Establishing the risk of Toxoplasmosis associated with the consumption of pork and pork products

Final Report
APL Project 2011/1017.401

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Executive Summary

Toxoplasma gondii is a two-host meat borne protozoan parasite found in all warm-blooded animals that causes lifelong infection in humans. Its ability to cross the placental barrier and infect the foetus, as well as its ability to emerge from hibernation within muscle cysts during periods of immune suppression in the host (e.g. AIDS patients, patients treated with corticosteroids) has led to its status as a pathogen of increasing public health concern. In addition, there are indications that it is associated with schizophrenia and risk taking behaviour, resulting in increased risk of car accidents. Warm blooded animals (including humans) can get infected from the faeces of an infected cat (the primary host) or from consumption of undercooked infected meat (including pork). Overseas competitors are focusing on reducing the level of *Toxoplasma* in their pigs with some apparent success. In 1983-84 the US national seroprevalence in sows was 42% (Dubey et al, 1991). Subsequent national surveys have documented a decline in Toxoplasma seroprevalence in sows from 20% in 1990 to 15% in 1995 and to 6% in 2000 (Patton et al., 1996, 1998, 2002)

None-the-less, a recent US report, based on 2013 data from the U.S. Department of Agriculture's (USDA's) Economic Research Service, estimated the annual cost to the US of the 15 foodborne pathogens responsible for "more than 95% of the illnesses and deaths from foodborne illnesses in which the Centres for Disease Control and Prevention (CDC) can identify a pathogen cause". For Toxoplasmosis, the cost was US$3.3 billion, second only to nontyphoidal Salmonellosis at US$3.7 billion (Dan Flynn, Oct. 2014). In 2013 there were 86,686 cases of Toxoplasmosis in the US with 327 deaths. Only 16 cases were congenital.

This project was jointly funded by Australian Pork Limited and Meat & Livestock Australia.

Key outcomes

- Collaborations were established with not one but two (2) Australian based Toxoplasma experts - Professor John Ellis (University of Technology Sydney) and Dr Milton McAllister (University of Adelaide, School of Animal and Veterinary Sciences). Professor Ellis provided ongoing input into the initial design and later modification of the project. His laboratory also provided the initial toxoplasma infected material. The collaboration with Dr McAllister provided a valuable additional resource with the advantage of local accessibility to expertise, infective material and a Dubey pig strain of T. gondii. Dr McAllister's laboratory is developing its own Toxoplasma qPCR which could prove useful for further work.
- A refined molecular test was established to detect Toxoplasma in meat.
    - ❖ DNA extraction protocols were compared and demonstrated to be effective in providing sufficient *T. gondii* DNA for testing by PCR. The extracted DNA can also be used for future genotyping by a specialised multiplex nested PCR if required.
    - ❖ A nested PCR to detect infection was established and validated and a mouse housekeeping gene was incorporated to act as a positive control. This provided confirmation that the PCR was working appropriately and confidence that a negative result was due to an absence of Toxoplasma DNA.
    - ❖ It was demonstrated that both the nested PCR and the qPCR were effective in detecting Toxoplasma DNA.
    - ❖ The qPCR was compared to the nested PCR and although more expensive, was found to be more sensitive (10 x), less time consuming (approx. 1/3rd the time) and was able to more accurately quantify the amount of DNA present (and by extension the number of Toxoplasma organisms).
- The Dubey digestion/centrifugation technique was validated to improve sensitivity and the recovery rate investigated. It was further demonstrated that there was less inhibition seen

with the qPCR of the digested product than with the qPCR of the straight sample. It must be remembered, however, that the spiked mince simply had *T. gondii* organisms added while naturally infected meat would contain the organisms within cysts within the muscle structure itself, which may reduce the recovery rate.

