bp amplicon. Cultured samples were scored positive on the presence of motile trichomonads with multiple anterior flagella, a posterior flagellum, a visible undulating membrane and characteristic jerky motility upon microscopic examination.

Sample transport was simulated by storing the inoculated InPouch™ TF media and animal samples for up to 5 days at ambient temperatures for smegma or mucus, and at 4 °C for urine. Samples for 5′ *Taq* nuclease assays, conventional PCR assays and selective culture were processed as described above at time 0, and after 2 and 5 days of storage. To examine changes in cell numbers under these storage conditions, quantified cell estimates were compared using 5′ *Taq* nuclease assay results of the 10^4 cells/ml spiked samples.

2.9. Field diagnostic comparison

Smegma and mucus samples were collected from 129 bulls and 30 cows as described above, from herds in north eastern Australia with confirmed culture/microscopy positive animals. Urine was also collected randomly from 71 animals (48 bulls, 23 cows) as described above, to confirm the usefulness of urine for *T. foetus* screening. Specimens were assayed by both *T. foetus* culture/microscopy and the 5′ *Taq* nuclease assay as described above. Fisher's exact test (Steel and Torrie, 1980) was used to determine the statistical significance of the results obtained with the traditional culture/microscopy methods and the *Taq* nuclease assay.

3. Results

3.1. Assay specificity and sensitivity

The sequence of the species-specific amplicon from *T. foetus* strain YVL-W was 100% homologous with the following GenBank ITS accessions: *T. foetus* (AF339736), *T. suis* (U85966), and *T. mobilensis* (U86612). 5′ *Taq* nuclease assay of genomic DNA from a range of related organisms and bovine venereal microflora (Table 1) did not produce any non-specific amplification.

The sensitivity of the assay using genomic DNA preparations of *T. foetus* as template was consistent at 3 fg DNA and C_T values ≥ 40 are negative (Fig. 1). Cell equivalents were compared to standard curves derived from crude cell lysates of serial dilutions of *T. foetus* cells (Fig. 1). Inter-assay variability was evaluated through eight assays of *T. foetus* genomic DNA samples, with a standard deviation of 1.21 cycles between the C_T values obtained for a given sample. Both *T. foetus* DNA and the *T. foetus* cell lysate templates were used as assay positive controls to compensate for any variation in C_T values between real time PCR experiments and maintain assay integrity. The 5′ *Taq* nuclease assay detected a single cell equivalent per assay from laboratory-spiked preputial smegma, vaginal mucus and urine (Table 2), with less than a cell equivalent per assay being reliably detected from several heat-lysed specimens.

When techniques used for preparing clinical samples for 5′ *Taq* nuclease assay were compared, processing the samples using the heat lysis method provided as good or better sensitivity than the QIAamp DNA minikit (Table 2). A number of the samples collected from healthy animals contained visible contamination with feces, semen and blood. Microscopic examination revealed high levels of bacteria, semen, blood, pus, epithelial cells and plant material in many genital specimens. The sensitivity limits and quantitative estimates observed using the 5′ *Taq* nuclease assay

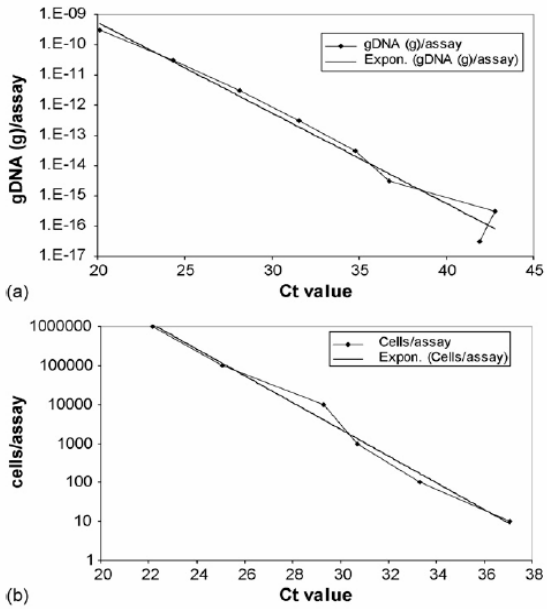

Fig. 1. Cycle threshold (C_T) values demonstrating the sensitivity of the *Tritrichomonas foetus*-specific 5′ *Taq* nuclease assay for *T. foetus* genomic DNA and crude cell lysates of serially diluted cells. (a) Sensitivity of detection for genomic DNA. Trendline: R^2 value = 0.9759; $y = 0.0005\ e^{-0.6897x}$. (b) Sensitivity of detection for crude cell lysates. Trendline: R^2 value = 0.9863; $y = 4E + 13\ e^{-0.7899x}$.

Table 2
Detection limits for *T. foetus* from laboratory-spiked stored smegma, mucus and urine using microscopy following selective culture, conventional PCR assay (Felleisen et al., 1998) and 5′ *Taq* nuclease assay

Sample type	Storage time (days)[a]	Selective culture/microscopy (cells/inoculum)[b]	Assay post-enrichment culture[c]		Conventional PCR	5′ *Taq* nuclease assay	
			5′ *Taq* nuclease assay	Conventional PCR	QIAamp	QIAamp	Heat lysis
Smegma	0	>25000	50	500	1	1	0.1
	2	2500	50	500	1	1	1[d]
	5	>25000	50	5000	1	1	0.1
Mucus	0	25000	50	500	1	1	0.1
	2	250	50	500	1	1	0.1
	5	25000	50	500	1	1	0.1
Urine	0	5000	ND[e]	ND	ND	1	0.1
	2	500	ND	ND	ND	1	1
	5	500	ND	ND	ND	1	0.1

[a] Smegma and mucus samples for 5′ *Taq* nuclease assay were stored at ambient temperatures in physiological saline. Urine samples were stored at 4 °C. Samples for culture were stored in InPouch™ TF test kits at 37 °C.
[b] Sensitivity limits for both conventional PCR and 5′ *Taq* nuclease assay are given in cell equivalents/assay. Selective culture/microscopy sensitivity limits are indicated as the minimum number of cells/inoculum of InPouch™ TF media required for motile *Tritrichomonas*-like protozoans to be visible on subsequent microscopic examination.
[c] DNA was extracted from InPouch™ TF media for 5′ *Taq* nuclease and conventional PCR assay using a commercial kit (QIAamp).
[d] Positive assay results were possible for the log dilutions below this concentration, but were not reliably obtained.
[e] Not done.

did not change in the presence of these potentially inhibitory substances. These same crude heat lysis extracts did not amplify reliably in conventional PCR which thus required kit purified DNA as reliable PCR template (data not shown). Assay of selectively cultured specimens by conventional PCR detected as few as 500 cells inoculated into the InPouch™ TF test kit, substantially improving upon the sensitivity of culture/microscopy.

Culture/microscopy-based detection was markedly less sensitive than PCR, giving positive identification of motile trichomonads following inoculation with a minimum of 2500 cells. Spiking experiments demonstrated that smegma and mucus required the inoculation of high cell numbers to obtain a positive identification by culture/microscopy compared to that observed for urine (Table 2).

3.2. Sample storage and transport

Several trends were observed throughout the course of storage. Selective culture/microscopy-based diagnosis of *T. foetus* was most sensitive after 2 days, and several InPouch™ test kits with visible motile trichomonads after 2 days, which were subsequently not visible after 5 days (Table 2). Estimates of the numbers of *T. foetus* cells present in each sample using the 5′ *Taq* nuclease assay were generally lower with longer storage/transport up to 5 days (Table 3), but detection limits did not change (Table 2).

