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Cysticercus bovis - enhanced classification of suspect lesions identified at meat inspection

Abstract

The meat-borne zoonotic tapeworm *Taenia saginata* causes little harm to humans, however the parasite poses significant costs to the Australian meat industry in terms of inspection and the condemnation of carcases destined for export markets. Most research agrees that the positive predictive value of meat examination by incision and visual inspection is poor due to a high rate of false identifications resulting from morphologically similar cysts in beef muscle caused by other parasites (hydatid cysts or *Sarcocystis* spp.). A risk-based approach to inspection has been proposed which includes the use of a fast, molecular-based diagnostic test that will improve the management of *Cysticercus bovis*, the larval stage of *T. saginata*, in Australia. The aim of this research was to develop a real-time PCR assay for the identification of *C. bovis* in lesions submitted from abattoirs. After comparison of two different real-time PCR designs using either SYBR green dye or hybridisation probes, a Taqman MGB probe assay that simultaneously detects *C. bovis* DNA and a bovine internal control in a single reaction, using the respective mitochondrial CO1 gene of each species, was selected. The Taqman assay is species-specific and able to detect viable, calcified and degenerated cysts, although is unable to detect the most highly degenerated examples. The Taqman assay has a rapid turnaround time, ease of interpretation and demonstrated transferability across laboratories.

Executive Summary

A sensitive and specific probe-based molecular assay has been developed and evaluated to improve detection of bovine cysticercosis caused by *Taenia saginata* in slaughtered Australian beef cattle. Two real-time PCR approaches, including a SYBR green dye and Taqman MGB hybridisation probe assay, were compared for their diagnostic utility. The Taqman MGB probe assay was selected for further test performance and validation studies based on its fast turnaround time, ease of interpretation and high levels of specificity and sensitivity.

Why was the work done?
Cysticercus bovis is the larval stage in the life cycle of *Taenia saginata*, a tapeworm for which man is the definitive host. The public health implications of *T. saginata* are relatively minor however export markets still regard it as a food safety issue and significant economic costs are associated with its management and control. In Australian abattoirs, all slaughtered cattle are examined for *C. bovis* by incision and visual inspection, with carcasses of affected animals being excluded from the food chain. In lightly infected animals, the Australian Standard allows for trimming of the detected cysts, plus freezing of the carcass for an extended period to render remaining cysts non-viable.

The visual inspection approach to manage *C. bovis* in Australia has been questioned due to a lack of precision and efficiency. The parasite is rare in Australia but the cost of inspecting animals is high while the positive predictive value (PPV) of meat inspection is poor. Meat inspection relies on the gross, morphological appearance of cysts for diagnosis as *C. bovis* which may be confused with other cestode larvae (hydatid cysts), tissue parasites (e.g. *Sarcocystis* spp.) and occasionally neoplastic lesions. Similarly, laboratory confirmation of suspect lesions is currently based on histopathological examination which in itself has drawbacks as results are equivocal in degenerated lesions.

An alternative system of managing *C. bovis* in Australia has been proposed and entails a risk-based post-mortem inspection program that would result in savings to the industry by reducing inspection levels in identified low-risk animals. Due to the poor accuracy of traditional inspection and diagnostic methods, this project's objective was to develop a molecular assay to rapidly and conclusively establish the true status (and incidence) of inspected lesions which would in turn allow an accurate assessment of risk. Real-time PCR was selected as the best molecular strategy as it offers high degrees of sensitivity and specificity compared to traditional diagnostic methods, reduced risk of contamination and fast turnaround times.

What was achieved?
Two real-time PCR technologies (SYBR Green dye and Taqman MGB hybridisation probes) were used to design diagnostic tests that could sensitively and specifically detect bovine cysticercosis. Both tests simultaneously identified a *C. bovis*-specific target and an internal control, to confirm the presence of amplifiable DNA, in a single reaction using either mitochondrial (cytochrome oxidase subunit 1) or nuclear genes (18S ribosomal RNA). The tests were evaluated on their analytical specificity, sensitivity and ease of interpretation of the test results, before selection of the primary assay.

The Taqman MGB probe assay was selected for further evaluation of its diagnostic performance. The test sensitivity showed the assay is able to detect viable, calcified and degenerated *C. bovis* lesions, although is unable to detect highly degenerated lesions. The assay is highly specific against other parasites that may occur in beef muscle and an evaluation of the test's performance using the results of a previously published conventional PCR as the true status of 27 suspect lesions, showed the test could positively identify true *C. bovis* lesions (PPV) 100% of the time. The assay's implementation across two different laboratories also showed it was highly reliable and reproducible. Major advantages of the assay over current conventional PCR protocols are the objective, easy interpretation of results, greatly reduced risk of sample contamination and its rapid turnaround time. The entire process of DNA extraction, PCR set-up, run-time and analysis takes

only 6 hours to complete, of which roughly 2.5 hours is labour. The cost per sample, including a full component of labour, is $122.90. If samples were batched, up to 12 samples could be processed concurrently at $23.50/sample including labour.

The SYBR green assay was not selected as although it outperformed the Taqman MGB probe assay in terms of sensitivity and could detect more of the highly degenerated lesions examined in this study, the turnaround time was greater (8.5 hours) and the interpretation of results more subjective due to a high degree of variability in the melt curve analyses used to identify lesions. The specificity was also conditional on stringent PCR cycling protocols and a cost analysis showed there were no significant savings using this usually more economical real-time-PCR option ($122.05/sample).

When and how industry can benefit from the work?

The meat and livestock industries greatest benefit from this work is the enhanced classification of suspect lesions identified at meat inspection over the relatively inaccurate traditional diagnostic methods. Histopathology failed to identify any *C. bovis* lesions in the submissions examined in the study, and there was non-concordance between this and molecular methods test, underscoring the difficulties in identification when lesions have degenerated. The beef abattoir industry can also benefit from the rapid turnaround time of the real-time PCR test. Immediate savings can be achieved for the abattoir where carcasses being held aside in the processing chain can re-enter the chain when confirmed as negative. Industry can benefit from the work immediately as most diagnostic laboratories are equipped with real-time PCR thermocyclers and the reagents are easily obtainable from commercial sources. Sufficient DNA standards for ~5000 runs are prepared with an estimated shelf life of 5 years with correct storage. In the long term, abattoirs will also benefit from this work by reducing costs associated with adoption of a risk-based *C. bovis* inspection program.

Who can benefit from the results?

In addition to beef abattoirs, beef producers and the livestock industry as a whole are most likely to benefit from the results of this study. The Australian meat industry's reputation for excellence in food safety and quality control will be enhanced by the inclusion of a highly specific and sensitive molecular assay as part of an overall program of *C. bovis* management. Beef producers benefit from the results of this study as timely traceback investigations to determine the original source of *C. bovis* infection at the farm/feedlot level can prevent future cases and disadvantages to the producer at subsequent cattle sales.

Recommendations for future actions

Due to the poor response rates for suspect *C. bovis* cyst submissions during the study period, an insufficient number of cysts were collected to give statistical reliability when establishing the diagnostic parameters for the developed test. The original proposal estimated 100 suspect lesions were required in order to allow a 10% error at the 95% confidence level, from which only 27 (27%) have been collected. In order to improve the precision of the current test performance estimates, as well as to facilitate publication of this research, we recommend MLA keep the suspect lesion submission request open to AQIS-supervised abattoirs until at least 50 suspect lesions (estimated precision 13%) are collected. We propose to continue testing at the UQ School of Veterinary Science laboratory with a cost/sample incurred to MLA. As sufficient reagents to perform the DNA extraction, reference and real-time PCR procedure have been retained, the main costs incurred will be of labour, sequencing and histopathology. Furthermore, as samples could be stored and processed in batches during this period, an extrapolated cost of 12 samples would apply. The total estimated cost incurred to MLA for a further 23 (n=50) samples processed in 2 batches is $1365. A breakdown of this amount can be found in Appendix 3.

Contents

1. Background

Cysticercus bovis is the larval (metacestode) stage in the life cycle of *Taenia saginata*, a zoonotic parasitic tapeworm of cattle and humans. The public health implications of *T. saginata* are relatively minor, nevertheless export markets still regard it as a food safety issue and condemned meat and offal results in economic losses. It is a requirement that all cattle slaughtered in Australia are visually examined for evidence of *C. bovis* with the carcasses of affected animals excluded from the food chain. In the case of lightly infected animals, the Australian Standard allows for trimming of the detected cysts plus freezing of the carcass for an extended period to render any remaining cysts non-viable.

The visual inspection approach to management of *C. bovis* in Australia has been questioned because of the lack of precision and efficiency. The parasite is rare in Australian cattle, but the cost of inspecting all animals is high while the predictive value of positive inspection findings is poor. Previous investigations into the sensitivity of post-mortem examinations have shown that although the method can be effective in detecting heavily infected carcasses, it is not as reliable in detecting light infections [1]. Furthermore, precision of the inspection method is often hampered by misidentification of lesions due to degeneration of *C. bovis* cysts or macroscopic, morphological lesions caused by other cestode larvae (hydatid cysts) or tissue parasites such as *Sarcocystis* spp..

Recently, the usefulness of PCR assays to objectively and sensitively identify *C. bovis* lesions from bovine tissues have been investigated. Generally, molecular assays have demonstrated good sensitivity and specificity by correlating with abattoir and laboratory diagnosis, although PCR sensitivity decreases with increasing levels of lesion degeneration [2, 3]. Currently described molecular assays utilise standard PCR methods where reagents and equipment are relatively low in cost, however they require post-amplification processing and often two rounds of PCR to attain adequate levels of sensitivity. These steps increase turn-around times, the risk of contamination and set-up and running costs.

Real-time PCR assays are now commonly employed in detection of parasite DNA from host material [4-6]. The chemistries of these assays result in significantly superior sensitivities compared to standard PCRs which increase the likelihood of detecting *C. bovis* DNA from degenerated cysts and therefore their utility as an enhanced method of identification. Real-time PCR assays also do not require post-amplification processing which further reduces the risk of contamination, turn-around time and labour costs.

Two common methods in real-time PCR are SYBR green based detection assays which utilise a non-specific fluorescent dye that intercalates with double-stranded DNA, and sequence-specific fluorescent hybridisation probes. SYBR green assays provide the simplest and most economical format for detection as the absence of costly probes reduces set-up and running costs. In a multiplex assay, primers are specifically designed to amplify different sized products which can then be differentiated on the basis of the melting temperature of each product (melt curve analysis). Disadvantages include the potential for false positive signals as the dye binds non-specifically to any double-stranded DNA sequences, and the potential complexity of interpretation of the melt curve analysis when multiple PCR products are amplified in a single reaction.

Hybridisation probes involve the hybridisation of sequence-specific probes to DNA targets which can significantly reduce background noise and false positive signals, although there is a loss in sensitivity compared to SYBR green dyes. Probes can be labelled with different reporter dyes allowing recognition of specific amplification products in multiplexed reactions. Manufacturing and labelling of probes can increase running costs of the assay and multichannel thermocyclers are costly.

2. Project Objectives

The project had two main objectives, each with target goals:

1. To develop, document and describe a molecular assay suitable for detecting DNA specific for *C. bovis* in lesions submitted from abattoirs. The specific goals were to:

    - Test a DNA extraction protocol for *C. bovis* lesions *in situ* to ensure sensitivity and minimise time and cost

    - Compare the diagnostic utility of two real-time PCR approaches including:

        i. A cost-effective SYBR-green based assay where species-specific primers amplify different sized products using mitochondrial genes of *C. bovis* and *Echinococcus granulosus*. These products can then be differentiated on the basis of melt curve analysis

        ii. A *C. bovis*-specific Taqman MGB labelled probe-based assay

2. Select one real-time PCR approach and test on suspect *C. bovis* lesions to establish the diagnostic performance and reproducibility of the test

3. Methodology

3.1. Source of samples

3.1.1. Positive reference samples

For the development of the real-time PCR assay, 31 *C. bovis* lesions were used as positive reference samples. Fourteen of these lesions originated from individual animals identified at meat inspection in July 2010 and were forwarded chilled and *in situ* to the University of Queensland's School of Veterinary Science (UQSVS). They were banked for 8 months at -20^{o}C until further examination. Seventeen lesions originated from a single heavily-infected animal identified at inspection and submitted in April 2012. These lesions were processed soon after collection.