- An effective mouse bioassay was developed which enabled:
    - ❖ the production of known positive Toxoplasma material that was used to validate both the nested and qPCR.
    - ❖ the sensitivity of PCRs to be established by direct comparison. Serially diluted PCR positive brain material was compared to its ability to cause infection.
    - ❖ confirmation that digestion/centrifugation did not appear to inhibit *T. gondii* viability by demonstrating that spiked, PCR positive digested mince still caused infection.
    - ❖ the future verification of the infectivity of meat samples of concern found to be PCR positive (if required to facilitate further studies e.g. UCFM)
    - ❖ the capability to multiply toxoplasma strains of interest to obtain DNA for genotyping if needed in future work (e.g. to compare meat isolated strains with those from human infection).
- A sub-sample of stored sheep tongues from serologically positive animals was tested by both nested and qPCR and by direct sampling and following pepsin digestion/centrifugation. No *T. gondii* DNA was detected, presumably due to DNA degradation during the 6 year storage period.
- The Toxoplasma methods developed have been formally written up as five SARDI standard test methods and laboratory procedures, covering Toxoplasma DNA extraction (FS1.04), Toxoplasma isolation from muscle using digestion/centrifugation (FS14.01), Toxoplasma nested PCR (FS14.03), Toxoplasma qPCR (FS14.04) and Toxoplasma bioassay in mice (FS14.05). The SARDI procedures can be found in Appendix 6.

Project 2011/1017.401: Establishing the risk of Toxoplasmosis associated with the consumption of pork and pork products

Establish a reliable capacity for detection and molecular typing of Toxoplasma gondii in meat

1. Background

Toxoplasma gondii is a two-host meat borne protozoan parasite found in all warm-blooded animals that causes lifelong infection in humans. Its ability to cross the placental barrier and infect the foetus, as well as its ability to emerge from hibernation within muscle cysts during periods of immune suppression in the host (e.g. AIDS patients, patients treated with corticosteroids) has led to its status as a pathogen of increasing public health concern. In addition, there are indications that it is associated with schizophrenia and risk taking behaviour, resulting in increased risk of car accidents. Warm blooded animals (including humans) can get infected from the faeces of an infected cat (the primary host) or from consumption of undercooked infected meat (including pork). Overseas competitors are focusing on reducing the level of *Toxoplasma* in their pigs with some apparent success. In 1983-84 the US national seroprevalence in sows was 42% (Dubey et al, 1991). Subsequent national surveys have documented a decline in Toxoplasma seroprevalence in sows from 20% in 1990 to 15% in 1995 and to 6% in 2000 (Patton et al., 1996, 1998, 2002)

None-the-less, a recent US report, based on 2013 data from the U.S. Department of Agriculture's (USDA's) Economic Research Service, estimated the annual cost to the US of the 15 foodborne pathogens responsible for "more than 95% of the illnesses and deaths from foodborne illnesses in which the Centres for Disease Control and Prevention (CDC) can identify a pathogen cause". For Toxoplasmosis, the cost was US$3.3 billion, second only to nontyphoidal Salmonellosis at US$3.7 billion (Dan Flynn, Oct. 2014). In 2013 there were 86,686 cases of Toxoplasmosis in the US with 327 deaths. Only 16 cases were congenital.

In 1997/98, the Pig Research and Development Corporation (PRDC) funded pilot study (Hamilton *et al.,* 2000) found that approximately 1.3% of 310 finishers and up to 10% of 243 culled sows carried antibodies, a prevalence relatively low by international standards. A follow up survey (APL Project 2019) on 601 bloods from the APL 2000 blood bank , using the same serological test, indicated that the finisher prevalence was 4.6% (Hamilton *et al.,* 2006). This represented a significant increase ($p=0.016$), raising the spectre of a proportional increase in culled sows.

A subsequent 2008 serological survey of 419 culled sows in APL Project 2198 using three commercially available test kits, gave conflicting results, suggesting a prevalence between 6.9 and 22.9%. This inconsistency highlighted the need to investigate the genetic makeup of the *T. gondii* strains infecting Australian pigs to characterise infection and identify the most useful testing approach (Hamilton *et al.,* 2009).

In APL Project 2009/2306, matched blood and muscle samples (tongue, heart, skeletal and diaphragm) were collected from 138 culled sows from the five mainland states to compare serological and molecular techniques. None of the 473 sow tissue samples tested definitively positive by PCR for *T. gondii* DNA using the B1 gene. The B1 gene, although of unknown function, is widely exploited in a number of diagnostic and epidemiological studies because of its specificity and sensitivity. It was assumed that the poor DNA detection rate was due to the known relatively low concentration of Toxoplasma cysts in porcine muscle tissue.

Of the 136 sera tested by the three test kits only one sample tested positive by all serological tests. While the apparent serological prevalence estimate (between 0.74 and 7.36%) supports the findings from 2008, there remains considerable uncertainty about the sensitivity and specificity of the various serological tests when applied to the Australian pig herd.