Following enrichment in InPouch™ TF test kits, both the 5′ *Taq* nuclease assay and conventional PCR assay improved upon the detection sensitivity of selective culture/microscopy, although sensitivity was markedly reduced when compared to 5′ *Taq* nuclease assay or conventional PCR assay without enrichment culture (Table 2).

3.3. Field diagnostic comparison

Of the 159 samples tested from animals from culture positive herds (129 female, 30 male), 14 samples (13 smegma and 1 mucus) were positive in the 5′ *Taq* nuclease assay with 3 of the smegma samples confirmed positive by microscopic examination of the InPouch™ TF test kits. For 1 herd group of 20 animals, the selective culture/microscopy was performed in an accredited veterinary diagnostic laboratory during routine herd management. Quantitation of *T. foetus* density in the clinical specimens against clinical samples spiked with directly enumerated *T. foetus* was performed by 5′ *Taq* nuclease assay, although the data was unable to be related to numbers of parasites present in the host due to variability in both the volume of genital mucus collected and the site of collection within the genital tract. All 145 negative samples were negative by both 5′ *Taq* nuclease assay and culture/microscopy. The Fisher's exact test indicated statistically significant results ($P < 0.001$) confirming higher sensitivity of the 5′ *Taq* nuclease assay compared with culture/microscopy. Incomplete urine sampling was undertaken and no correlating urine samples from smegma and mucus *T. foetus* positive animals were collected. All other urine samples collected tested

Table 3
A comparison of quantitative average cell/ml estimates of *T. foetus* by 5′ *Taq* nuclease assay of stored smegma, mucus and urine inoculated with 10^4 cells

Sample type	Storage time (days)[a]	5′ *Taq* nuclease assay post-enrichment culture[b,c]	5′ *Taq* nuclease assay	
			QIAamp	Heat lysis
Smegma	0	3431	5820	3435
	2	3353	664	2480
	5	5408	79	157
Mucus	0	1727	4667	7682
	2	2434	11755	21185
	5	148	6994	11554
Urine	0	ND[d]	3343	191
	2	ND	494	427
	5	ND	7957	1387

[a] Smegma and mucus samples for 5′ *Taq* nuclease or conventional PCR assay were stored at ambient temperatures in either PBS or physiological saline. Urine samples were stored at 4 °C. Samples for culture were stored in InPouch™ TF media at 37 °C.
[b] DNA was extracted from InPouch™ TF media for 5′ *Taq* nuclease assay via a commercial kit (QIAamp).
[c] Quantitative 5′ *Taq* nuclease assay estimates are given in cells/ml.
[d] Not done.

negative in both 5′ *Taq* nuclease assay and culture/microscopy.

4. Discussion

The 'gold standard' diagnostic test for trichomoniasis in either male or female cattle is the cultivation of live organisms from smegma or cervicovaginal mucus until parasites propagate to detectable numbers to allow the recognition of *T. foetus* organisms microscopically (Clark et al., 1971; Reece et al., 1983). Traditional culture/microscopy diagnosis is more effective for smegma (81–91%) than mucus (58–75%) and PCR methods have been developed to differentiate *T. foetus* from non-pathogenic trichomonads (Bryan et al., 1999; Campero et al., 2003; Clark et al., 1971; Felleisen et al., 1998; Fitzgerald et al., 1954; Lun et al., 2000; Parker et al., 1999; Reece et al., 1983; Skirrow, 1987; Skirrow et al., 1985). Increased specificity and sensitivity for the detection of *T. foetus* infection in cattle will improve both the accuracy of prevalence studies and the understanding of disease epidemiology, thereby improving disease management practises (Felleisen, 1997; Levy et al., 2003; Rae, 1989). Real time PCR-based techniques have been successfully used for the clinical diagnosis of a wide range of pathogens from a variety of sources, including *Campylobacter jejuni* from human stools (Iijima et al., 2004; Nogva et al., 2000) and *T. vaginalis* from female urine and genital secretions and male urine (Hardick et al., 2003; Jordan et al., 2001). These assays provide greater sensitivity and specificity compared to selective culture and direct microscopic examination, and diagnoses can be obtained in significantly less time. As there is no need for post-amplification processing, real time PCR techniques improve the sensitivity and specificity of conventional-PCR assays and are also less labour intensive (Wilhelm and Pingoud, 2003). This is the first report of a novel real time probe-based PCR assay for the specific and sensitive detection of *T. foetus* directly from clinical samples.

The 5′ *Taq* nuclease assay for *T. foetus* described here is based on the ITS-1 region shown previously to be conserved and reliable as a target for conventional PCR (Cobo et al., 2003; Felleisen et al., 1998; Hoevers et al., 2003; Parker et al., 2003). Previous reports using the Felleisen et al. (1998) *T. foetus* PCR assay have described the amplification of non-specific fragments (BonDurant et al., 2003); the ITS-1 based 5′ *Taq* nuclease assay described here proved specific and robust with no non-specific detection. The *in silico* matches of the primers and probe described here with ITS-1 sequences of *T. suis* and *T. mobilensis* are consistent with current phylogenetic understanding of the Tritrichomonadinae subfamily which indicate that these organisms belong to the same species (Felleisen, 1997; Kleina et al., 2004; Tachezy et al., 2002). The Felleisen et al. (1998) assay based on ITS-1 has also been adapted to detect *T. foetus* in cat feces (Gookin et al., 2002), further demonstrating the specificity of this target to detect this species from a range of clinical sources.

Sensitivity of detection was markedly improved compared to culture and microscopy, with the reliable detection of a single organism equivalent per assay from smegma, cervico-vaginal mucus or urine. Our observations concluded that *T. foetus* loses viability in culture, due to the absence of visible motile trichomonads in InPouch™ TF test kits within a few hours of inoculation. The reason for this death is unclear, although isolate-specific antimicrobial susceptibility may play a significant role. The low sensitivity of microscopic examination following selective culture indicates that this is not the most effective diagnostic tool for *T. foetus* infection, leading to false negative results. For example, a sheath scrape from an infected bull is reported to contain an average of 141 organisms/ml (Mukhufhi et al., 2003), which is well below the sensitivity limits of selective culture/microscopy. In addition, the presence of non-*T. foetus* trichomonads cultured from the preputial mucosa of bulls can confound positive identification of *T. foetus* (Hayes et al., 2003). The poor sensitivity of microscopy following selective culture was confirmed in our field study where only 3 of the 14 infected animals that were detected by 5′ *Taq* nuclease assay detected as positive by culture/microscopy. This study clearly demonstrates that PCR direct detection from clinical specimens is more sensitive compared with PCR following culture enrichment.