3.1.2. Suspect lesion collection

To evaluate the validity of the real-time PCR assay and estimate the diagnostic sensitivity and specificity parameters, suspect *C. bovis* lesions identified at routine meat inspection were collected. Attempts were made to obtain the lesions from both overseas and domestic sources. Overseas collaborators from Bombay Veterinary College (India), the University of Udayana (Bali) and University of the Punjab (Pakistan) were contacted to source suspect *C. bovis* lesions from local domestic abattoirs as the prevalence of the parasite is greater in these countries than Australia [7, 8]. Preparation and storage conditions involved excising the lesion from bovine tissue, bisecting the lesion with one half stored in 70% ethanol and the other in 30% formalin. Sample submission sheets asked for animal-related information such as muscle location of the cysts (heart, masseter, tongue etc), number of lesions, lesion size, presence of a cavity/fluid and colour requested to be reported.

A letter requesting the co-operation of meat inspectors and Government on-plant veterinarians (OPVs) to submit suspect lesions to the research project and outlining the project's objectives, was circulated nationally to 48 AQIS-supervised export beef abattoirs in January, June and December 2011 (Appendix 1). Export beef abattoirs were considered a good source of lesions as OPVs with experience in identifying larval cysts in cattle were present on-site and the majority of Australian

cattle are slaughtered at these establishments [9]. If a lesion was encountered in beef muscle during inspection, excised or *in situ* lesions were forwarded chilled to the UQSVS. Each lesion was then photographed and size, colour, the presence of a cavity or fluid and the muscle location recorded. The lesions were then bisected, with one half submitted for histopathology and the other half stored at -20^{o}C or +4^{o}C until further examination. No more than two lesions per animal were included in the study. The period of collection was from January 2011 to June 2012.

Veterinary pathologists from the University of Sydney and the NSW and Queensland Department of Agriculture, Fisheries and Forestry (QDAFF) were enrolled in the study and asked to forward any suspect lesions submitted to their departments. These lesions were paired with the respective department's histopathology report and either ethanol-preserved or chilled lesions were stored at -20^{o}C until further examination.

3.1.3. Negative reference samples

Negative reference samples included non-target genomic DNAs (gDNA) of species taxonomically similar to *C. bovis* or that may occur in bovine muscle tissue. The panel included other *Taenia* and cestode species (*Taenia solium*, *Echinococcus granulosus*/hydatid cysts, *Taenia hydatigena*, *Spirometra erinacei*), ascarids (*Toxocara vitulorum*, *Toxocara canis*), protozoan parasites (*Sarcocystis cruzi*, *Neospora caninum* and *Toxoplasma gondii*) and bacteria (*Actinobacillus lignieresii*/wooden tongue). Non-target organisms were sourced from the UQSVS parasite collection or the Queensland Alliance for Agriculture and Food Innovation (QAAFI) (Table 1). Each DNA was either extracted from ethanol-preserved adult or metacestode stages or was provided previously purified from other research purposes. All non-target DNAs were confirmed by species-specific PCR and DNA sequencing of the PCR product.

Table 1. Sources of reference negative samples

Species	*n*	Host	Geographical origin	DNA origin
E. granulosus	G1 (*n*=2)	Macropod	Queensland, Australia*	Protoscoleces/ germinal membrane
	G1 (*n*=7)	Varied	Pakistan[Ŧ]	Protoscoleces/ germinal membrane
Taenia hydatigena	1	Sheep	Australia	Metacestode
Spirometra erinacei	1	Cat	Australia	Adult worm
Toxocara vitulorum	1	Cattle	Laos	Adult worm
Toxocara canis	1	Dog	Australia	Adult worm
Sarcocystis cruzi	1	Cattle	Australia	Bradyzoite in muscle
Toxoplasma gondii	1	unknown	USA	RH Strain
Neospora caninum	1	Dog	USA[§]	Nc-1 strain
Actinobacillus lignieresii	1	Cattle	Australia	Bacterial plate culture

*sample originally described in [10]
[Ŧ]samples originally described in [11]
[§]samples originally described in [12]

3.2. Classification and PCR identification of lesions

The 14 positive reference *C. bovis* lesions collected in 2010 were frozen before the study which precluded the use of histopathology to identify them. Instead, they were examined macroscopically and classified as either viable, calcified or degenerated based on criteria in Table 2. Only a subset of the 17 positive reference lesions collected in 2012 underwent histopathology but all were examined macroscopically. Lesions were viable if they contained a fluid-filled cavity, calcified or degenerating if they were soft, pulpy, caseous and lacking a cavity, or highly degenerated if their appearance was solely granular material in muscle.

Table 2. Classification of *C. bovis* cysts in muscle tissue by macroscopic examination

Lesion classification	Description
Viable, immature	small white lesions (cysticerci 2-3 weeks post infection)
Viable, mature	clear, transparent bladders 5x10 mm (infective cysticerci, 12-15 weeks post-infection
Calcified	opaque and pearl-like (over 15 weeks post-infection)
Degenerating	degeneration, caseation and calcification (over 12 months post-infection)
Highly degenerate	degenerative myocarditis

All suspect *C. bovis* lesions submitted from Australian abattoirs underwent histopathology and were classified as either a) viable immature/mature cysts of *C. bovis* if a definitive *C. bovis* structure was identified, b) degenerate cysts of parasitic origin, c) true negative samples e.g. neoplasm or identifiable hydatid cyst or d) non-specific granulomas (unknown). For histopathological examination, cysts were bisected and preserved in 10% neutral buffered formalin. Tissue was then embedded in paraffin, sectioned at 5µm and stained with haematoxylin and eosin.

A previously evaluated conventional PCR for the identification of bovine cysticercosis was included as a reference method as histopathology results were generally equivocal. All lesions were characterised by nested PCR based on a 253 bp fragment of the *T. saginata COI* gene using an assay developed by Chiesa et al. [2]. Both rounds of the reference PCR were carried out in a 25 µl reaction using 10x PCR Buffer, 0.5U of Hotstar *Taq* polymerase (both QIAGEN), 12.5 pmols of primer and 0.25 mM each dNTP. Up to 100 ng of DNA was added for round 1 and 2.5 µl of amplification product for round 2. Water and DNA from bovine, *E. granulosus* and *Sarcocystis cruzi* were included in all rounds of PCR as negative controls. The reaction was performed twice for each lesion and amplified products purified using an Invitrogen Purelink Purification kit (Invitrogen) were submitted for sequencing.

3.3. DNA Extraction

At the study start, there were insufficient confirmed *C. bovis* lesions with which to optimise the DNA extraction method, therefore a routine method in the UQSVS was modified for use. DNA was extracted from bisected *C. bovis* lesions after removing as much bovine tissue as possible from the lesion. Samples preserved in ethanol were soaked in 1 mL of Tris-EDTA buffer overnight prior to extraction. The lesion was enclosed in a piece of heavy duty alfoil and frozen in liquid nitrogen (LN_2) for 1-2 mins. The lesion in foil was then mechanically disrupted using a hammer before being transferred to a clean 2.0 mL microcentrifuge screw-cap tube. The tube was alternately boiled at 90^{o}C and frozen in LN_2 three times. A QIAGEN DNeasy Blood and Tissue DNA extraction kit was used for the remainder of the protocol from step 2, which included the addition of 180 µl buffer ATL and 20 µl proteinase K. The samples were incubated for 3 hours at 56^{o}C with vortexing at 1 hr intervals. Each sample was eluted in 50 µl of elution buffer. DNA quantity was measured using a Nanodrop spectrophotometer and the DNA concentration adjusted to 50 ng/µl.

3.4. DNA standards

DNA standards for PCR positive controls were prepared for *C. bovis* and internal control targets for both real-time PCR assays. Purified PCR products produced by conventional PCR were cloned into the TOPO-TA cloning vector according to the manufacturer's instructions (Invitrogen, Carlsbad, CA). Clones were then sequenced using the respective forward primer and diluted to 0.1 ng/ul (which corresponded to a Ct score of ~12-14) and stored at -20C. A clone of the correct sequence could not be obtained for the SYBR green assay internal control target, however easily obtainable bovine gDNA can be used alternatively. Glycerol stocks of *E. coli* bacterial cultures containing the vector and insert were prepared and stored at -80C.

3.5. Real time PCR assay development

3.5.1. SYBR green assay

3.5.1.1. Description of the test

The proposed SYBR green real-time PCR assay involved a multiplex real-time PCR and melt curve analysis based on the differential amplification of either mitochondrial or nuclear genes for the diagnosis of *C. bovis* and *E. granulosus* (hydatid) cysts as well as an internal control for the presence of amplifiable DNA in each reaction. *E. granulosus* was selected for differential diagnosis as these parasites constitute the greatest number of misdiagnosed *C. bovis* cysts at meat inspection [2, 9, 13]. A bovine target was selected as an internal control as bovine DNA was likely to be included in any DNA extraction process of lesions from beef tissue.

3.5.1.2. Primer selection and optimisation

Primer sets that targeted various genes for *C. bovis*, *E. granulosus* and *Bos taurus* were either designed manually using aligned sequences or selected from published assays. Each primer set was then evaluated for similar biophysical properties and dimer formation with Oligoanalyzer 3.1 (IDT, Leuven, Belgium) and blasted against the NCBI nucleotide database to confirm a lack of homology with non-target organism sequences.

The primers selected for the SYBR green assay are summarised in Table 3. For the specific amplification of *C. bovis*, a fragment from the *COI* gene was amplified using the forward primer (Tsag) designed by Yamasaki *et al*. [14] and the nested *COI* reverse primer designed by Chiesa *et al*. [2] were used. Four primer sets targeting mitochondrial genes of *E. granulosus* were evaluated however none were suitable for use in the assay due to hybridisation of primers, lack of specificity and product melting temperatures (T_m) that were not discernable from the *C. bovis* target. Three published bovine-specific primer sets targeting the *18S rRNA*, *RPLPO* or *GAPDH* loci were evaluated, with the *18S rDNA* primers [15] selected for use. Primer set concentrations were optimised in a combination matrix of 50 nM, 100 nM, 300nM, 600 nM and 900 nM. The combination resulting in the highest sensitivity and specificity was selected.

Table 3. Oligonucleotide primers used in the SYBR green PCR assay for detection of *C. bovis*

Target	Oligonucleotide name	Sequence	Product length	Product T_m (^oC)
C. bovis	Tsag F Nested COI R	5'-TTGATTCCTTCGATGGCTTTTCTTTTG-3' 5'-ATTAATAGAACTAAAAATTCTAGACG-3'	131 bp	78.3
B. taurus	18S rRNA F 18S rRNA R	5'-GTAACCCGTTGAACCCCATT-3' 5'-CCATCCAATCGGTAGTAGCG-3'	151 bp	81.3

3.5.1.3. Amplification and detection

The assay was conducted using a Rotor-Gene 6000 (Corbett Research) with the 72-well reaction ring. The reaction volume was 10 µl containing 5 µl of SensiMix Plus SYBR® Green (Quantace Ltd.), 300 nM for *C. bovis*-specific primers, 75 nM for internal control primers and 150 ng of genomic DNA. All reactions were duplicated and all runs included negative and positive controls. Amplification involved touchdown PCR to increase specificity: 10 mins at 94^{o}C, followed by 10 cycles of 94^{o}C for 30 secs, with annealing temperature starting at 65^{o}C for 60 secs (decreasing 0.9^{o}C/cycle) and 72^{o}C for extension. This step was followed by 30 cycles of 30 secs at 94^{o}C, 56^{o}C for 60 secs and 72^{o}C for 60 secs. Fluorescence was measured in the green channel and data acquired at the extension step. A melt-curve analysis was performed post-run in order to match amplicons with positive controls and confirm specificity and involved a 0.1^{o}C increase in temperature every 10 seconds between 75^{o}C and 87^{o}C. The run time was approximately 3 hours.