The aim of this project was to increase the effectiveness of molecular detection of *T. gondii* DNA by investigating a method of muscle cyst concentration using pepsin digestion and centrifugation. The

project was established as a collaboration between the pork and red meat industry, jointly funded by APL and Meat and Livestock Australia (MLA).

Ultimate Industry Outcome

To have available in Australia a validated molecular test (with known limits of detection) for identifying *Toxoplasma gondii* infected meat, in order to provide greater certainty with regard to consumer risk and to better inform risk management decisions.

1) Aims

- Establish collaborations with local Australian researchers having appropriate expertise with *Toxoplasma gondii.*
- Establish a refined molecular test to improve the accuracy of detection and strain identification in Australian livestock.

  Technical elements of achieving this include;

  - Establish a PCR for detection of the Toxoplasma organism
  - Evaluate sensitivity of nested PCR against a commercial qPCR (that became available during the project)
  - Evaluate digestion/centrifugation to improve sensitivity of detection in meat
  - Develop a bioassay for the following capabilities/purposes;
    - ❖ Produce positive material to validate PCRs
    - ❖ Establish sensitivity of PCRs
    - ❖ Establish whether digestion/centrifugation inhibits *T. gondii* viability
    - ❖ Verify infectivity of PCR positive meat samples (to facilitate further studies e.g. uncooked fermented meat (UCFM) products)
    - ❖ Assess bioassay sensitivity for confirmation of *T. gondii* viability in meat following molecular detection
    - ❖ Enrich *T. gondii* samples to obtain sufficient DNA for future genotyping
- Compare human genotypes with those isolated from meat
  - ❖ Develop the potential capability for this to be done if required.
- Test previously collected sheep tongue samples by PCR to compare with serology

2. Methods

As this project was exploratory, it was divided into a number of logical, but flexible, sequential go/no go steps at which proposed modifications were discussed with the joint funders APL and MLA.

Experimental Research Plan Overview

- Identify an Australian based specialist collaborator with extensive experience with *Toxoplasma* molecular techniques and with a public health focus to provide expert advice on the research plan development and execution
- Obtain viable *Toxoplasma* material for detection methodology development and to validate tests and bioassay models

- Mix infective material with 50g minced pork
- Recover and concentrate the *Toxoplasma* organisms from the mince by pepsin digestion and centrifugation (Dubey 1998)
- Confirm the presence of *Toxoplasma* DNA in the recovered material by PCR
- Use mouse bioassay to confirm the viability of the recovered material and as the gold standard of detection for *T. gondii*
- Determine the limits of detection of the methodology

2.1: Establishment of Collaboration

The initial step was to identify an appropriate human specialist parasitologist willing and able to collaborate in this project. The specialist needed to have a public health focus and be able to refine existing molecular tests to be more effective in pigs, sheep and cattle.

The Australian Society for Parasitology was contacted to ascertain who in Australia has capability in this area. Ultimately, decisions about collaboration were based on identification of the most suitable collaborator.

Face to face discussions were held with Prof John Ellis from the School of Medical and Molecular Biosciences, University of Technology in Sydney (UTS), the researchers and MLA and a collaboration approach established. UTS agreed to provide expert critique of the experimental protocol, viable *Toxoplasma* organisms for the project, and to conduct parallel molecular testing when required.

As the project involved bioassay in mice, it was necessary to obtain approval from both the SARDI and SA Pathology Ethics Committees and the SA Pathology Biohazard Committee before proceeding. This proved to be a lengthy process, taking 3-4 months, which compromised the project timetable as it ran into the Christmas period.

Issues developed with this collaboration (UTS Ethics Committee issues, UTS personnel illness, logistic issues with supply of fresh infective material, UTS sabbatical leave) but fortunately the collaboration was able to be extended to Dr Milton McAllister, a new lecturer at the Roseworthy Veterinary School, who had conducted *Toxoplasma* research in the United States and was establishing his laboratory at Roseworthy. This collaboration proved a valuable addition to the project as he was able to supply fresh infective material on demand and was locally available for hands on assistance.

2.2: Molecular Methods

"… it is important to emphasize that molecular diagnostics, being a constantly improving modern methodology, is not standardized even among the world's leading laboratories. The differences are substantial and numerous, and they extend to all segments of the methodology such as target genes for parasite detection and markers for genotyping, equipment manufacturers and different protocols (various sets of primers and probes and their concentration, different internal controls, etc...)." (Ivovic et al 2012)

2.2.1 DNA extraction

Using the Wizard Genomic DNA Extraction Kit (Promega, Wisconsin USA) and following the manufacturer's instructions, two alternative DNA extraction protocols included with the kit were trialled in order to identify the approach that would provide the maximum DNA yield.