Using purified DNA samples prepared directly from spiked clinical specimens, we demonstrated equal sensitivity of the 5′ *Taq* nuclease with the conventional Felleisen et al. ITS-1 PCR (1998). The 5′ *Taq* nuclease assay provided a higher level of sensitivity when using crude heat lysis extracts as template, detecting less than a single cell equivalent compared to a single cell equivalent when purified DNA prepared directly from the clinical sample was used. It is likely that this is to due to the potential of 12 copies of ITS1 target per *T. foetus* genome (Chakrabarti et al., 1992). However, the conventional PCR assay was not amenable to using crude heat lysis extracts as template, limiting its sensitivity to 1 cell equivalent per assay. The detection limit of less than one cell equivalent/assay when assaying heat lysed clinical

specimens by the 5′ *Taq* nuclease assay is approximately 10-fold that observed when assaying purified DNA using the conventional PCR assay. Assay sensitivity has been reported to decline when assaying DNA extracted directly from clinical specimens compared to DNA extracted from cultured specimens (Chen and Li, 2001; Felleisen et al., 1998). This was probably due to the presence of a range of inhibitory substances present in the clinical samples that were not removed during DNA isolation (Al-Soud and Radstrom, 2001; Felleisen et al., 1998; Inglis and Kalischuk, 2003). It is not known if new assays such as that described by Grahn et al. (2005) provide equivalent sensitivity to the assay described here as cell equivalents were not reported. However, based on concentrations of genomic DNA tested, the assay described here demonstrated higher sensitivity (0.003 pg/assay) compared with the detection limit described by Grahn et al. (2005) at 0.1 pg/assay.

5′ *Taq* nuclease assays such as the one described here allow accurate estimation of the number of copies of the target DNA in the clinical sample if performed with appropriate quantitative standards. Such standards were used in this study to estimate *T. foetus* numbers within the clinical samples, but these estimates were unable to be related to the density of *T. foetus* in the infected animal due to variability in the volume and physical localisation of genital mucus collected. The development of a rigorously standardised methodology for the collection of genital mucus, allowing collection of a specified volume of mucus from an area that accurately reflects variations in the *T. foetus* population of infected animals, will allow the assay described in this study to be used to estimate the levels of *T. foetus* infecting a host.

5′ *Taq* nuclease assays do not require viable organisms for detection, and are capable of detecting target sequences despite high levels of non-target organisms or cells. As viable cells are not needed, transport from the field to the laboratory becomes greatly simplified, without the need for complex transport enrichment media. The presence of a high proportion of contaminating organisms or host cells in a specimen did not provide any impediment to an accurate diagnosis in this study. In addition, the use of a bull rasper has been found to simplify the collection of genital specimens from both male and female cattle (Tedesco et al., 1977), and is expected to provide a suitable specimen for either selective culture/microscopy or 5′ *Taq* nuclease assay as demonstrated in a related *C. fetus* subsp. *venerealis* study (McMillen et al., 2006). Urine has been used as a specimen for the diagnosis of human trichomoniasis (Schwebke and Lawing, 2002) and was thus screened here to determine whether *T. foetus* could be detected in urine submitted to diagnostic laboratories for the detection of other pathogens, i.e. *Leptospira* spp. Although feasible *in vitro*, the field sampling in this study was inconclusive to confirm whether urine can be used as diagnostic specimen for *T. foetus* detection. A similar study describing real time PCR to target *C. fetus* subsp. *venerealis* found that urine was not a suitable diagnostic specimen for the diagnosis of the related venereal disease campylobacteriosis (McMillen et al., 2006). As female cattle mostly clear *T. foetus* within 3 months and bulls can carry the organism for >3 years, our study supports the recommendation to screen smegma from bulls as the most reliable diagnostic sample for the detection of *T. foetus* within a herd (BonDurant, 2005).

5. Conclusion

The 5′ *Taq* nuclease assay described here is a reliable, sensitive and specific detection method for *T. foetus* in bovine venereal diagnostic specimens. This assay reliably detected 1 cell equivalent per assay, and was able to readily discriminate between the target organism and other venereally localised trichomonads. Specimen collection from male and female cattle using a bull rasper was found to be simple and efficient, and specimens suspended in physiological saline proved to be stable during transport at ambient temperatures. Diagnostic specimens could be processed by a simple and rapid heat lysis technique with no loss of sensitivity. Marked improvements were observed in both sensitivity and specificity over those obtained with selective culture-based and conventional PCR techniques. Thus real time PCR technologies offer many advantages as a diagnostic tool in clinical and veterinary diagnostic laboratories (Mackay, 2004) and *T. foetus* detection could be determined simultaneously with the detection of *C. fetus* subsp. *venerealis* (BonDurant, 2005; McMillen et al., 2006). Stringent controls (DNA and cell lysates) were used here, however it will be essential to standardise inter-laboratory internal positive and negative control protocols for the monitoring of assay integrity. A multi-centre evaluation of the specimen collection, transport, processing and assay procedures should prove valuable as well as the establishment of certified laboratories to undertake this testing.

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VETERINARY MICROBIOLOGY

A 5' *Taq* nuclease (TaqMan®) assay for detecting pathogenic *Leptospira* in cattle

B. Corney[a,*], L. Smythe[b], A.E. Lew[a], L. McMillen[a], I. Diallo[a], L. Wright[a], G. Hewitson[a], M. Dohnt[b], M. Symonds[b], L. Barnett[b], M. Kelly[a], V.J. Doogan[a], S. Fenwick[c], E. Taylor[c], P. Wai'in[c], P. Phuektes[c], S. Reid[c], A. Vivas-Marfisi[d], T. Berg[e], B. Rodwell[a].

[a]Animal Research Institute, Queensland Department of Primary Industries and Fisheries, Locked Mail Bag 4, Moorooka, Queensland 4105, Australia.
[b]WHO/FAO/OIE Collaborating Centre for Reference and Research on Leptospirosis, P.O. Box 594, Archerfield, Queensland 4108, Australia.
[c]School of Veterinary and Biomedical Sciences, Murdoch University, South Street, Murdoch, Western Australia 6150.
[d]Animal Health Laboratories, Agriculture Western Australia, Locked Mail Bag 4, Bentley Delivery Centre, Bentley, Western Australia, 6983, Australia.
[e]Regional Veterinary Laboratory, Elizabeth Macarthur Agricultural Institute, New South Wales Department of Primary Industries, Private Mail Bag 8, Camden, New South Wales 2570, Australia.

Corresponding author. Tel.: +617 3362 9578; fax +617 3362 9457.
E-mail address: bruce.corney@dpi.qld.gov.au.

Abstract
Bovine leptospirosis is currently diagnosed using serology. Although pathogenic *Leptospira* can be isolated from infected animals, culture is labour intensive and takes several weeks to complete, thus making it unsuitable for routine diagnostic use.
To improve the diagnosis of bovine leptospirosis, a TaqMan® assay originally designed for use in diagnosing human leptospirosis was modified and validated for use on bovine material. The composition of the reaction mixture and the cycling conditions were modified to suit the Corbett Rotor-Gene platform, and to compensate for the often dirty nature of bovine samples collected under field conditions. The assay was assessed in terms of analytical sensitivity and specificity, and was compared to *Leptospira* culture using urine, vaginal mucus and kidney samples from experimentally infected heifers, and urine, vaginal mucus and preputial smegma samples collected from Queensland beef herds. Urine and vaginal mucus samples collected from a *Leptospira*-free heifer before and after vaccination were tested to determine whether a commonly-used commercial vaccine would interfere with the TaqMan® assay. The robustness of the assay was demonstrated using a small range of instrumentation platforms in four different laboratories.
Only pathogenic *Leptospira* reacted in the TaqMan® assay. Detection limits of the modified assay were 1-10 *Leptospira* cells/ml of PBS, urine, vaginal mucus and preputial smegma. Vaccination of *Leptospira*-free animals with a commercial leptospirosis vaccine did not give rise to false positive results.
The assay was slightly less sensitive than culture when used on samples from the experimentally infected heifers. In contrast, 12.2% of the field urine samples were either suspect or positive in the TaqMan® assay, but none of the urine samples were positive on culture. Little evidence of *Leptospira* presence was found in vaginal mucus or preputial smegma from the beef herds or from the experimentally infected heifers.
The modified TaqMan® assay has potential for diagnosing bovine leptospirosis, especially on a herd basis. Urine and kidney were the most appropriate sample types examined in the study.