At the end of the run, the negative first derivative of the change in fluorescence as a function of temperature was plotted using the Rotorgene software to produce melt peaks for each sample. The threshold was set to 0.5 and the temperature threshold set to 76^{o}C. The run was invalid if either positive control did not produce a melt peak above the threshold or if there was a reaction in the no-template negative control. A sample was a true *C. bovis* positive if there were two peaks within a 2.5^{o}C range of the *C. bovis* standard T_m and 1^{o}C of the internal control T_m. The sample was *C. bovis* negative if there was a single peak at the internal control T_m only and the reaction failed if there was no peak at all. To assess the reliability of the SYBR green assay melt curve analysis for use as a diagnostic tool, the degree of T_m variation among *C. bovis* isolates was examined in 31 positive control *C. bovis* lesions.

3.5.2. Taqman MGB probe assay

3.5.2.1. Description of the test

The probe-based assay was designed with Taqman minor groove binding (MGB) detection probes. Two targets including *C. bovis* and *Bos taurus* mt*COI* DNA were selected for a duplex assay as the Rotor-Gene 6000 thermocycler at UQSVS had a reading capacity of only two channels and an *E. granulosus* target could not be incorporated. An upgrade of this machine to read 3 or more channels was not anticipated at the project's start and was not possible due to costs outside its scope.

3.5.2.2. Primer and probe design and optimisation

PCR primers and MGB probes were designed using Primer Express software (Applied Biosystems, Foster City, CA) and the mtCOI sequences of *C. bovis/T. saginata* (Accession no. AB533172.1) and *Bos taurus* (Accession no. AF493541.1). The *C. bovis* sequence was then aligned with corresponding mtCOI sequences from the non-target reference panel and the primer and probe sequences manually checked for base pair mismatches to ensure non-crossreactivity (Figure 1). The *C. bovis*-specific primers, MGB Tsag forward and MGB Tsag reverse (Geneworks, Australia), amplified a 131 bp fragment and the 6FAM-labelled probe, MGB Tsag Probe (Applied Biosystems, Foster City, CA) was used to detect the product (Table 4). The internal control primers, MGB *Bos* forward and MGB *Bos* reverse (Geneworks) amplified a 150 bp product and the VIC-labelled probe MGB *Bos* Probe (Applied Biosystems) detected the internal control product. Primer sets were optimised using SYBR green dye in combination matrices of 100, 300 and 600 nM for the *C. bovis*-specific primers and 50, 75 and 100 nM for the internal control primers. Presence or absence of primer dimers was evaluated by melt curve analysis. Three different mastermixes were evaluated including QuantiTect Multiplex PCR Buffer (Qiagen), Kapa Probe Fast Universal (Kapa Biosystems, Woburn, MA) and RealMastermix Probe (5Prime, Germany). The optimal annealing temperature (Ta) of the reaction was tested between 55 ^{o}C and 58 ^{o}C.

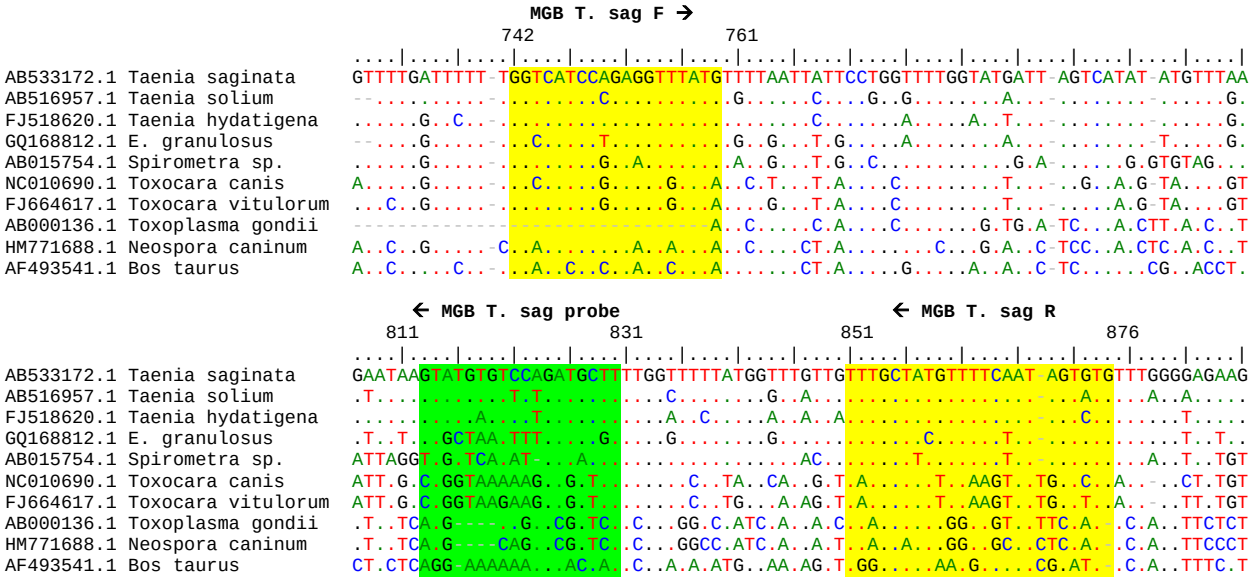


Figure 1. Multiple sequence alignment of the *Taenia saginata* COI sequence and non-target organisms for which sequence was available showing base pair mismatches in the primer and probe oligonucleotide sequences. Dots indicate homology, dashes indicate the presence of a gap.

Table 4. Oligonucleotide primers and detection probes used in the Taqman MGB probe assay for detection of *C. bovis* and an internal control

Target	Oligonucleotide name	Sequence	Genbank Accession No.	Amplicon
C. bovis	MGB Tsag F	5'-GGTCATCCAGAGGTTTATG-3'	AB533172.1	131 bp
	MGB Tsag R	5'-CACACTATTGAAAACATAGCAAA-3'		
	MGB Tsag Probe	6FAM-AAGCATCTGGACACATAC-MGBNFQ*		
B. taurus	MGB *Bos* F	5'-TGAGCCCACCATATATTCA-3'	AF493541.1	150 bp
	MGB *Bos* R	5'-AGCAGGAGACCATTTGATA-3'		
	MGB *Bos* Probe	VIC-CACACGAGCCTACTTCA-MGBNFQ*		

*MGBNFQ is MGB non-fluorescent quencher

3.5.2.3. Amplification and detection

The assay was conducted using a Rotor-Gene 6000 (Corbett Research) with the 72-well reaction ring. The reaction volume was 10 µl containing 4 µl of RealMastermix Probe with primer and probe concentrations as follows: 600 nM for MGB Tsag Forward and Reverse primers and 200 nM for MGB Tsag Probe, 75 nM for MGB Bos Forward, 100 nM for MGB Bos Reverse, 50 nM for MGB Bos Probe and 150 ng DNA as the input. All reactions were tested in duplicate or triplicate and negative and positive controls were run with all samples. Amplification conditions included an initial denaturation step of 95 ^{o}C for 1 min, followed by 40 cycles of 95 ^{o}C for 10 secs, 56 ^{o}C for 10 secs and 68 ^{o}C for 20 secs. Fluorescence was measured in the green and yellow channels and data acquired at the extension step. The run time was approximately 1 hour. For SYBR green analysis during primer optimisation, the same cycling profile and primer concentrations were used and a melt curve analysis performed at the end of the run.

At the completion of each run, the data was analysed using the Rotorgene 6000 software. The green and yellow channel were simultaneously selected in the Allelic Discrimination analysis

window and normalisation methods and threshold settings applied according to Table 5. In the Outlier Removal window, the NTC threshold was set to 10%, which allows the operator to distinguish between samples with minor changes in fluorescence and genuine reactions.

Table 5. Parameters of the Rotorgene 6000 software for Taqman MGB probe assay data analysis

Parameter	Value/Function
Threshold	0.01
Begin normalising	Cycle 1
Normalisation method	Dynamic tube, slope correct enabled
NTC threshold	10%
Reaction efficiency threshold	disabled

The genotypes were set as described in Table 6. The samples were then assessed according to the following criteria: the run was invalid if either of the green or yellow channel positive controls did not react in their respective channels or if there was a reaction in the negative control. A sample was invalid if one of the duplicated reactions did not amplify. A sample was considered a true *C. bovis* positive if there was a reaction in both the green and yellow channel. A sample was negative if there was a reaction in the yellow channel only. The sample was negative if there was a reaction in the green channel only, but should be repeated and/or sequenced for confirmation.

Table 6. Genotype designation in an allelic discrimination analysis of the Taqman MGB assay.

Genotype	Reacting Channel		Result
C. bovis positive – PCR Fail	Cycling A Green		**False positive (repeat)**
C. bovis positive – PCR Pass	Cycling A Green	Cycling A Yellow	**True positive**
C. bovis negative – PCR Pass		Cycling A Yellow	**True negative**

3.5.2.4. Efficiency and limit of detection

The efficiency and limit of detection of the Taqman probe assay was determined using eight 10-fold dilutions of purified plasmid DNA containing the *C. bovis* COI insert. The dilutions ranged from $2.2x10^8$ to 22 copies of target and were tested in three different runs in both singleplex and duplex reactions. In addition, samples containing 100 ng of bovine gDNA spiked with plasmid DNA were run to assess any changes in efficiency and limit of detection parameters.

The efficiency for the *C. bovis* primer/probe reaction was assessed using the standard curve and slope generated from the serial dilutions by the Rotorgene 6000 software. Two-tailed t-tests were performed in Excel to determine if there were significant differences between the efficiencies and linearity (R^2) values of the runs. The assay's limit of detection was determined as the DNA concentration in which the COI *C. bovis* or bovine target was reliably detected, or when 3/3 of triplicated reactions in all three runs gave a positive signal with a total coefficient of variation (total CV) less than 5%.

3.5.2.5. Repeatability and reproducibility

To evaluate the reliability of the Taqman MGB probe assay, the repeatability (intra-laboratory agreement) and reproducibility (inter-laboratory agreement) were evaluated using the measured outcome (Ct value) of positive *C. bovis* samples in different laboratories. The agreement of the

assay using the dichotomous result (+/-) for positive and negative samples was also assessed. Reliability was assessed using a modified hierarchical design outlined in Jordan et al. [16] and included 10 positive and 10 negative samples identified by the reference PCR. Each sample was divided into 10 replicates, and distributed between the two laboratories with 5 replicates each to be run on 5 different days. The samples were blinded in respect to their disease status or identity. Quantitative estimates of both validation criteria were derived as a reliability coefficient in R using linear mixed models to generate the variance estimates used in its calculation [16, 17].

3.6. Analytical specificity of the tests

The specificity of both the SYBR green and Taqman probe assay primers to produce a single gene-specific amplicon in the target gDNA was checked by melt curve analysis using SYBR green dye. The specificity of each primer set to produce a species-specific amplicon was evaluated in 3 separate runs against the negative reference panel of DNAs (refer to Table 1) including host DNA, *T. solium*, *Echinococcus granulosus* Genotype 1 (*n*=7), *Taenia hydatigena* (*n*=1), *Spirometra erinacei* (n=1), *Toxocara canis* (n=1), *Toxocara vitulorum* (n=1), *Sarcocystis cruzi* (n=2), *Neospora caninum* (n=1), *Toxoplasma gondii* (n=1), *Actinobacillus lignieresii* (n=1) and *Bos taurus*.