We compared the 2 approaches using both a saline suspension of brain tissue (modified Mouse Tail Protocol) and straight brain tissue (Mouse Liver & Brain Protocol).

Result

Although neither approach identified Toxoplasma DNA in the mouse samples tested, the detection of mouse housekeeping genes confirmed the effectiveness of both methods. Based on the PCR band strength it appeared that less DNA was recovered using the brain suspension, however the difference was marginal. Details of the methodology can be found in Appendix 1 (1. A & 1.B).

2.2.2 Nested PCR – establishment and validation

Conventional Polymerase Chain Reaction (PCR) involves the extraction & purification of the genome followed by amplification of a specific region of the DNA that is characteristic of the target (in this case *T. gondii*), the 35-fold-repetitive B1 locus. Although it is not known what function it performs, the B1 gene is widely used diagnostically due to its sensitivity and specificity for *T. gondii*.

The initial conventional PCR was based on the method published by Burg et al 1989 (Appendix 2 Study 1a). Subsequently, it was decided to trial a nested PCR as described by Grigg and Boothroyd 2001 (Appendix 2 Study 1b), reported to improve sensitivity (at this stage a *T. gondii* qPCR was not available in Australia). Details of both methods can be found in Appendix 2.

The nested PCR that was used requires 2 sequential PCR runs, with the final amplified DNA then being run on an agarose gel for detection. The quantity of DNA is estimated by comparison of the band intensity to a known control, but there is a degree of subjectivity in this.

The nested PCR was further refined by the introduction of DNA extraction controls. The original nested PCR method lacked controls to either verify DNA extraction and purification efficiency or to check for the presence of inhibitors in the sample, an important adjunct if Toxoplasma DNA is not detected. The controls involved a second PCR being conducted in parallel to the nested PCR on the same DNA to detect particular mouse housekeeping genes. Therefore, as part of the development, two murine house-keeping genes Cxxc1 (encoding CpG binding protein) and Mrpl48 (encoding mitochondrial ribosomal protein L48) were selected as useful potential control candidates as both are present and stable across mouse strains and tissues (Frericks and Esser 2008). The detection of either gene in extracts from mouse tissue negative for *T. gondii* is useful to ensure the result is due to absence of the toxoplasma DNA sequence, rather than the presence of inhibitors in the sample.

Results

Both genes were detected in the extracted DNA (Figure 1). It should be noted that estimating relative amounts of DNA is largely qualitative, relying on a judgement re relative band widths and intensity. (compare qPCR which became commercially available late in the study).

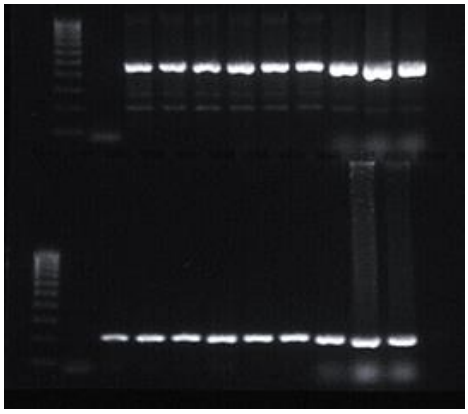

Figure 1. Gel from nested PCR showing housekeeping genes Cxxc1 bands (top lanes) & Mrpl48 bands (bottom lanes). (Lane1:100-1000bp marker; Lane 2: No template control; Lanes 3-8: Murine brain DNA; Lanes 9-11 Murine liver DNA)

2.2.3 Quantitative PCR (qPCR) - establishment and validation

Quantitative PCR (qPCR) or real time PCR enables detection & quantitation in the same procedure. This saves considerable time and reduces the potential for cross contamination.

During the project, a commercial real-time kit (TaqVet® kit) for *T. gondii* from animal tissue became available in Australia which was then trialled and compared with the nested PCR.

http://www.lifetechnologies.com/order/catalog/product/TXP50

The TaqVet® kit also detects the sequence encoding the B1 gene specific to the genus *Toxoplasma*. The kit includes the ready-to-use mastermix with one set of primers for the B1 gene and another primer set for detection of an internal positive control (IPC). The IPC is an endogenous gene present in organs and enables verification of the DNA extraction efficiency and the presence or absence of inhibitors in the same sample tube or well.