Keywords: Bovine, Leptospirosis, TaqMan®, Real-time PCR, Validation

1. Introduction

Bovine leptospirosis is currently diagnosed by serology. The definitive serological test for leptospirosis is the microscopic agglutination test (MAT) (Chappel, 1993). However, MAT results are often difficult to interpret, and anergic chronically infected animals are often missed (Chappel, 1993). An IgM ELISA is also available, and may offer some advantages over the MAT in differentiating between recent infections and residual titres from earlier infections (Cousins *et al.*, 1985).

Leptospira can be cultured from the urine and kidneys of infected animals. *Leptospira* may also be isolated from milk (Thiermann, 1982) the reproductive tract (Ellis *et al.*, 1986) and aborted foetuses (Ellis *et al.*, 1982). However, although isolation of *Leptospira* provides indisputable evidence of infection, culture is labour intensive and takes several weeks to complete, and is therefore unsuitable as a diagnostic test. For these reasons, it is often very difficult to diagnose leptospiral sub-fertility with any degree of certainty. PCR offers a sensitive and rapid alternative to culture for unequivocally demonstrating current infection with *Leptospira*.

Although many PCRs for pathogenic *Leptospira* are described in the literature, only a few have been used on clinical or veterinary samples (Bal *et al.*, 1994; Merien *et al.*, 1993; Merien *et al.*, 1995; Richtzenhain *et al.*, 2002). Some have been used successfully in assessing various treatment regimens for leptospirosis (Alt *et al.*, 2001; Gerritsen *et al.*, 1993; Gerritsen *et al.*, 1994). Also, PCR-based typing procedures have been developed to simplify serovar identification and taxonomy (Barocchi *et al.*, 2001; Brown and Levett, 1997; Corney *et al.*, 1993; Corney *et al.*, 1997).

A more recent refinement of PCR in which product accumulation is monitored during amplification (real-time PCR) has also been used in diagnosing leptospirosis (Smythe *et al.*, 2002; Levett *et al.*, 2005). The PCR developed by Smythe *et al.* (2002) is a TaqMan® assay designed for detecting *Leptospira* in human blood and urine samples. Twenty-three pathogenic and six saprophytic leptospiral serovars were tested by Smythe *et al.* (2002) in this assay, of which only the pathogenic serovars reacted. Equivalent sensitivities were demonstrated for the TaqMan® assay using two *Leptospira* serovars commonly found in cattle: Hardjo and Pomona. In this paper, we assess a modified version of this assay for use in diagnosing bovine leptospirosis.

2. Materials and methods

2.1. PCR

The TaqMan® assay of Smythe *et al.* (2002) was performed on a Rotor-Gene 3000 (Corbett Research), with the threshold set at a normalised fluorescence of 0.1. The assay was modified by increasing the annealing and extension temperature from 60 °C to 64 °C to improve discrimination between negative and weak positive samples, reducing the probe concentration in the assay from 200 nM to 50 nM and by extending the number of cycles from 40 to 50. The modified assay utilised 2 μl of template per 25 μl reaction, and used Platinum Quantitative PCR Super Mix-UDG (Invitrogen). All samples were tested in duplicate and results were expressed as the mean threshold cycle (C_T).

2.2. Leptospira *and other organisms*

L. interrogans serovar Pomona and *L. borgpetersenii* serovar Hardjo type hardjobovis were used for method development and determinations of analytical sensitivity. The *L. interrogans* serovar Pomona reference strain (Pomona) was obtained from the WHO/FAO/OIE Collaborating Centre for Reference and Research on Leptospirosis, Coopers Plains, Queensland, Australia. *L. borgpetersenii* serovar Hardjo type hardjobovis (93/94451-3) was isolated from a dairy herd in north Queensland,

Australia, and was stored in liquid nitrogen at the Biosecurity Sciences Laboratory, Yeerongpilly, Queensland, Australia.
The non-pathogenic *L. biflexa* serovar Patoc and DNA from eight pathogenic *Leptospira* were also obtained from the WHO/FAO/OIE Collaborating Centre for Reference and Research on Leptospirosis. These organisms were used in assessing the analytical specificity of the modified TaqMan® assay. Full details of these organisms are in Table 1.
Leptospira cultures were grown at 30 °C in either Difco EMJH (Becton and Dickinson) supplemented with 0.9% w/v sodium pyruvate or in Bovuminar PLM-5 (Intergen Biomanufacturing Corporation).
Leptospira were counted as follows. One drop of formalin was added to 2 ml of a suitable dilution of culture in PBS (pH 7.4), and incubated for a minimum of 30 min at room temperature. The diluted culture was then loaded into a haemocytometer, and cells were counted using a dark field microscope.
Table 1 shows the origins of the other organisms used in this study.

2.3. Sample preparation
Urine, vaginal mucus, preputial smegma and kidneys were used as samples for the TaqMan® assays.
Midstream urine samples were collected from the first or second void following either palpation or administration of the diuretic Frusemide (Troy Laboratories). Vaginal mucus and preputial smegma were collected into 5 ml of saline using a bull rasper as described in McMillen *et al*. 2006.
Up to 5 ml of urine was centrifuged for 30 min at 2000 × *g*. All but 0.5 ml of the supernatant was discarded. The pellet was resuspended in the remainder of the supernatant, transferred to a 1.5 ml microfuge tube, then centrifuged for 10 min at 4600 × *g* in a microfuge. The final pellet was resuspended either in 50 μl of sterile reverse-osmosis (RO)-purified water by vigorous pipetting or in 180 μl of ATL buffer (QIAGEN).
Up to 1.5 ml of vaginal mucus or preputial smegma (in saline) was centrifuged for 10 min at 4600 × *g* in a microfuge. The supernatant including mucus was removed and the pellet was resuspended either in 50 μl of sterile RO-purified water by vigorous pipetting or in 180 μl of ATL buffer.
Suspensions in ATL buffer were processed using a QIAamp DNA Mini Kit (QIAGEN) as per the manufacturer's instructions. DNA was eluted from the QIAamp spin columns in 50 μl of AE buffer (QIAGEN). Suspensions in water were processed using a heat lysis procedure (McMillen *et al.*, 2006). Briefly, the suspensions were allowed to sit at room temperature for a minimum of 2 min, then heated for 10 min at 95 °C, and stored at -20 °C. The lysates were briefly centrifuged at 15800 × *g* in a microfuge, and the supernatant was tested in the TaqMan® assays.
Tissue samples (1-8 pieces of approximately 8 mm^3) were processed using a QIAamp DNA Mini Kit. The tissue samples were either ground in 2 ml of PBS with a mortar and pestle or digested for up to 20 h at 56 °C in 180 μl of ATL buffer containing 2 mg/ml of proteinase K (QIAGEN). For the ground tissue, 200 □l of the PBS suspension was centrifuged for 10 min at 4600 × *g* in a microfuge, and the pellet was suspended in 180 □l of ATL buffer. Twenty □l of proteinase K (20 mg/ml, QIAGEN) was added, and the mixture was incubated for 10 min at 56 °C. The DNA in the ATL digests was purified on a QIAamp DNA Mini column as per the manufacturer's instructions. DNA was eluted in 50 μl of AE buffer.
DNA was extracted from bacterial cultures and bovine herpesvirus type 1 using either a High Pure PCR Template Preparation Kit (Roche Applied Science) or a QIAamp DNA Mini Kit as per the manufacturers' instructions.