3.7. Diagnostic sensitivity of the tests

The degree of lesion viability has previously been reported as a factor in PCR sensitivity. In order to estimate the lowest degree of viability of a *C. bovis* lesion that could be detected by either real-time PCR assay, the diagnostic sensitivity was investigated using 31 positive control *C. bovis* lesions that were classified as either viable, calcified or degenerate. As there were no highly degenerated lesions in this group, three lesions of this classification from the suspect lesion group were also included in the analysis. All lesions were prepared and tested according to the DNA extraction and PCR protocols described previously, and run in both the Taqman MGB probe and SYBR green assay. Prior to the analysis, all 34 lesions were confirmed as *C. bovis* using the reference PCR.

3.8. Validation of the Taqman MGB probe assay using suspect lesions submitted from abattoirs

The diagnostic performance of the Taqman MGB probe assay was evaluated using the 27 abattoir submitted lesions collected during the study period. The level of agreement between the Taqman assay and the reference PCR was measured by calculating the kappa coefficient in WinEpiscope ver 2.0. [18]. Sensitivity and specificity of the Taqman MGB probe assay were estimated using the results of the reference PCR as the true status of lesions (gold standard).

4. Results and discussion

4.1. Suspect lesion collection

Between January 2011 and June 2012, 24 suspect lesions from 18 animals were submitted from beef export abattoirs. An additional 3 lesions from 3 animals were submitted by veterinary pathologists, bringing the total number of lesions for use in the field evaluation study to 27 from 21 animals. From overseas sources, only 4 lesions were forwarded from Bali for which histopathology could not be performed due to poor quality of the formalin-preserved sample. Following DNA extraction of ethanol preserved specimens, 3 of 4 lesions produced DNA of low quantity and quality thus all 4 samples were removed from the study.

4.2. Classification and PCR identification of lesions

4.2.1. Positive reference samples

The morphological classification of the positive reference *C. bovis* lesions resulted in 3 viable lesions and 28 lesions ranging between 'calcified' and 'degenerated'. There were no lesions that could be classified as highly degenerate. All lesions produced a single PCR product of the expected size in the first round of the reference PCR except one lesion which was positive in the second round. Each sample was confirmed as *C. bovis* when its nucleotide sequence was analysed using a BLASTN sequence similarity search at the NCBI database. Figure 2 shows a subset of these amplification products on an agarose gel generated by the first round of the reference PCR.

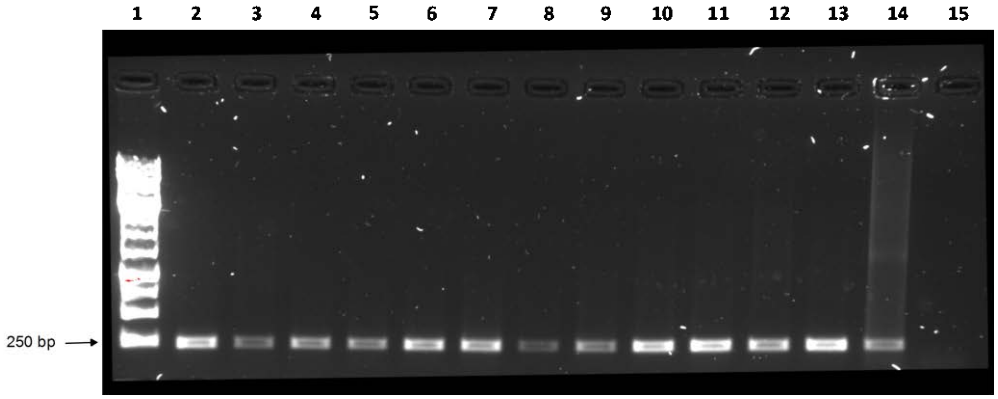


Figure 2. 1% agarose gel showing 253 bp amplification products of positive control *C. bovis* lesions from 2010 following round 1 of the reference PCR. Lane 1 is a Generuler 1 kb DNA Ladder (Fermentas), lanes 2-14 are *C. bovis* lesions, lane 15 is the no-template control

4.2.2. Suspect Lesions

The classification of suspect lesions using histopathology and the reference PCR are summarised in Table 7. Most lesions originated from the heart (66%) and masseter (26%). None of the submitted lesions could be identified unequivocally as *C. bovis* by histopathology and the most common classification was non-specific granuloma or 'unknown'. All lesions classified as true negatives were sarcomas. In contrast, the reference PCR showed 14/27 (52%) of the lesions were positive for *C. bovis* when both PCR rounds were applied and all were confirmed as *T. saginata/C. bovis* following sequence analysis of the 130 bp PCR product. Two lesions, originating from the same animal, were classified by histopathology as true negatives due to the presence of well-demarcated, neoplastic masses yet were confirmed as *C. bovis* in the nested round of the reference PCR. In this case, It is possible that the neoplastic and infectious lesions were co-existing but separate entities in this case, however there is increasing evidence that some parasitic diseases could predispose to the development of neoplasia. Possible pathogeneses include parasite-induced immunosupression compromising immune system surveillance for tumour cells, and transfer of DNA between parasite and host leading to neoplastic transformation. Increasingly, chronic inflammation with its ongoing cellular replication and mediator release has been associated with neoplastic transformation and this scenario could also be considered where parasitic and neoplastic lesions are occurring together [19]. A further three suspect lesion samples were equivocal/unreproducible in the reference PCR in that they were positive in only 1 of 2 PCR runs and they were called as negative. Ten samples were considered negative as they did not amplify in any round and there was no amplification in any of the negative controls.

The reference PCR was previously validated using 171 *C. bovis* lesions identified at meat inspection and demonstrated a sensitivity of 94.7% (162/171) following both PCR rounds. In this study, only four of the twenty-seven (15%) abattoir submitted lesions were positive for *C. bovis* in

the first PCR round with an additional 10 lesions positive in round 2, resulting in an estimated sensitivity of 52%. The reduced level of sensitivity of the PCR shown here could be attributable to the increased level of degeneration seen in many of the submitted lesions or an increased prevalence of sarcomas, hydatid or *Sarcocystis* spp. lesions in Australian beef which are misidentified as *C. bovis* at meat inspection.

Table 7. Classification and identification of suspect *C. bovis* lesions by histopathology and reference PCR

		PCR results		
		Chiesa PCR		
Histopathology classification	**No. of samples**	**Round 1**	**Round 2**	**Negative**
Viable *C. bovis*	0	-	-	-
Degenerate cyst of parasitic origin	5	0	3	2
True negative	7	0	2	5
Non-specific granuloma	15	4	5	6
Total	27	4	10	13

4.3. Assessment of the SYBR green and Taqman probe assay detection and analysis

4.3.1. Analytical specificity of the tests

Dissociation melt curves produced for both the SYBR green and Taqman MGB assay primer sets, in either a singleplex or duplex format, showed a single amplicon generated per set and the absence of primer dimer formation or extraneous products after 40 amplification cycles (Figure 3). Both assays showed 100% specificity when challenged with DNA from other species that may occur in beef muscle, with no non-specific amplification from three different runs using the conditions described in the amplification and detection protocols (Figure 4. SYBR Green assay; Figure 5. Taqman assay). This high degree of specificity offers confidence to end-users in preventing false-identifications of macroscopic lesions caused by other parasitic infections.

Under some conditions, there was non-specific amplification or artefacts in both assays. In the SYBR green assay, positive signals occurred in DNA samples of *Taenia hydatigena* and *Toxocara canis* when standard (not touchdown) cycling parameters or less stringent (lower) annealing temperatures were used (data not shown). In the Taqman assay there was weak but consistent amplification in DNA of the closely related *Taenia solium* attributable to close homology between the MGB T. sag primer/probe oligonucleotide and *T. solium* COI sequence (refer to Figure 1). The cross-reactivity of the assay in this species is not of concern however as the biological relevance of this swine-specific parasite is negligible. Sequence analysis of another closely related bovine-specific species, *T. asiatica* (data not shown) indicates this species is also likely to cross-react in the assay. Although this species occurs in the liver of cattle and has not been reported in Australia to date, the potential amplification of *T. asiatica* in the Taqman assay is an advantage as the zoonotic potential of this parasite means beef carcasses infected with this parasite should also be withheld.

The Taqman assay also occasionally generated positive results from non-target DNAs after data was normalised using the dynamic tube and slope correct normalisation settings. These false-positive signals resulted from minor changes in the background fluorescence of samples and were confirmed as non-sigmoidal or linear changes in fluorescence in the raw channel data. Non-sigmoidal or linear changes in fluorescence are regarded as negative reactions and may be due to probe autohydrolysis in later cycles or inconsistency/differences in the inherent background fluorescence of a sample. To circumvent these artefacts, the NTC threshold in the Outlier Removal

Window of the Rotorgene software was set to 10%, allowing the operator to distinguish between samples with minor changes in fluorescence and genuine reactions. All non-specific signals that crossed the threshold were then eliminated.