As a positive control with a known number of copies of the Toxoplasma B1 gene (present as a plasmid) was included with the kit, it was possible to produce a standard curve (Figure 2). As it is known that each *T. gondii* genome (organism) contains 35 copies of the B1 gene, an estimation of the number of Toxoplasma organisms present in a sample could be calculated. Details of the method of estimation can be found with an example of the calculation in Appendix 4.

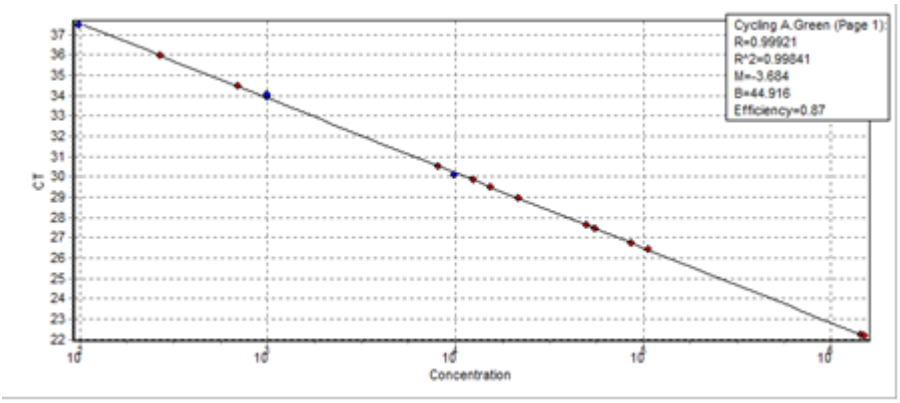

Figure 2. Standard curve with the plasmid positive control dilutions (blue) and samples (red)

2.2.4 Comparison of nested PCR & qPCR

A dilution series of both purified ME49 DNA and mouse brain infected with Dubey pig strain *T. gondii* were tested by both nested and qPCR, in order to compare their efficacy.

The Toxoplasma strain ME49 was prepared as serial dilutions from 10^{-2} to 10^{-6} in TE buffer. It was then tested by both nested and qPCR. The nested PCR results are shown in Figure 3. The ME49 DNA was detected to the 10^{-4} dilution in one of the 2 replicates.

The mouse brain was tested both undiluted and at 10^{-1}. *T. gondii* DNA and the Mouse Housekeeping gene (Cxxc1) were detected in both sets of replicates