2.4. Leptospira *isolation*

Midstream urine, vaginal mucus and preputial smegma were collected as described for sample preparation. One ml of urine was immediately added to 10 ml of semisolid EMJH (EMJH broth containing 0.9% w/v sodium pyruvate, 0.01% w/v 5-fluorouracil and 0.15% w/v agar), and chilled for transport to the laboratory. One ml of preputial smegma or vaginal mucus was immediately added to 3 ml of semisolid EMJH, which was chilled for transport to the laboratory. Cultures were incubated at 30 °C for six weeks. They were examined by dark field microscopy on arrival in the laboratory and at three and six weeks. Cultures were also subcultured into fresh semisolid EMJH on arrival and at three weeks.

Urine samples were also cultured using a floating filter method (Myers, 1985), in an attempt to minimise overgrowth by contaminants. A 0.2 μm cellulose nitrate filter was floated on 5–6 ml of semisolid EMJH in a 45 mm diameter petri dish. Approximately 0.2 ml of urine was placed on the filter, and the petri dish was incubated in the dark at room temperature for 6–24 h. The filter and inoculum were then removed, and the contents of the petri dish were added to 10 ml of semisolid EMJH. The process was continued as described above.

Pieces of kidney of approximately 1 mm^3 were placed in 10 ml of semisolid EMJH, which was cultured as described above.

2.5. Serology

Leptospiral antibodies were assayed using the MAT (Chappel, 1993).

2.6. Analytical sensitivity

Ten-fold serial dilutions of actively growing *L. borgpetersenii* serovar Hardjo type hardjobovis or *L. interrogans* serovar Pomona cultures were prepared in urine and vaginal mucus from *Leptospira*-free heifers, PBS, and preputial smegma which had been submitted for purposes not related to leptospirosis. QIAamp DNA Mini extracts were prepared from 5 ml of each dilution (0.5 ml for vaginal mucus), and were tested in the modified TaqMan® assay.

DNA preparations of a *L. interrogans* serovar Pomona dilution series in first void urine from a *Leptospira*-free heifer were similarly used to evaluate the analytical sensitivity of the modified TaqMan® assay using heat lysis instead of a QIAamp DNA Mini Kit.

To further assess the effect of sample extraction method on TaqMan® analytical sensitivity, a suspension containing 2×10^3 cells/ml of *L. interrogans* serovar Pomona was prepared in vaginal mucus from a *Leptospira*-free heifer. DNA was extracted from two 0.5 ml aliquots of the mucus using either the QIAamp DNA Mini Kit or heat lysis, and was tested in the modified TaqMan® assay.

2.7. Analytical specificity

Eight pathogenic *Leptospira* serovars of likely veterinary relevance, one saprophytic serovar, seven bacterial, two protozoal and one viral pathogen implicated in bovine reproductive diseases were tested for reactivity in the modified TaqMan® assay (Table 1). Leptospiral DNA was tested at approximately 25-50 ng/μl. *Tritrichomonas foetus*, *Neospora caninum* and other bacterial DNAs were tested at approximately 1-50 ng/μl. Bovine herpesvirus type 1 DNA corresponding to 8×10^4 $TCID_{50}$ was tested in the modified TaqMan® assay.

2.8. Experimental infections

Four Hereford heifers, approximately nine months old, were selected on the basis of negative MAT reactions to the following *Leptospira* serovars: Pomona, Hardjo, Tarassovi, Grippotyphosa, Celledoni, Copenhageni, Australis, Zanoni, Robinsoni, Canicola, Kremastos, Swajizak, Medanensis, Bulgarica, Cyanopteri, Ballum, Bataviae, Djasiman, Javanica, Panama and Shermani. Two heifers (designated I1 and I2) were inoculated on four consecutive days via the conjunctiva with 0.5 ml of a

broth culture containing approximately 10^7 cells/ml of *L. borgpetersenii* serovar Hardjo type hardjobovis as described by Smith *et al.* (1997). On the first day only, the heifers were also inoculated by vaginal instillation with 5 ml of culture as described by Ellis *et al*. (1985). The other heifers (C1 and C2) were retained as controls, and were housed in a separate building to the infected heifers. Urine and vaginal mucus samples were collected from all four heifers before inoculation commenced (day 1) and at regular intervals thereafter. First and second void urine samples and vaginal mucus were tested in the TaqMan® assays. Second void urine samples and vaginal mucus were also cultured for *Leptospira*. Culture samples were transferred to the laboratory and processed immediately after collection from the heifers. *Leptospira* isolates were typed using the MAT, and were also compared with the inoculum strain by random amplified polymorphic DNA fingerprinting (RAPD, Corney *et al*. 1993). Blood samples were collected on day 57 for MAT.
At day 64, C1 was vaccinated with Ultravac 7 in 1 vaccine (CSL). Urine and vaginal mucus samples were collected from C1 and C2 before C1 was vaccinated, and at one and seven days after vaccination of C1. These samples were tested by TaqMan® assay only.
At the conclusion of the experiment, all four heifers were necropsied. Urine (from the bladder after the heifers were euthanized) and kidney samples were collected for PCR and *Leptospira* isolation.
DNA extracts were prepared using a QIAamp DNA Mini Kit as described above. Initially, TaqMan® assays were performed using an annealing and extension temperature of 60 ºC, and most assays incorporated probe at 200 nM as described by Smythe *et al.* (2002). These TaqMan® results were used to assess the suitability of first and second urine voids as samples for leptospirosis testing and assessing the sensitivity of the TaqMan® assay relative to culture.
To establish criteria for interpreting C_T values, selected QIAamp extracts from the experimental infection trial were retested in the modified TaqMan® assay. The extracts were from first and second void urine samples and from vaginal mucus samples from the control heifers, and culture-positive second void urine samples from the infected heifers. C_T ranges corresponding to negative, suspect and positive results were determined by relating the modified TaqMan® assay result to the infection status of the animal and the culture result for each sample.

2.9. Field trial
Two hundred and twenty-two urine samples (first void only), 132 preputial smegma and 95 vaginal mucus samples were collected from beef cattle from 25 Queensland beef herds. The samples were chilled, and sent to the laboratory for testing in the modified TaqMan® assay and for *Leptospira* isolation. Transportation generally took several days. Sera from the animals were stored at -80 °C for testing by MAT.
The QIAamp DNA Mini Kit was compared with heat lysis for preparing urine as follows. The extracts from both methods were tested in the modified TaqMan® assay, and were classified as negative, suspect or positive. The results for each method were then compared using the χ^2 test (Fleiss, 1981). Also, a paired t-test was used to compare the C_T values obtained for the two extraction methods. C_T values > 50 were regarded as equalling 50 for the paired t-test.