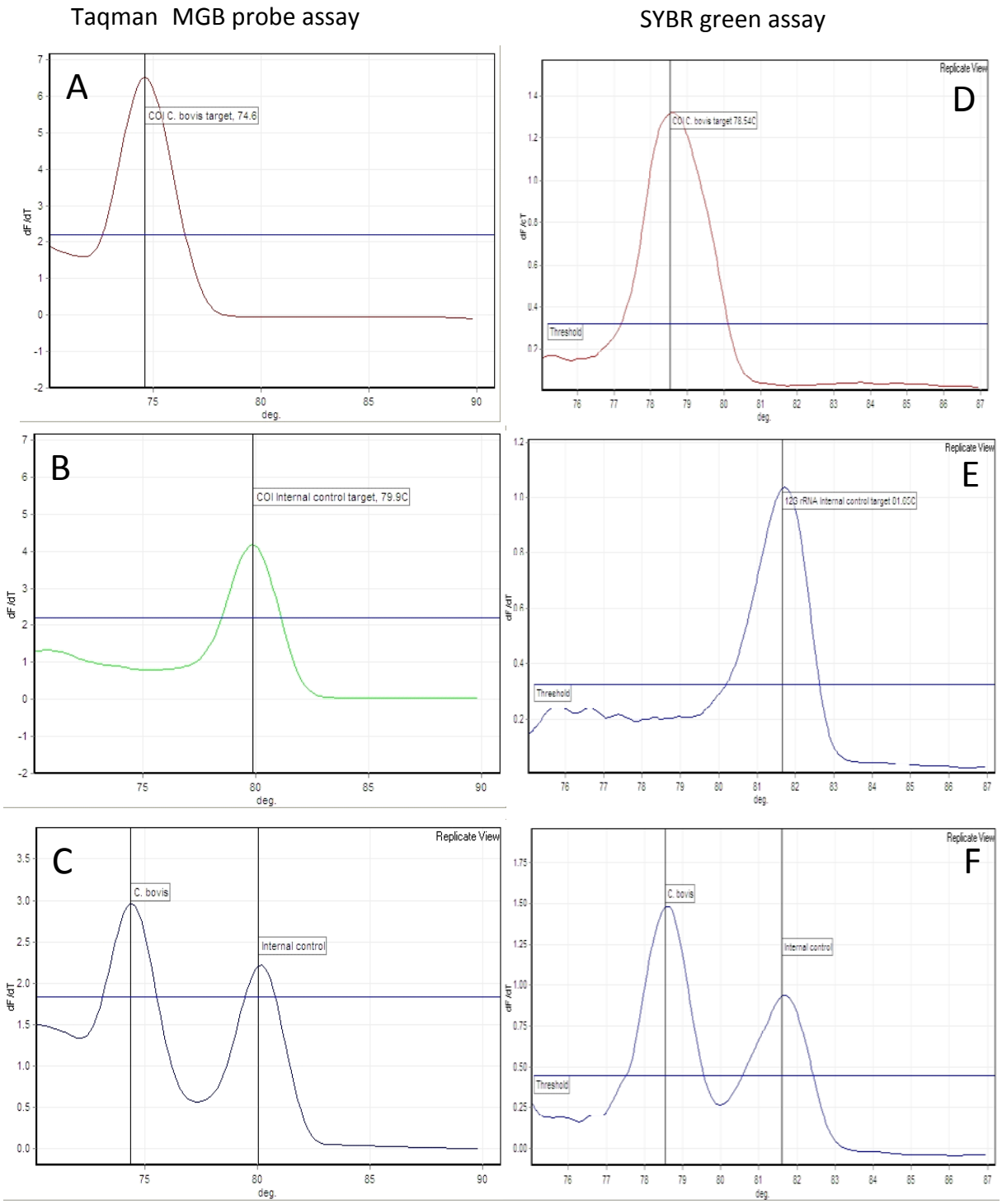
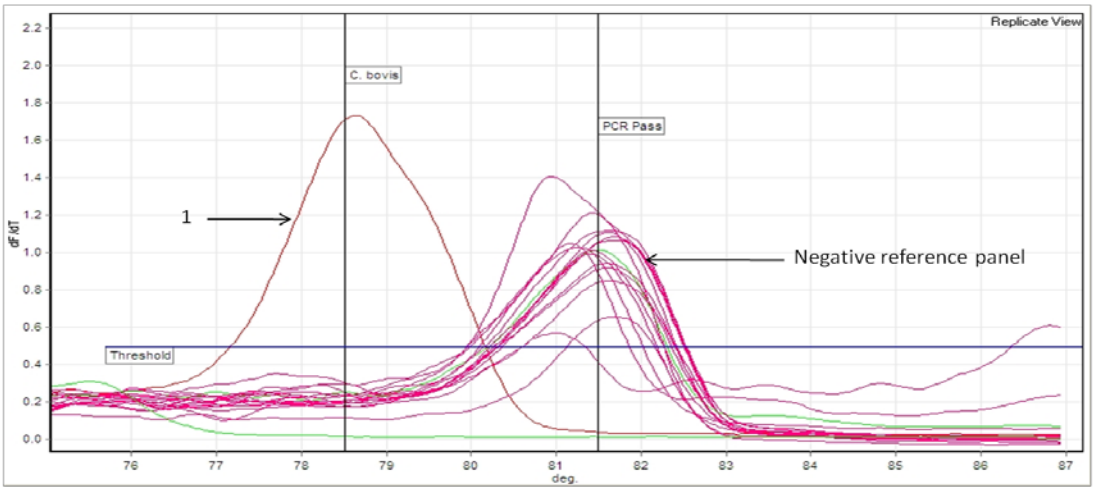
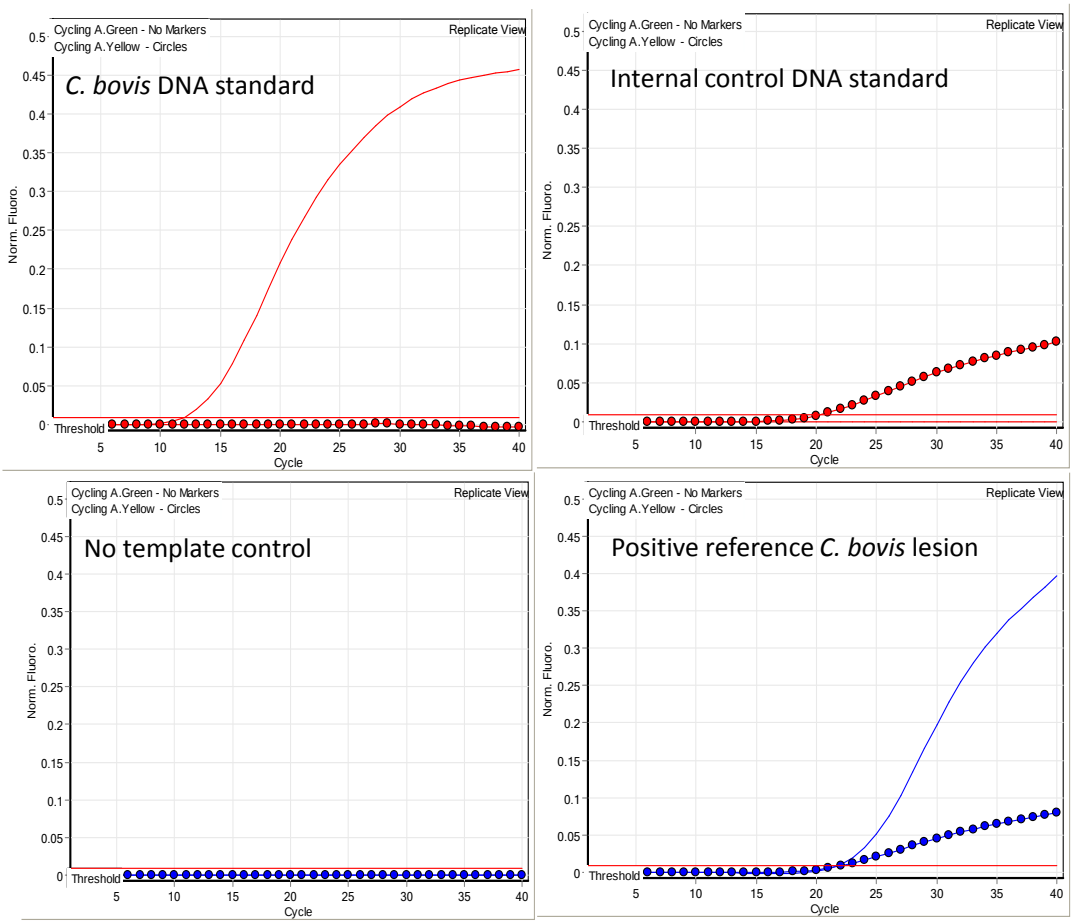


Figure 3. The SYBR green and Taqman MGB probe assay primer sets amplify a single gene-specific product for target and internal control DNA. Single amplicons shown by dissociation (melt) curve analysis: A & D are *C. bovis* targets; B & E are internal control targets; C & F are duplexed primer sets



Sample	Peak 1	Peak 2
SYBR T. sag	78.70 (C. bovis) 78.58 (C. bovis)	
Bovine gDNA		81.47 (PCR Pass) 81.52 (PCR Pass)
Toxocara vitulorum		81.43 (PCR Pass) 81.40 (PCR Pass)
Toxoplasma gondii		81.50 (PCR Pass) 81.70 (PCR Pass)
Taenia hydatigena		81.62 (PCR Pass) 81.60 (PCR Pass)
Toxocara canis		81.55 (PCR Pass) 81.67 (PCR Pass)
E. granulosus 1		81.18 (PCR Pass) 81.17 (PCR Pass)
E. granulosus 2		81.05 (PCR Pass) 86.83
E. granulosus 3		81.70 (PCR Pass) 81.68 (PCR Pass)
E. granulosus 4		81.27 (PCR Pass) 81.25 (PCR Pass)
E. granulosus 5		81.40 (PCR Pass) 81.40 (PCR Pass)
E. granulosus 6		81.63 (PCR Pass) 81.62 (PCR Pass)
E. granulosus 7		81.43 (PCR Pass) 81.38 (PCR Pass)
Sarcocystis cruzi		81.55 (PCR Pass) 81.65 (PCR Pass)
Neospora caninum		80.95 (PCR Pass) 80.95 (PCR Pass)
Actinobacillus lignieresii		81.68 (PCR Pass) 81.72 (PCR Pass)
Spirometra erinacei		81.78 (PCR Pass) 81.63 (PCR Pass)
dH20		

Figure 4. Graphical interpretation and tabulated results showing the specificity of the SYBR green assay to non-target species. The graph shows the dissociation melt curve analysis of the SYBR *C. bovis* DNA standard (1) and the negative reference panel using touchdown cycling parameters and annealing temperatures 65-56^{o}C. The tabulated results show how genotypes for *C. bovis* and PCR Pass are ascribed when bins are set.



Name	Genotype	Green	Yellow
C. bovis standard	*C. bovis*, PCR Fail	Reaction	No Reaction
Internal control standard	PCR Pass	No Reaction	Reaction
Positive reference *C. bovis*	*C. bovis,* PCR Pass	Reaction	Reaction
Taenia solium	*C. bovis*, PCR Fail	Reaction	No Reaction
Taenia hydatigena	PCR Pass	No Reaction	Reaction
Echinococcus granulosus 1	PCR Pass	No Reaction	Reaction
Echinococcus granulosus 2	PCR Pass	No Reaction	Reaction
Echinococcus granulosus 3	PCR Pass	No Reaction	Reaction
Echinococcus granulosus 4	PCR Pass	No Reaction	Reaction
Echinococcus granulosus 5	PCR Pass	No Reaction	Reaction
Echinococcus granulosus 6	PCR Pass	No Reaction	Reaction
Echinococcus granulosus 7	PCR Pass	No Reaction	Reaction
Spirometra erinacei	No amp	No Reaction	No Reaction
Toxocara canis	No amp	No Reaction	No Reaction
Toxocara vitulorum	No amp	No Reaction	No Reaction
Toxoplasma gondii	No amp	No Reaction	No Reaction
Neospora caninum	No amp	No Reaction	No Reaction
Sarcocystis cruzi	PCR Pass	No Reaction	Reaction
Actinobacillus sp.	No amp	No Reaction	No Reaction
Bos taurus	PCR Pass	No Reaction	Reaction
dh20	No amp	No Reaction	No Reaction

Figure 5. Graphical interpretation and tabulated results of genotype mapping and specificity analysis in the Taqman MGB probe assay. In the graphs, amplification traces with circles indicate the internal control reaction (yellow channel), and no markers indicate the *C. bovis* reaction (green channel). The table shows the mean results of an Allelic Discrimination analysis. A 'No amp' result indicates the absence of both bovine and *C. bovis* DNA.

*DNA extracted from a *T. hydatigena* cyst in sheep liver gives a positive result in the yellow channel due to cross-reactivity in ovine DNA with the MGB Bos primers.

4.3.2. Diagnostic sensitivity of the tests

Thirty-four *C. bovis* lesions classified according to their degree of viability were used to evaluate the diagnostic sensitivity of both the SYBR green and Taqman probe assay. All 34 lesions were positive in the reference PCR with 30 lesions positive in the first round of the PCR and an additional 4 lesions, including the 3 highly degenerate lesions, positive in the second round. The SYBR green assay demonstrated the better sensitivity of the two newly developed tests, although the Taqman probe assay was only marginally less sensitive (Table 8). In both the SYBR green and Taqman probe assay, the highly degenerated lesions were most commonly not detected which was consistent with degree of viability as a factor in PCR sensitivity.

Table 8. Sensitivity analysis of the SYBR green and Taqman probe real-time PCR assays for detection of *C. bovis* using the results of a reference PCR as the true disease status of 34 lesions.

Sensitivity analysis of two real-time PCR assays						
Assay type	**Reference PCR/Real-time PCR assay**					**Relative Sensitivity (95% Confidence Limit)**
	+/+	+/-	-/+	-/-	Total	
SYBR green	32	0	2	0	34	94.118 % (86.209 – 100.00)
Taqman MGB	31	0	3	0		91.176 % (81.672 – 100.00)

4.3.3. SYBR green assay melt curve analysis

Table 9 summarises the average T_m and 99% confidence interval ranges for multiple PCR positive *C. bovis* isolates that were amplified using the SYBR green assay in a single run. Figure 6 illustrates the melting curve graphs and the characteristic dual melt peaks for both targets. Melting curve analyses can be reliable methods for identifying and differentiating targets in multiplex PCR as long as the T_ms of each product are sufficiently different from each other. The *C. bovis* and internal control targets could be easily differentiated based on their different T_m values and the 99% confidence interval ranges did not overlap. However, although low variability amongst isolate T_m values for the *C. bovis* target indicated the melt curve analysis could be a reliable diagnostic tool for *C. bovis*, there was a greater than 0.5 ^oC difference in the average T_m of the *C. bovis* DNA standard compared to the diagnostic samples. As a result, the peak bin-width for the *C. bovis* genotype needed to be increased to 2.5 ^oC to correctly map all the *C. bovis* isolates to the standard resulting in a upper T_m only marginally less than the lower confidence interval of the internal control.