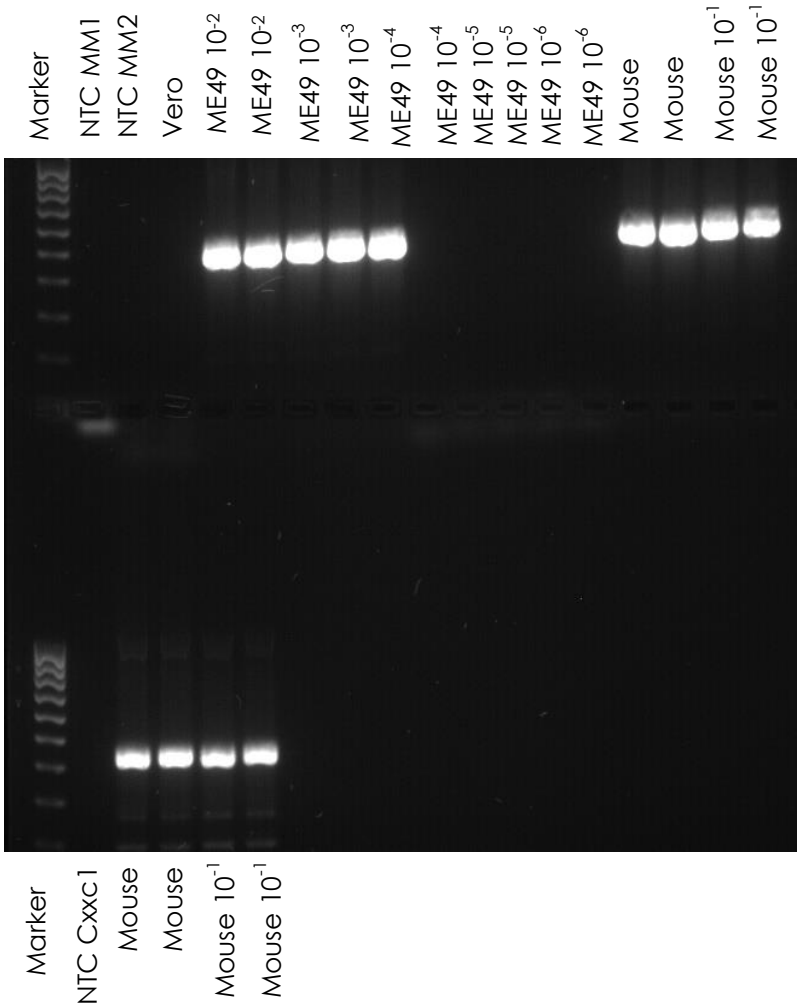

Figure 3. Gel of nested PCR products showing *T. gondii* B1gene (top lanes) & housekeeping gene Cxxc1 (bottom lanes) bands. (Note NTC Cxxc1, NTC MM1 & NTC MM2 are blank controls with no DNA added but contain PCR reagents. Vero is the negative control.).

The results for the qPCR are shown in Table 1. The ME49 DNA was detected to 10^{-5} (both replicates). The two mouse brain replicates tested positive for Toxoplasma DNA.

Results

In this study the qPCR was considerably quicker to perform (approximately 1/3 of the time), was quantitative and appeared to be more sensitive (able to detect *T. gondii* DNA at a 10 fold dilution).

Table 1. Data from qPCR Qiagen Rotorgene Q series TaqVet for B1 gene outlining the calculated concentration in copies/µL of the ME49 DNA and mouse brain (pig strain),

Sample.	Name	Type	Ct	Av Ct	Given Conc (copies/ul)	Calc Conc (copies/ul)	Av Conc
1	NC	Unknown					
2	mQ blank	Unknown					
3	EPC Toxo	Standard	30.1		9.90E+03	1.05E+04	1.05E+04
4	1/10	Standard	33.95	33.98	9.90E+02	9.48E+02	9.30E+02
5	"	Standard	34.01		9.90E+02	9.11E+02	
6	1/100	Standard		37.46	9.90E+01		1.05E+02
7	"	Standard	37.46		9.90E+01	1.05E+02	
8	1/1000	Standard			9.90E+00		
9	"	Standard			9.90E+00		
10	Vero DNA	Unknown					
11	ME49 10^{-2}	Unknown	22.15	22.19		1.51E+06	1.48E+06
12	"	Unknown	22.23			1.44E+06	
13	10^{-3}	Unknown	27.44	27.52		5.55E+04	5.29E+04
14	"	Unknown	27.59			5.03E+04	
15	10^{-4}	Unknown	30.51	30.17		8.12E+03	1.03E+04
16	"	Unknown	29.83			1.25E+04	
17	10^{-5}	Unknown	35.97	35.21		2.68E+02	4.84E+02
18	"	Unknown	34.44			6.99E+02	
19	10^{-6}	Unknown					
20	"	Unknown					
21	Mouse 5.05.14	Unknown	26.39	26.56		1.07E+05	9.67E+04
22	"	Unknown	26.73			8.64E+04	
23	10^{-1}	Unknown	28.94	29.21		2.17E+04	1.86E+04
24	"	Unknown	29.48			1.55E+04	

3. Study 1. Recovery of Toxoplasma gondii from spiked pork mince

Objectives:

To spike pork mince with *Toxoplasma* infected mouse brains and recover using the Dubey pepsin digestion/ centrifugation method. Confirm recovery by PCR and assess the sensitivity of the PCR and the impact of the process on *Toxoplasma* viability using mouse bioassay.

Methodology & Results

UTS infected 20 laboratory mice with tissue culture tachyzoites from one of 2 strains of *Toxoplasma gondii*: an international laboratory strain (ME49), and a strain isolated from an Australian canine (Dog strain). After 8 to 12 weeks (to allow time for the encysted bradyzoite stage to develop) the mice were euthanized and the carcases transported from NSW to SARDI in South Australia under refrigeration, where they were processed within 7 days of being euthanized. A number of the ME49 infected mice (8/10) either died or had to be euthanised prematurely by UTS for welfare considerations. This meant in total we were able to utilise 2x ME49 infected and 10x dog strain infected mice.