2.10. Multicentre evaluation of the modified TaqMan® assay
DNA extracts were prepared from 21 urine samples collected from a *Leptospira*-positive beef cattle herd. The extracts were prepared using heat lysis if the urine samples were reasonably clean, or a QIAamp DNA Mini Kit if dirty. The extracts were tested using the modified TaqMan® assay at four laboratories around Australia, three of which used Rotor-Gene 2000 or 3000 platforms, whilst the other used an iCycler (Bio-Rad). The iCycler data were analysed with the threshold adjusted manually so that the C_T value for the positive control was similar to that obtained by Laboratory 1. Correlations between the C_T values for the three laboratories using the Rotor-Gene platform were

calculated, ignoring samples with C_T values > 50. The results were also classified as positive, suspect, or negative using the criteria established using samples from the experimentally infected heifers, and the degree of agreement between the laboratories was assessed using the Kappa statistic (Fleiss, 1981). A Kappa statistic ≥ 0.75 indicates excellent agreement, and a Kappa statistic between 0.40 and 0.75 indicates fair to good agreement.

2.11. Animal ethics

All work involving experimental and field animals was approved by the Animal Research Institute Animal Ethics Committee, Yeerongpilly, Queensland, Australia.

3. Results

3.1. Analytical sensitivity

The analytical sensitivity results using DNA prepared with QIAamp DNA Mini kits are shown in Table 2. The modified TaqMan® assay detected down to 10 cells/ml in PBS, 1-10 cells/ml in urine, 10 cells/ml in vaginal mucus and 1 cell/ml in preputial smegma. In these fluids, 10 cells/ml corresponded to C_T values ranging from 35 to 38, whereas 1 cell/ml corresponded to C_T value ranging from 38 to 40, depending on diluent used for the cells.

Using DNA prepared by heat lysis, the modified TaqMan® assay detected to at least 1 *Leptospira* cell/ml of urine (Table 3), comparable to the analytical sensitivity obtained using the QIAamp DNA Mini Kit for DNA extraction. The C_T values for urine containing 1 and 10 cells/ml were similar to those obtained using the QIAamp DNA Mini kits.

The results of the comparison of heat lysis and QIAamp DNA Mini extraction for Leptospira in vaginal mucus are also shown in Table 3. Heat lysis performed poorly in comparison to the QIAamp DNA Mini Kit, with a 5 cycle increase in C_T. Heat lysis was not tested on preputial smegma.

3.2. Analytical specificity

All eight pathogenic *Leptospira* serovars reacted in the modified TaqMan® assay whereas the other organisms did not react (Table 1).

3.3. Experimental infections

By day 57, heifers I1 and I2 had seroconverted to Hardjo (MAT titres were 1600 and 400 respectively). *Leptospira* was isolated from I1 and I2 second void urine samples collected from day 17 to the end of the trial, and also from their kidneys. All vaginal mucus samples from the infected heifers were culture-negative. The isolates obtained from I1 and I2 most resembled serovar Hardjo in the MAT, and their RAPD fingerprints were identical to those of the inoculum culture (results not shown). Up to 3.5 weeks were required for growth to be detected in positive cultures.

Heifers C1 and C2 remained negative for Hardjo in the MAT, and all urine, vaginal mucus and kidney samples from these heifers were culture-negative.

In an initial screen using the TaqMan® assay as described by Smythe *et al.* (2002) and applying their interpretation criteria (positive = $C_T \leq 40$), seven urine samples from the infected heifers were both culture and TaqMan®-positive, and two were culture-positive and TaqMan®-negative. Leptospiruria was detected in the infected heifers using the TaqMan® assay from day 28 (I1) or 17 (I2) through to the end of the trial. Four urine samples and four vaginal mucus samples from the control heifers had C_T values in the range 33–40. Kidney samples from the infected heifers were culture-positive and TaqMan®-positive. Kidney samples from the control heifers (C2 without vaccination and C1 postvaccination) were negative in the TaqMan® assay.

Urine samples collected from heifer C1 prevaccination and at one and seven days postvaccination were negative in the TaqMan® assay, whereas the vaginal mucus sample collected at one day postvaccination had a CT value of 39. The other vaginal mucus samples were negative.
Similar C_T values were obtained for first and second void urine samples from each collection from the infected heifers (Table 4), with differences of < 2 cycles in most cases. These results show that either first or second void urine give equivalent results in the TaqMan® assay.
When retested in the modified TaqMan® assay, two urine extracts from the control heifers, and two vaginal mucus extracts from the control heifers had C_T values (mean of duplicate reactions) in the range 37–40, and an additional vaginal mucus sample from a control heifer had a single reaction C_T value in this range (the duplicate reaction had a C_T value > 50). Thirty-one urine extracts and nine vaginal mucus extracts from the control heifers had C_T values of > 40, of which 18 urine extracts had C_T values > 50. Extracts from three culture-positive urine samples had C_T values (mean of duplicate reactions) ranging from 29 to 32, and a fourth had a C_T value of 40.8. Extracts from three additional culture-positive urine samples had single reaction C_T values of up to 44.8 (the duplicate reactions had C_T values > 50). Two other urine samples which were initially positive by culture and TaqMan® assay were not retested in the modified TaqMan® assay. Because of the overlap of C_T values from known positive and negative samples, the following criteria for interpreting C_T values were set: positive ≤ 37, 37 < suspect ≤ 40, negative > 40. Samples for which one reaction had C_T value of ≤ 40, and the other reaction a C_T value of > 50 were retested or scored as suspect. The detection limits of the assay (1- 10 cells/ml) fell within the suspect C_T range. Applying these criteria, kidney samples from the infected heifers were positive, and all postvaccination urine and vaginal mucus samples from heifer C1 were negative in the modified TaqMan® assay. The comparison of first and second void urine samples was not repeated using the modified TaqMan® assay.

3.4. Field trial

In the field trial, 222 urine samples, 95 vaginal mucus samples and 132 preputial smegma samples were analysed using the modified TaqMan® assay.
Twenty-seven urine samples (12.2 % of the total) were positive or suspect in the modified TaqMan® assay. These comprised the 26 samples which were analysed using both QIAamp DNA Mini and heat lysis extracts (Table 5), and one sample which was analysed using a QIAamp DNA Mini extract only. Cultures from 20 TaqMan®-positive or suspect urine samples were culture-negative, whilst those from the other seven were overgrown by contaminants. Twenty-two of the animals from which these samples were taken had MAT titres ≥ 50 for at least one of the serovars Hardjo, Pomona and Tarassovi. One was negative for these serovars, and the other four were not tested.
Six vaginal mucus samples (6.3% of the total) were suspect in the modified TaqMan® assay (C_T values were between 38 and 40), whilst all of the preputial smegma and the remainder of the vaginal mucus samples were negative. Two of the animals from which these samples were collected had MAT titres ≥ 50 for at least one of the serovars Hardjo, Pomona and Tarassovi. One of the animals was negative for all three serovars, and two were not tested. Urine samples from the six animals were negative in the modified TaqMan® assay. Three of the TaqMan®-positive/suspect vaginal mucus samples were overgrown by contaminants when cultured, whereas the other three were not cultured. None of the other samples grew *Leptospira* when cultured.
Animals not producing suspect or positive reactions in the modified TaqMan® assay were not tested using the MAT.