Table 9. Mean melting temperatures (T_m) and 99% confidence intervals for real-time PCR products amplified from *C. bovis* isolates and DNA standard using the SYBR green assay

Species	**n**		**Mean T_m**	**Std. Dev.**	**Confidence interval (99%)**
DNA standard	6*****		78.89	0.13	78.65 – 79.12
C. bovis isolates	32	*C. bovis* COI	78.21	0.14	78.14 – 78.28
		Internal control	81.52	0.18	81.43 – 81.60

* measurement calculated from a single DNA standard over 6 independent runs

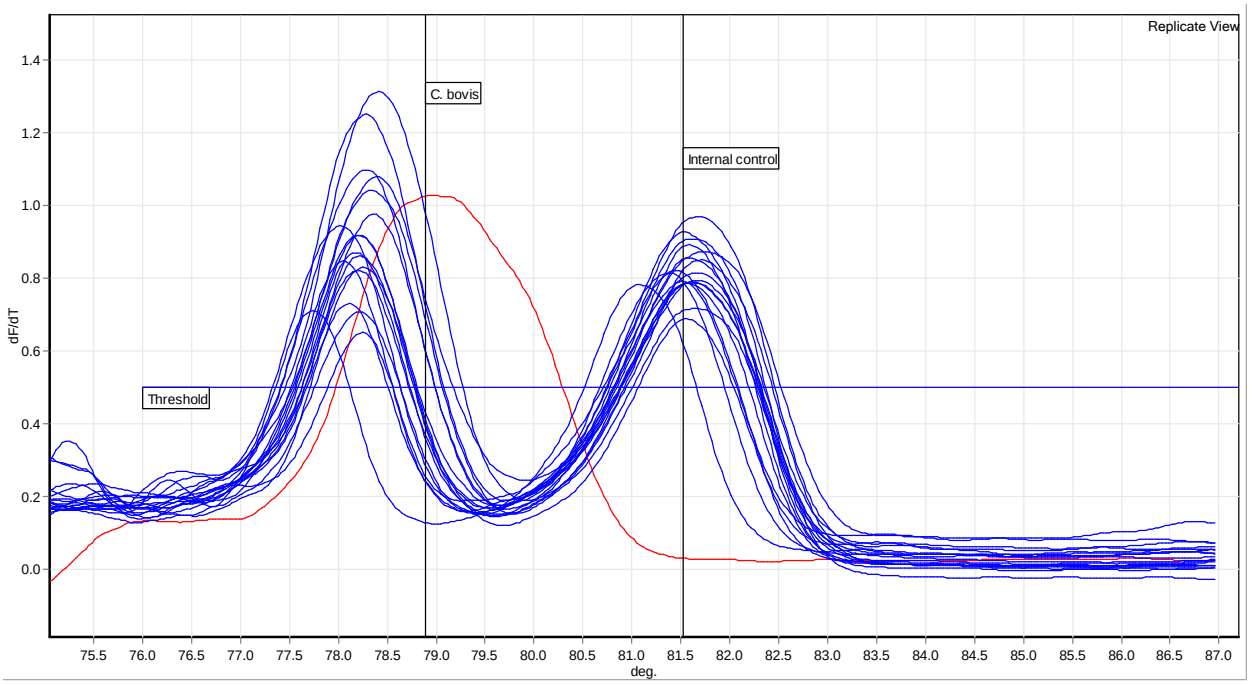


Figure 6. Dissociation melt curve analysis illustrating the characteristic dual melt peaks in *C. bovis* isolates that also contain amplifiable DNA (internal control) in replicate view. Temperature (^{o}C) is plotted along the x-axis and the negative first derivative of the change in fluorescence as a function of temperature is plotted along the y-axis. Note the 0.5 ^{o}C difference in T_m between the *C. bovis* DNA standard and diagnostic samples.

4.4. Taqman MGB probe assay performance

4.4.1. Efficiency and limit of detection

The Taqman assay, when pure plasmid DNA containing the COI *C. bovis* target was used in a singleplex or duplex format, showed high real-time PCR efficiency and linearity in the investigated range (Table 10). There was no change (P>0.05) in the duplex or singleplex reaction efficiency when plasmid DNA spiked with bovine DNA was the input although there was a significant reduction (P=0.0002) in linearity in the duplex format (although still within the recommended range of 0.95). The lack of difference between the efficiencies of singleplex or duplex reactions confirms the high level of MGB T. sag primer specificity to its target and the absence of primer dimers that would reduce efficiency. The efficiency of the internal control reaction was less than optimal, although the linearity was acceptable. The efficiency of the internal control was deliberately not optimised so as not to subtract from the efficiency of the *C. bovis* reaction.

Table 10. Comparison of the mean efficiency parameters of the Taqman MGB probe assay from three runs in either singleplex or duplex formats or with pure or spiked DNA inputs.

Primer set	DNA input	Single-plex			Duplex		
		Efficiency	R^2	Slope	Efficiency	R^2	Slope
MGB T. sag	Pure plasmid	94%	0.996	-3.475	104%	0.992	-3.237
MGB T. sag	Plasmid + Bovine gDNA	100%	0.992	-3.316	102%	0.958	-3.282
MGB Bos	Pure plasmid	Not done			67%	0.967	-4.48

The limit of detection when pure plasmid DNA was the input was as little as 22 copies of the *C. bovis* target or 100 attograms of DNA (Ct 31.85 ± 0.54). There was a 10-fold reduction in sensitivity in the bovine-spiked plasmid DNA reactions and therefore the reproducible level of sensitivity for the assay is 220 copies of target gene or 1 fg of *C. bovis* DNA corresponding to a Ct value of 30.47 ±0.66 (Figure 7). There was no difference in the limit of detection between the singleplex or duplex format, indicating a lack of competition for PCR reagents between the *C. bovis* and internal control reaction. The reduction in sensitivity is most likely attributable to decreased sample homogeneity due to the presence of large amounts of non-target DNA. Lastly, the reliable detection limit for the MGB Bos internal control was 1 pg (Ct 28.06 ± 0.35) of bovine DNA or $2.2x10^5$ copies of target (data not shown).

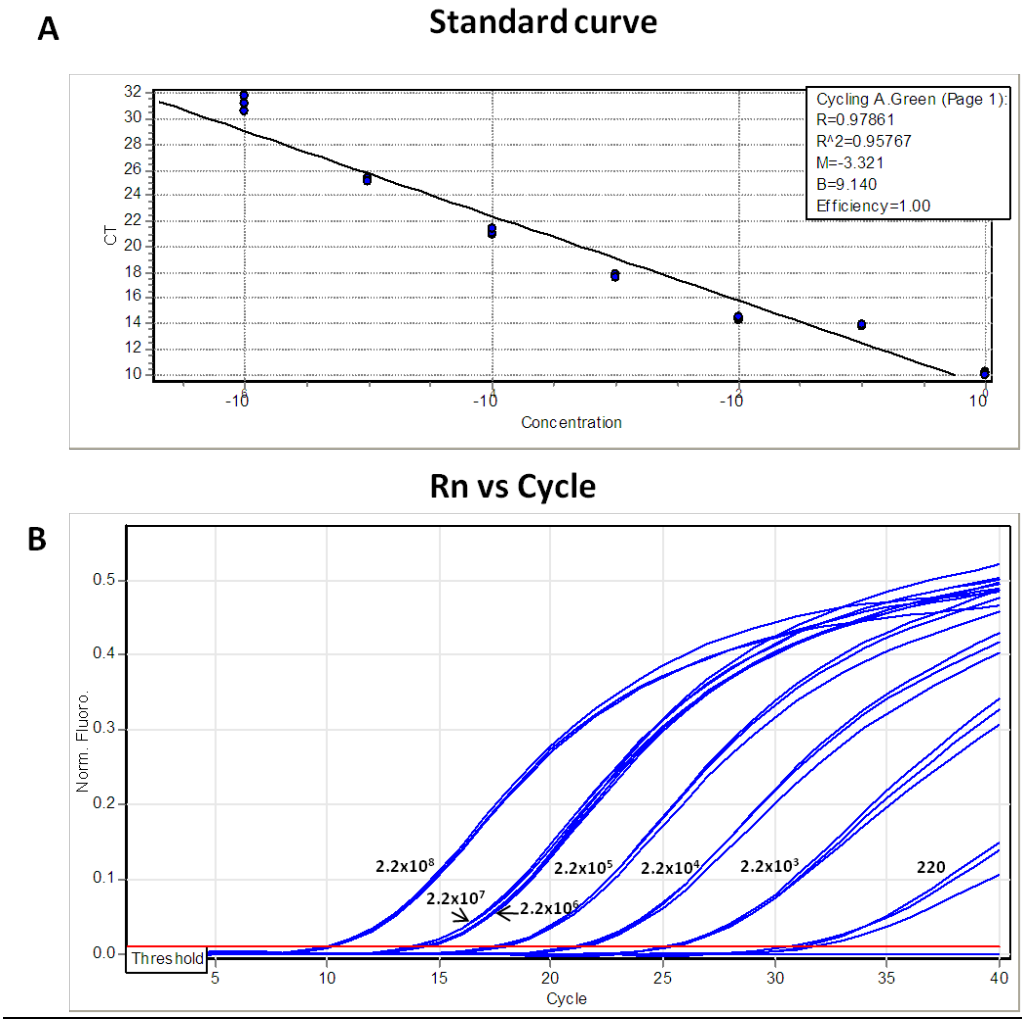


Figure 7. The *C. bovis* DNA standard in the duplexed Taqman probe assay spiked with host DNA has a 7-log linear dynamic range from $2.2x10^8$ to 220 copies of template. A: Standard curve generated by ten-fold serial dilutions in triplicate reactions of purified template mixed with 100 ng of bovine gDNA. B: Amplification plot of serial dilutions in the Taqman probe assay.

4.4.2. Repeatability and reproducibility

The assay showed good repeatability and reproducibility with high estimates of the reliability coefficient within and among laboratories using the Ct scores of positive samples (Table 11). The majority of variation within both laboratories was due to differences between samples rather than variation between runs or random noise. Typically, there was greater variability in Ct score among samples that were weakly positive than samples that were strongly positive (Figure 8).

Table 11. Variance components and reliability coefficients of *C. bovis* positive samples using linear mixed models to estimate repeatability and reproducibility of the Taqman MGB probe assay.