3.5. Comparison of heat lysis and QIAamp DNA Mini Kit for preparing field trial samples

QIAamp extracts and heat lysis extracts from 173 urine samples collected in the field trial were compared in the modified TaqMan® assay. Both types of DNA extract were negative for 147 urine samples. The C_T values for the other 26 urine samples are shown in Table 5. Overall, there was no significant difference between C_T values obtained using QIAamp and heat lysis (mean difference = 2.256; t = 1.57 with 25 degrees of freedom; P = 0.129). A two-way comparison of the classified results for QIAamp and heat lysis was performed with positive and suspect results combined to obtain a sufficiently large data set (Table 6). The results for the two extraction methods indicated a strong association between QIAamp extraction and heat lysis (χ^2 = 54.4; P < 0.001). A comparison of the proportions positive using QIA amp (12.7%) and heat lysis (8.7%) indicated no difference between the 2 methods (P=0.222).

3.6. Multicentre evaluation of the modified TaqMan® assay

The C_T values obtained from the four laboratories and their classifications are shown in Table 7. There was good agreement in terms of C_T classification between the three laboratories which used the Rotor-Gene platform (Kappa = 0.70, P < 0.001). Furthermore, the correlations between these laboratories in terms of C_T values were highly significant (r = 0.898-0.978; P < 0.0010).

The results from Laboratory 4 (iCycler) were not included in the statistical analyses, as the use of a different platform had an obvious effect on the C_T values. In particular, the samples classified as negative by the other laboratories had lower C_T values when tested on the iCycler at Laboratory 4.

4. Discussion

This report describes the modification and partial validation of the TaqMan® assay of Smythe *et al.* (2002) for use in diagnosing bovine leptospirosis. Initially, the TaqMan® assay was performed as described by Smythe *et al* (2002), but reoptimisation of the probe concentration was necessary due to the use of a Rotor-Gene instead of an ABI Prism 7700 Sequence Detector as described in the original paper. The TaqMan® assay was developed for use on specimens of human origin, which are normally collected under clean conditions. However, bovine urine and vaginal mucus samples are often contaminated with faeces and dust, which are a likely cause of the occasional weak positive reaction observed with known negative samples. Increasing the annealing and extension temperature was necessary to minimise these reactions. At 64 °C annealing and extension temperature, the non-specific reactions were confined to C_T values > 37. Therefore samples yielding C_T values > 37 but ≤ 40 were scored as suspect rather than positive.

All samples were tested in duplicate in the TaqMan® assays. It was not unusual for samples with high C_T values ($C_T \geq 37$) to have one of the duplicate reactions with a C_T value > 50. This reflects the random distribution of limiting amounts of template between the duplicate reactions. This effect is discussed by Svenstrup *et al*. (2005).

The modified TaqMan® assay was sensitive and specific. As few as one *Leptospira* cell could be detected per ml of urine, and only the pathogenic *Leptospira* reacted in the assay.

Culture performed better than both the original TaqMan® assay as described by Smythe *et al.* (2002) and the modified TaqMan® assay with the samples from the experimentally infected heifers. In the field trial, culture attempts were unsuccessful and were often overgrown by contaminants, whereas 27 urine samples were scored as positive or suspect in the modified TaqMan® assay, which was consistent with the serology results for most of these animals. -Consequently no recognised “gold standard” was available for comparison. This was attributed mainly to the time required for transporting the samples from remote areas to the laboratory, resulting in failure of any *Leptospira* to survive and overgrowth of the samples by other bacteria. Thus culture is very sensitive under the ideal conditions experienced during the experimental infections, but is totally unsuitable for use as a

diagnostic tool on samples from naturally infected herds. Under these conditions the modified TaqMan® assay is a useful diagnostic tool. Even under ideal conditions, culture has the additional disadvantage of taking several weeks to obtain a result and is therefore seldom used as a veterinary diagnostic test.

A major factor in the improved performance of culture with samples from the experimentally infected heifers is the relatively large sample volume (0.2-1 ml) used to inoculate the cultures. In contrast, each TaqMan® reaction receives 2 µl of DNA extract, corresponding to a maximum of 0.2 ml of the original sample. The fact that culture performed better than the modified TaqMan® assay in this situation suggests that the latter may not always detect very low levels of *Leptospira* excretion in urine from infected cattle, despite having an analytical sensitivity of 1-10 cells/ml of urine. For this reason, the modified TaqMan® assay would be most useful as a herd test.

No numerical data comparing the modified TaqMan® assay with culture were obtained from the field trial. However, the fact that all urine samples from the experimental infections that reacted in the modified TaqMan® assay were also culture-positive, and that only two of the 31 urine samples from the control heifers had C_T values in the suspect range, allows reasonable confidence in the specificity of the modified TaqMan® assay. Furthermore, comparing the modified TaqMan® assay with culture using samples from the experimental infection trial allowed us to establish criteria for interpreting C_T values, also assisting the identification of infected animals and herds with reasonable confidence.

Both methods of extracting DNA from urine field samples yielded similar C_T values in the modified TaqMan® assay. However, the heat lysis method is simpler to perform and requires less time than the QIAamp method, and is therefore recommended for routine use.

Leptospira were not detected in vaginal mucus from the experimentally-infected heifers by TaqMan® assay or by culture. Similarly, there was no definite evidence of the presence of *Leptospira* in field samples of vaginal mucus or preputial smegma. On this basis, urine is the sample of choice for diagnosing bovine leptospirosis using the modified TaqMan® assay.

Samples from the vaccinated heifer did not react in the modified TaqMan® assay, suggesting that vaccination does not lead to false positive results. As samples from only one heifer were used, however, further testing may be required to confirm the suitability of post-vaccination samples for testing in the modified TaqMan® assay.

Limited data were obtained for tissue samples in the TaqMan® assays. The available data show that Leptospira can be detected in kidney tissue by culture and by both TaqMan® assays, and demonstrate that the modified TaqMan® assay can be used for detecting *Leptospira* in tissue samples.

The modified TaqMan® assay was also trialled in three other laboratories using a standard set of samples. The laboratories used three different platforms; two different versions of the Rotor-Gene and an iCycler. No significant difference was observed between the results from the laboratories using the Rotor-Gene. However, the results obtained using the iCycler were noticeably different from those obtained using the Rotor-Genes, especially for the negative samples. Modifying the criteria for interpreting C_T values would probably be sufficient to allow the modified TaqMan® assay to be run on this platform with confidence. If not, optimising the assay for the iCycler would be necessary as the assay described in this paper was specifically optimised for the Rotor-Gene. Spackman and Suarez (2005) similarly observed that optimisation of a TaqMan® assay for avian influenza virus (AIV) for individual platforms was essential to maintain consistency across the platforms. Although the AIV TaqMan® assay is a reverse transcriptase PCR, the same appears to apply to at least some degree to the modified TaqMan® assay for *Leptospira.* Overall, the assay was sufficiently robust to

allow transfer between laboratories using either version of the Rotor-Gene, and to allow its use on other platforms with some modification.

5. Conclusion

The data demonstrate that the modified TaqMan® assay of Smythe *et al.* (2002) has potential for use in diagnosing bovine leptospirosis, especially as a herd test. Urine (first or second void after administering a diuretic) and tissue samples are suitable for the modified TaqMan® assay. Urine samples may be prepared using either the QIAamp DNA Mini kit, or heat lysis, although the latter is preferred due to its speed and simplicity. The assay was sufficiently robust to allow its use by other laboratories.

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Table 1.
Origins of cultures and specificity of the *Leptospira* TaqMan® assay.