Type of Reliability	Lab		Variance	Reliability coefficient
Repeatability	1	Sample	27.04093	0.995
		Run	0.000000	
		Residual	0.12626	
	2	Sample	32.78345	0.980
		Run	0.39749	
		Residual	0.25031	
Reproducibility	Both	Sample	735.4981	0.948
		Lab	10.3244	
		Lab X run	15.4628	
		Sample X Lab	9.8467	

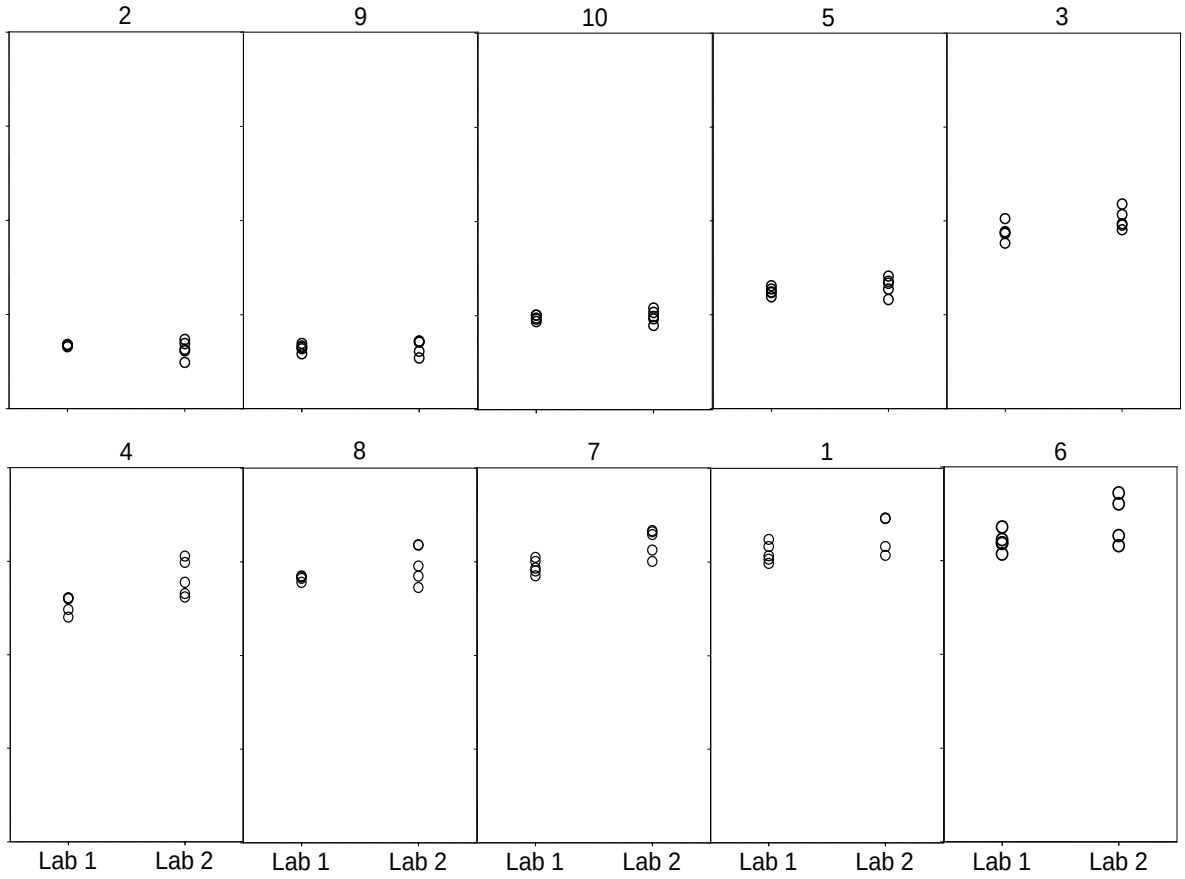


Figure 8. Mean Ct scores of PCR duplicates for ten *C. bovis* positive samples tested in five different runs and two laboratories using the Taqman MGB probe assay. Sample plots are arranged in increasing mean Ct score (strong-weak positive) and panel headers indicate sample number. The y axis is Ct score.

The dichotomous (positive or negative) outcome of both the positive and negative samples and the agreement of results between laboratories is presented in Figure 9. In total, there were 4 disconcordant results (results where a positive sample tested negative or vice versa) occurring among the total 200 recorded observations. Three of these results were due to errors in the Ct estimation of weakly positive samples (Ct > 30) which were close to the assay's limit of detection. The fourth disconcordant result was due to poor selection of sample as this sample was in fact a true *C. bovis* lesion that was only weakly positive in the reference PCR.

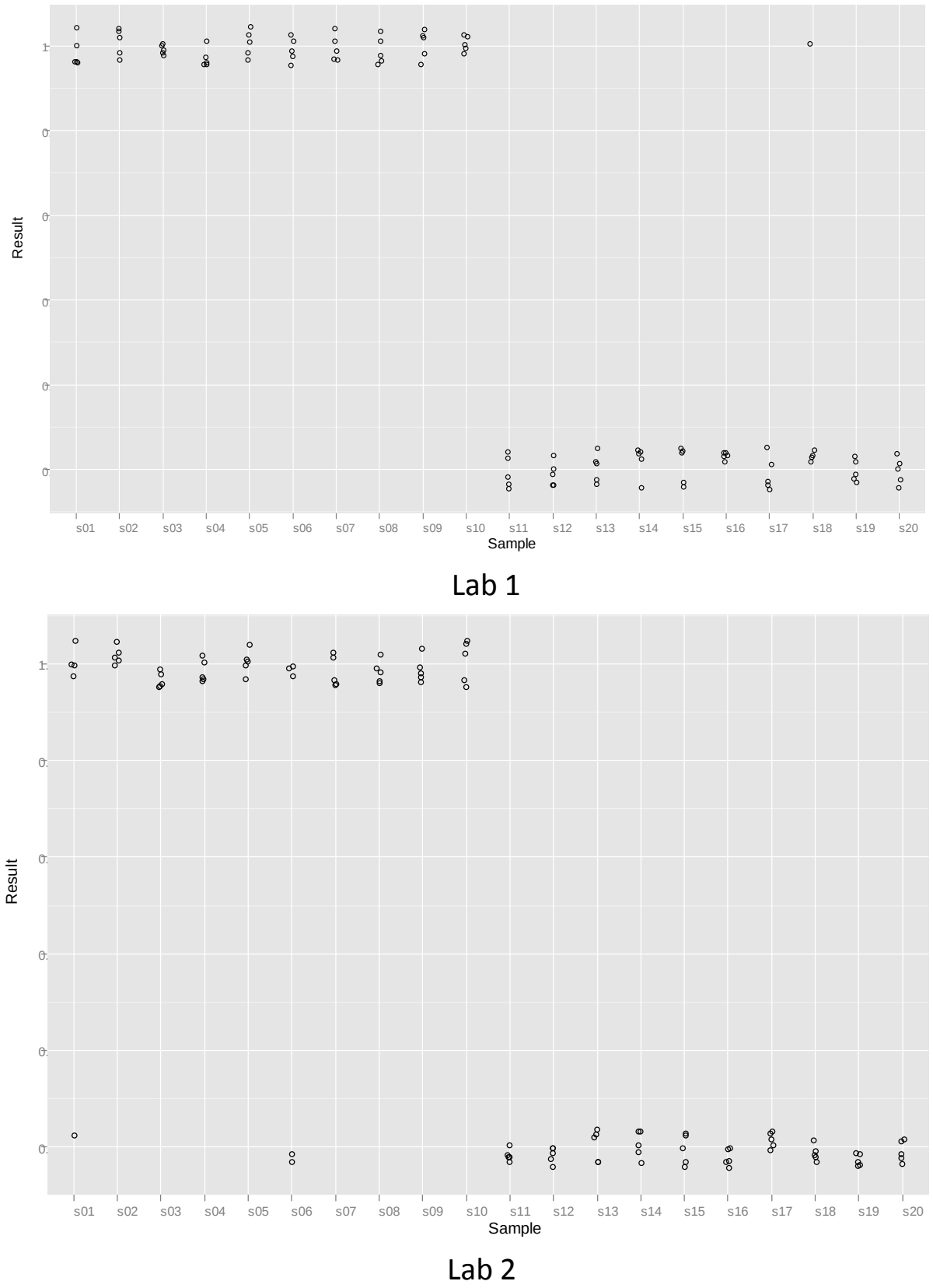


Figure 9. Agreement of the dichotomous (positive/negative) test result using the Taqman MGB probe assay for each Ct measurement of twenty samples tested 5 times in two different laboratories. On the y scale, 0 equals a negative result and 1 equals a positive result. On the x scale, s01-10 are positive samples and s11-20 are negative samples.

4.5. Validation of the Taqman MGB probe assay using abattoir submitted suspect lesions

The Taqman MGB probe assay's performance was validated using 27 suspect lesions submitted from Australian beef abattoirs. The kappa (κ) statistic at the 95% confidence interval, which estimates the level of agreement between the reference PCR and the Taqman probe assay was 0.638 (C.I. 0.374–0.903) indicating a moderate to substantial level of agreement [20]. The specificity of the test was 100%, although the sensitivity of the test was significantly less than the reference nested PCR at 57% (C.I. 31.220 – 83.066). This difference in sensitivity is a reflection of the greater sensitivity attained in nested PCRs albeit at an increased cost of labour, turnaround time and risk of contamination. In the reference PCR, the cumulative number of cycles in two rounds of PCR is 65 whereas in the Taqman assay only 40 cycles are performed by convention. If only results from the first round of the reference PCR are considered, then the sensitivity of the Chiesa PCR in one round is only 28.5% compared to 57% of the Taqman assay.

Table 11. Agreement, sensitivity and specificity of the Taqman probe assay using the reference PCR results of twenty-seven abattoir submitted suspect lesions as the true status of lesions

Sample size Taqman assay/Reference Test					**Results (95% Confidence Limit)**		**κ statistic**
+/+	+/-	-/+	-/-	Total			
8	0	6	13	27	Sensitivity:	57.153 (31.220 – 83.066)	0.638 (0.374 – 0.903)
					Specificity:	100.0 (100.00 – 100.00)	
					True Prevalence:	51.85 (33.005 – 70.699)	
					Predictive value +:	100.00 (100.00 – 100.00)	
					Predictive value - :	68.421 (47.520 – 89.322)	

5. Breakdown of assay costs

A breakdown of the costs involved in screening suspect lesions is presented in Table 12. DNA extraction using the commercially produced QIAGEN kit is a costly aspect of sample processing, however quality assurance and rapid extraction are advantageous. In regards to the PCR costs, both real-time PCR assay reactions were minimised and optimised to reduce costs and resulted in a total cost/sample for a duplicated reaction of less than $2.50. As expected, due to the increased costs of manufacturing probes, the Taqman probe assay was $0.85 more costly than the SYBR green assay per sample. The labour costs are the most costly aspect of processing samples and concurrent processing would be required to minimise the cost/sample. The most practical number of samples from which to concurrently extract DNA is 12 (most centrifuges are 12-place). An extrapolated cost per sample based on 12 concurrent samples results in a cost/sample of $23.50.

Table 12. Breakdown of labour and reagent costs associated with screening a single suspect *C. bovis* lesions and 12 lesions concurrently.

	Reagents	**Cost/reactions (rxn)**	**Cost per sample (2x PCR reactions)**	**Cost per 12 samples**
DNA Extraction	Qiagen DNeasy Kit (50 rxns)	$354.20/50 rxn	$7.08	
	Consumables (tips, tubes, gloves)		$1.00	
	Total for extraction		**$8.08**	**$96.96**
Taqman MGB PCR	Primers (*C. bovis* and *Bos)*	$80.0/5000 rxn	$0.05	
	Probe (*C. bovis*)	$566/3000 rxn	$0.37	
	Probe (Bos)	$566/12,000 rxn	$0.10	
	QIAGEN strip tubes	$157.00/1000 rxn	$0.32	
	Real-time PCR Master Mix	$477.90/1000 rxn	$0.95	
	Consumables (tips, tubes, gloves)		$0.50	
	Total for Taqman MGB PCR		**$2.30**	**$27.60**
SYBR Green PCR	Primers (*C. bovis* and Bos)	$80.00/5000 rxn	$0.05	
	QIAGEN strip tubes	$157.00/250 rxn	$0.32	
	Sensimix PCR master mix	$700.00/2500 rxn	$0.56	
	Consumables (tips, tubes, gloves)		$0.50	
	Total for SYBR green PCR		**$1.45**	**$17.40**
Taqman assay rate per hour	Procedure	Procedure Time 1 sample/12 samples		
	Sample preparation	0.75 hrs/1.0 hrs	$33.75	$45.0
$45.00/hour	DNA extraction	0.5 hrs/1.0 hours	$22.50	$45.0
	PCR set-up	0.75 hrs/1.0 hours	$33.75	$45.0
	Analysis/reporting	0.5 hrs/0.5 hours	$22.50	$22.50
	Total labour cost per sample		**$112.50**	**$157.50**
Total costs for samples processed in the Taqman MGB probe assay			**$122.88**	**$23.50**

6. Success in achieving objectives

The two main objectives were achieved during the course of this project in that a molecular assay suitable for detecting DNA specific to *C. bovis* in lesions submitted from abattoirs has been developed, and its diagnostic performance and reproducibility formally assessed. The Taqman probe assay greatly enhances the classification of suspect lesions identified at meat inspection by specifically reacting with *C. bovis* DNA and not other parasites that may occur in beef tissue in viable as well as degenerated lesions. The study underscored the need for a specific molecular tool for lesion classification by demonstrating the nonconcordance in positive identifications of *C. bovis* lesions at meat inspection and the true status of the lesions identified by reference PCR, as well as in the inability of histopathology to identify any non-viable *C. bovis* lesions. The Taqman probe assay lacked the capability to identify highly degenerated *C. bovis* lesions but as future

lesions screened in a diagnostic facility will not be bisected for concurrent histopathology testing, the test sensitivity is likely to increase with the potential doubling of parasite DNA.