Organism	Strain	Source	TaqMan® Result (C_T)
Pathogenic *Leptospira*:			
L. interrogans			
Copenhageni	M20	WHO/FAO/OIE	19.6
Robinsoni	Robinson	WHO/FAO/OIE	19.8
Pomona	Pomona	WHO/FAO/OIE	18.2
Zanoni	Zanoni	WHO/FAO/OIE	19.0
L. borgpetersenii			
Hardjobovis	LT1186	WHO/FAO/OIE	18.6
	93/94451-3	BSL	N
Tarassovi	Perepelicin	WHO/FAO/OIE	19.1
Ballum	Mus 127	WHO/FAO/OIE	18.3
L. kirschneri			
Grippotyphosa	Moskva V	WHO/FAO/OIE	21.1
Saprophytic *Leptospira*:			
L. biflexa			
Patoc	Patoc 1	WHO/FAO/OIE	> 50
Other organisms:			
Tritrichomonas foetus	ATCC 30003	ATCC	> 50
	YVL-W	BSL	> 50
Campylobacter fetus subsp. *fetus*	ATCC 15296	ATCC	> 50
	98/118432	BSL	> 50
C. fetus subsp. *venerealis*	ATCC 19438	ATCC	> 50
	98/109383	BSL	> 50
Neospora caninum	ATCC 50843	ATCC	> 50
	50843	BSL	> 50
Actinomyces pyogenes	99/145243	BSL	> 50
Corynebacterium renale		BSL/IFM*	> 50
Escherichia coli	ATCC25922	ATCC	> 50
Streptococcus bovis	S112	BSL	> 50
Streptococcus faecalis	S161	BSL	> 50
Bovine herpesvirus type 1	IBR-1	BSL	> 50

WHO/FAO/OIE, WHO/FAO/OIE Collaborating Centre for reference and Research on Leptospirosis, Coopers Plains, Queensland, Australia; BSL, Biosecurity Sciences Laboratory, Yeerongpilly, Queensland, Australia; N, not included in analytical specificity study; ATCC, American Type Culture Collection, Manassas, Virginia, USA; IFM, IFM Quality Services, Ingleburn, New South Wales, Australia

*This organism was isolated at BSL from material supplied by IFM as part of a veterinary bacteriology proficiency testing program.

Table 2.
TaqMan® titration of QIAamp DNA Mini extracts of DNA from *Leptospira* diluted in PBS, urine, vaginal mucus and preputial smegma.

Leptospira cells/ml	C_T				
	PBS	First void urine	Second void urine	Vaginal mucus	Preputial smegma
10^3	ND	ND	ND	ND	29.5
10^2	33.3	ND	ND	33.3	33.1
10^1	36.8	37.3	35.0	37.6	38.2
10^0	> 50	38.4	(37.0, > 50)*	> 50	39.5
10^{-1}	> 50	ND	ND	ND	ND

ND, not done.
*Only one of the duplicate reactions gave a C_T of < 50.

Table 3.
TaqMan® assay results for DNA extracts of urine and vaginal mucus containing *L. interrogans* serovar Pomona.

Leptospira cells/ml	C_T	
	First void urine	Vaginal mucus
10^2 Heat lysis	32.0	ND
10^1 Heat lysis	34.8	ND
10^0 Heat lysis	38.1	ND
2×10^3 QIAamp	ND	31.8
2×10^3 Heat lysis	ND	36.8

ND, not done.

Table 4.
C_T values for first and second void urine samples collected from two heifers experimentally infected with *Leptospira*.

Collection day	Heifer I1		Heifer I2	
	First void C_T	Second void C_T	First void C_T	Second void C_T
10	> 50	47.4	(41.0, > 50)*	(44.0, >50)*
17	43.7	42.5	38.5	40.6
28	33.6	31.8	32.9	31.5
38	31.3	30.3	30.5	27.3
57	38.7	38.6	26.1	26.5

*Only one of the duplicate reactions gave a C_T of < 50.

Table 5.
Comparison of C_T values obtained from positive and suspect field trial urines processed using the QIAamp DNA Mini Kit and heat lysis.

Sample number	QIAamp C_T	Heat lysis C_T	Sample number	QIAamp C_T	Heat lysis C_T
1	35.5	34.2	14	38.2	(44.0, > 50)*
2	35.8	35.8	15	37.6	(43.9, > 50)*
3	27.0	30.7	16	39.0	> 50
4	33.4	38.2	17	37.6	> 50
5	36.1	38.0	18	38.2	41.8
6	35.4	>50	19	(38.9, > 50)*	> 50
7	37.0	(43.0, > 50)*	20	(37.7, > 50)*	> 50
8	39.8	39.1	21	38.9	(49.1, > 50)*
9	37.8	37.7	22	39.0	(40.4, > 50)*
10	39.4	39.3	23	(47.3, > 50)*	36.7
11	(38.0, > 50)*	38.7	24	43.2	34.4
12	(39.9, > 50)*	38.7	25	> 50	37.5
13	37.9	38.4	26	(45.4, > 50)*	(39.3, > 50)*

*Only one of the duplicate reactions gave a C_T of < 50.

Table 6.
Comparison of TaqMan® assay results obtained from 173 urine samples prepared with either the QIAamp DNA Mini Kit or heat lysis.

	Heat lysis		
QIAamp	Positive/suspect	Negative	TOTAL
Positive/suspect	11	11	22
Negative	4	147	151
TOTAL	15	158	173

Table 7.
C_T values and classification of 21 urine samples tested by four Australian laboratories.

	Laboratory 1 (Rotor-Gene 3000)		Laboratory 2 (Rotor-Gene 3000)		Laboratory 3 (Rotor-Gene 2000)		Laboratory 4 (iCycler)
Sample Number	C_T	Classification	C_T	Classification	C_T	Classification	C_T
1	> 50	Neg	> 50	Neg	> 50	Neg	39.7
2	> 50	Neg	> 50	Neg	> 50	Neg	Not Tested
3	28.2	Pos	26.5	Pos	Not Tested	Not Tested	27.9
4	29.4	Pos	29.2	Pos	31.6	Pos	30.9
5	44.9	Neg	43.0	Neg	(44.2, > 50)*	Neg	32.6
6	38.4	Sus	37.1	Sus	(47.6, > 50)*	Neg	33.6
7	> 50	Neg	> 50	Neg	> 50	Neg	> 50
8	30.6	Pos	28.4	Pos	30.7	Pos	29
9	46.3	Neg	44.4	Neg	(49.7, > 50)*	Neg	37.7
10	41.9	Neg	43.4	Neg	(45.1, > 50)*	Neg	37.4
11	(43.7, > 50)*	Neg	44.7	Neg	> 50	Neg	36.5
12	> 50	Neg	> 50	Neg	43.7	Neg	> 50
13	> 50	Neg	> 50	Neg	> 50	Neg	> 50
14	> 50	Neg	> 50	Neg	> 50	Neg	> 50
15	38.6	Sus	38.1	Sus	43.1	Neg	33.9
16	38.7	Sus	37.1	Sus	44.5	Neg	33.8
17	33.1	Pos	32.5	Pos	38.2	Sus	35.5
18	34.8	Pos	34.5	Pos	36.7	Pos	34.3
19	37.6	Sus	37.1	Sus	38.6	Sus	33.4
20	37.4	Sus	35.1	Pos	37.6	Sus	32.7
21	37.6	Sus	Not Tested	Not Tested	39.8	Sus	35.4

*Only one of the duplicate reactions gave a C_T of < 50.