Limitations in the numbers of positive control samples at the study's onset precluded the optimisation of a DNA extraction protocol, although the extraction procedure used in this study resulted in high quantities of good quality DNA. The study also did not achieve its objective of incorporating an *Echinococcus granulosus*-specific target to discriminate *C. bovis* cysts from cystic hydatidosis into either the SYBR green assay or the Taqman assay. In the SYBR green assay, multiple primer sets specific for *Echinococcus granulosus* were evaluated but none were suitable for use due to sequence similarities with *C. bovis*, genotypic dissimilarities among *E. granulosus* and highly similar T_m values of PCR products that could not be differentiated in the melt curve analyses. The Taqman assay has the potential to incorporate an *E. granulosus* target using a thermocycler with greater than two channels, and this capability would further improve the negative predictive value (NPV) of the assay.

7. Impact on meat and livestock industry

- Enhanced classification of suspect lesions identified at meat inspection
- Savings for abattoirs as carcasses held aside in the processing chain re-enter the chain when confirmed as negative due to the rapid turnaround time of the assay
- Reduction in costs associated with the adoption of a risk-based *C. bovis* inspection program
- Enhanced reputation for excellence in food safety and quality control for the Australian Meat Industry with the inclusion of a highly specific and sensitive molecular assay as part of an overall program of *C. bovis* management
- Benefits for beef producers as timely traceback investigations to determine the original source of *C. bovis* infection at the farm/feedlot level can prevent future cases and disadvantages to the producer at subsequent cattle sales

8. Conclusions and recommendations

8.1. Conclusions

The *C. bovis*-specific real-time PCR assay enhances the classification of suspect *C. bovis* lesions identified at meat inspection and is a rapid and objective molecular tool for the meat industry's surveillance program for this meat-borne zoonosis. The cost per assay to screen a single lesion is under $125.00, although this amount includes a full component of labour which may not necessarily be incurred in a diagnostic laboratory. Without labour, the cost per sample is less than $11.00. The entire test can be performed in a single day and screening requires a facility with real-time PCR capabilities and basic training in molecular biology. By substantially improving the Negative Predictive Value (NPV) of the test used to identify suspect *C. bovis* lesions, the meat industry can accrue significant savings by returning withheld carcasses that furnish a negative result to the processing chain.

8.2. Recommendations

- Continue to collect suspect lesions identified at meat inspection in Australian abattoirs to improve the precision of the Taqman assay until a minimum of 50 samples has been collected
- Carry out additional research to integrate an *Echinococcus granulosus*-specific target into the assay to improve the NPV of the assay. A primer and probe combination targeting the COI

gene has already been designed and would enable end-users to differentiate between morphological lesions caused by *E. granulosus* parasites and highly generated *C. bovis* lesions

- ❖ Adopt the new *C. bovis*-specific real-time PCR assay for screening suspect lesions identified at meat inspection into the *C.bovis* abattoir surveillance program

- ❖ Explore facilities and capability of various government and private laboratories to conduct screening

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10. Appendices

Appendix 1. Letter circulated to Australian AQIS-supervised export beef abattoirs by Dr. Baden Pearse, AQIS on our behalf (January, June, December 2011):

Dear Inspector,

Cysticercus bovis, a meat-borne zoonosis, causes little harm to humans, however the parasite's main impact in Australia is the cost it poses to the meat industry in terms of meat inspection and the condemnation of carcases destined for the export market. A recent nation-wide survey revealed that the predictive value of positive meat inspection findings is poor. A risk-based approach to inspection has thus been proposed which will include the use of a highly sensitive molecular assay that will improve the management of C. bovis in the Australian meat industry.

The School of Veterinary Science, University of Queensland, Gatton in collaboration with the Department of Employment, Economic Development and Innovation, Queensland is undertaking a research project funded by Meat Livestock Australia, aimed at developing and validating a high-turnaround molecular-based diagnostic test for detecting DNA specific for *C. bovis* in lesions submitted from abattoirs. The test will be used in veterinary laboratories consistent with international OIE and SCAHLs guidelines for the stated purpose of "demonstrating freedom from *C. bovis* in individual animal products for trade purposes".

The success of this research project is highly dependent on the cooperation of the Australian beef industry in providing the research team with lesions that are classified as suspect *C. bovis* on post-mortem inspection. Due to the nature of this research project it is advised that submission of samples will not be for diagnostic but research purposes only and with this understanding, the research team will be highly grateful for any assistance offered.

If any suspect *C. bovis* lesions are observed at post-mortem inspection between December 2010 and October 2011, please incise a margin of at least 1 cm around the lesion(s) and place them in a sealed plastic bag on ice (chilled) (more details were provided by BP)

Shipping Address:

Leigh Cuttell/ Rebecca Traub
School of Veterinary Science
Building 8114, Level 0
Inner Ring Road, Gatton Campus
The University of Queensland
Gatton, QLD 4343

If you have any further questions please do not hesitate to contact Ms Leigh Cuttell, Postdoctoral Research Fellow on 0431 236 079 or (07) 54601090; l.cuttell@uq.edu.au

Appendix 2. Protocols for *C. bovis* Taqman MGB real-time PCR assay

DNA extraction reagents and materials

750 mL liquid nitrogen (LN_2), LN_2 dewar and scoop
Hammer
Heavy duty alfoil
Scalpel blades (1/sample)
Weigh boat (1/sample)
1.5 mL microcentrifuge tubes (2/sample)
Water bath heated to 90 ^oC
Waterbath heated to 70 ^oC
Waterbath heated to 56 ^oC
Microcentrifuge (adjustable speed to 14 000 rpm)
Qiagen DNeasy Tissue Kit (cat. 69504, 50 reactions) (1 column/sample)

DNA Extraction protocol

Homogenise lesion sample

1. Using a scalpel blade, excise as much of the cyst/lesion from bovine tissue as possible (if the cyst/lesion exceeds 25 mg, bisect and treat as two lesions)
2. Fold the lesion into a piece of heavy duty alfoil approximately 4cm x 4cm
3. Submerge lesion wrapped in foil in LN_2 for 30 secs or until frozen
4. Strike firmly with hammer 2-4 times to pulverise lesion
5. Transfer the pulverised lesion to a 2.0 mL screw cap tube
6. Add 200 µl buffer ATL from the Qiagen DNeasy tissue kit and vortex for 10 secs
7. Heat tube to 90 ^oC (waterbath) for 1 min
8. Transfer to LN_2 for 1 min, then repeat steps 7-8 a further 2 times
9. Heat tubes a final time to thaw contents

DNA extraction protocol

10. Cool sample to room temperature
11. Add 20 µl Qiagen proteinase K to sample and vortex for 10 secs
12. Incubate for 3 hours at 56 ^oC (waterbath) with vortexing for 10 secs at every 1 hour interval. After 3 hours, if there is incomplete digestion of the sample, continue incubation for a further hour.
13. Add 200 µl buffer AL to sample and vortex for 10 secs
14. Incubate sample at 70 ^oC (waterbath) for 10 mins
15. Add 200 µl 100% ethanol and vortex for 10 secs
16. Pulse centrifuge the sample to collect the sample
17. Transfer 600 µl of sample to a spin column
18. Centrifuge at 10 000 rpm for 1 min
19. Empty collection tube and add 500 µl Buffer AW1 to spin column
20. Centrifuge at 10 000 rpm for 1 min
21. Empty collection tube and add 500 µl Buffer AW2 to spin column
22. Centrifuge at 10 000 rpm for 3 mins
23. Replace collection tube with a new collection tube and centrifuge for a further minute at 10 000 rpm
24. Transfer Qiagen spin column to a clean, labelled 1.5 mL microcentrifuge tube
25. Add 50 µl Buffer AE to the spin column
26. Incubate at room temperature for 1 min
27. Centrifuge at 10 000 rpm for 1 min
28. Quantify DNA using a spectrophotometer and adjust DNA concentration to 50 ng/ul
29. Store DNA at -20 ^oC

Real-time PCR assay

Reaction components

	Volume per sample
Extracted lesion DNA (50 ng/ul)	3 µl
2.5 X RealMasterMix Probe (Eppendorf)	4 µl
C. bovis Forward primer (10 µM)	0.6 µl
C. bovis Reverse primer (10 µM)	0.6 µl
MGB-FAM Taqman *C. bovis* probe (2 µM)*	1.0 µl
Bos taurus Forward primer (10 µM)	0.075 µl
Bos taurus Reverse primer (10 µM)	0.1 µl
MGB-VIC Taqman *Bos taurus* probe (1 µM)*	0.5 µl
PCR-grade water	0.125 µl
Total reaction volume	10 µl

*Probe should be diluted from 100 µM stock solution immediately prior to run. Storage and re-use of diluted probe can give false signals in the assay.

To be included in every run:

- One positive control DNA reaction for each of:
  - 1 µl of pCRII-TOPO_*C. bovis*_COI (0.1 ng/µl)
  - 1 ul of pCRII-TOPO_*Bos_taurus*_COI (0.1 ng/ul)
- One negative control reaction of water

Cycling conditions

Thermocycle in a Rotor-Gene 6000 (Corbett Research) or equivalent

95 ^{o}C for 1 minute

Then, 40 cycles of
95 ^{o}C for 10 secs
56 ^{o}C for 10 secs
68 ^{o}C for 20 secs
Acquire green and yellow channels at end of the extension step

*Run time is approximately 73 minutes

Run analysis

At the completion of the run:
1. In the Analysis window, select Allelic Discrimination ('Other' Tab)
2. Select both the Green and Yellow channel, press 'show'
3. Normalise the background fluorescence and remove outliers using the following parameters:

Parameter	Value/Function
Threshold	0.01
Begin normalising	Cycle 1 (Ignore none)
Normalisation method	Dynamic tube, slope correct enabled
Outlier removal - NTC threshold	10%
Reaction efficiency threshold	disabled

4. Set Genotypes:

Genotype	Reacting Channel		Result
C. bovis positive - PCR Fail	Cycling A Green		**False positive (repeat)**
C. bovis positive - PCR Pass	Cycling A Green	Cycling A Yellow	**True positive**
C. bovis negative - PCR Pass		Cycling A Yellow	**True negative**

5. The run is valid if the following genotypes are ascribed to the controls:

    a. *C. bovis* DNA standard is **_C. bovis_, PCR Fail or PCR Pass**
    b. *Bos* DNA standard is **PCR Pass**
    c. The no template control has no reaction in either channel.

Appendix 3. Breakdown of costs associated with processing 23 additional *C. bovis* suspect lesions to achieve 50 samples required for better statistical reliability when assessing the Taqman assay performance characteristics.

	Sample	Batch (12 samples)	23 Samples	Batch multiplier x 2
Histopathology				
	$20		$460	
Reference PCR				
Labour (3 hours)		$135		$270
Reagents	NA	NA	NA	NA
PCR Purification				
Labour (1 hour)		$45		$90
Reagents	NA	NA	NA	NA
Sequencing				
	$10		$230	
Real-time PCR				
Labour *		$157.50		$315
Reagents	NA	NA	NA	NA
Totals				
			$690	$675
Total cost to MLA for further 23 samples				**$1365**

*includes DNA preparation and extraction procedures required for both reference and Taqman probe assay