At SARDI the brains were removed from the 12 mice. One half of each brain was used to produce slide smears which were examined under the microscope for the presence of typical *Toxoplasma* tissue cysts. The 12 remaining brain halves were processed and examined using conventional (not nested) PCR (Burg et al, 1989) for the presence of the *T. gondii* DNA, in particular the B1 gene (considered definitive for the *Toxoplasma* organism). The detailed PCR methodology can be found in Appendix 2 Study 1a.

After examination, the remaining brain material from each animal was blended with 12 x 50g of pork mince that had previously been frozen to render non-viable any *T. gondii* that may have been present in the mince from previous natural infection. Each spiked mince sample was digested with pepsin, filtered, centrifuged and re-suspended in 5mL saline following the protocol of Dubey et al (details in Appendix 1 Section 3 & Appendix 2 Study 1a).

The re-suspended material from the 12 digestions was examined by nested PCR (Grigg & Boothroyd, 2001) and then bio-assayed in naïve Balb C mice to assess the impact of the digestion recovery process on the viability of the *T. gondii* bradyzoites. In detail an estimate was made of the approximate number of Toxoplasma bradyzoites in 1 mL of suspension by estimating the number of copies of the B1 gene present (Appendix 1). Three serial dilutions were made of each of the re-suspended solutions, resulting in 36 solutions containing an estimated 200, 20, or 2 bradyzoites per mL. One mL of each of the 36 re-suspended *Toxoplasma* solutions was injected subcutaneously into one of 36 mice, resulting in 6 mice potentially infected with ME49 and 30 mice with Dog strain. An additional 3 control mice were injected with 1 mL of a similarly prepared solution derived from mince to which no brain material/*Toxoplasma* had been added.

After 8 weeks (to allow tissue cyst establishment) 6/39 mice (3 from each *Toxoplasma* strain) were euthanised. The brains were examined by microscopy for typical cysts and by nested PCR for *T. gondii* DNA (Appendix 2 Study 1b). All samples were negative, suggesting the inoculated dose was either too small, non-viable, or else the mice were resistant to *Toxoplasma*.

Following discussions with Prof John Ellis the remaining 33 mice were treated with Dexamethasone (5ug/mL) in the drinking water for 1 week, in order to suppress their immune system and assist the re-emergence of any *T. gondii* present (Djurkovle & Milenkovle, 2001).

After a further 8 weeks (to allow tissue cyst establishment) the remaining 33 mice were euthanised and the brains examined as before.

Results

Despite a number of mice exhibiting non-specific symptoms post dexamethasone treatment (i.e. ruffled, hunched, weight loss), all proved negative for *Toxoplasma*.

Logistically, sourcing the infective material from UTS interstate meant that it was generally 6 to 7 days post mouse euthanasia before it could be processed. Although theoretically this should not have affected the viability of the encysted *Toxoplasma*, it was seen as a possible confounder. This led to a modification of the protocol in the repeat study below.

4. Study 2. Modified recovery of Toxoplasma gondii from spiked pork mince

Aim: Repeat Study 1 using fresher *Toxoplasma* spiking material and include a newly available qPCR to compare with the nested PCR.

Coincidentally, at this time we established contact with a new lecturer at the Roseworthy Veterinary School, Dr Milton McAllister, who had conducted *Toxoplasma* research in the United States and was establishing his laboratory at Roseworthy. Following further discussions with Professor Ellis and Dr McAllister, the following modifications were made in Study 2 to address Study 1 issues:

- Dr McAllister provided fresh viable tissue culture tachyzoites locally, to inject into local mice to produce tissue cysts. This provided fresher (processed with hours of euthanasia) brain material containing *Toxoplasma* bradyzoites to spike mince samples
- The *Toxoplasma* strain was one obtained originally from pigs (compare ME49 or Dog strain in Study 1), considered possibly more relevant to the Australian pig industry
- The breed of mouse was changed from Balb C to Swiss Webster, considered to be more susceptible to *Toxoplasma* infection, thereby obviating the need to treat with Dexamethasone to enhance infection establishment
- As a commercial quantitative PCR (qPCR) kit for *Toxoplasma gondii* had become available in Australia, it was decided to purchase a kit and compare it with the nested PCR previously used in Study 1

Methodology & Results

A summary flow diagram for Study 2 is shown in Figure 4 and more details in Appendix 1.

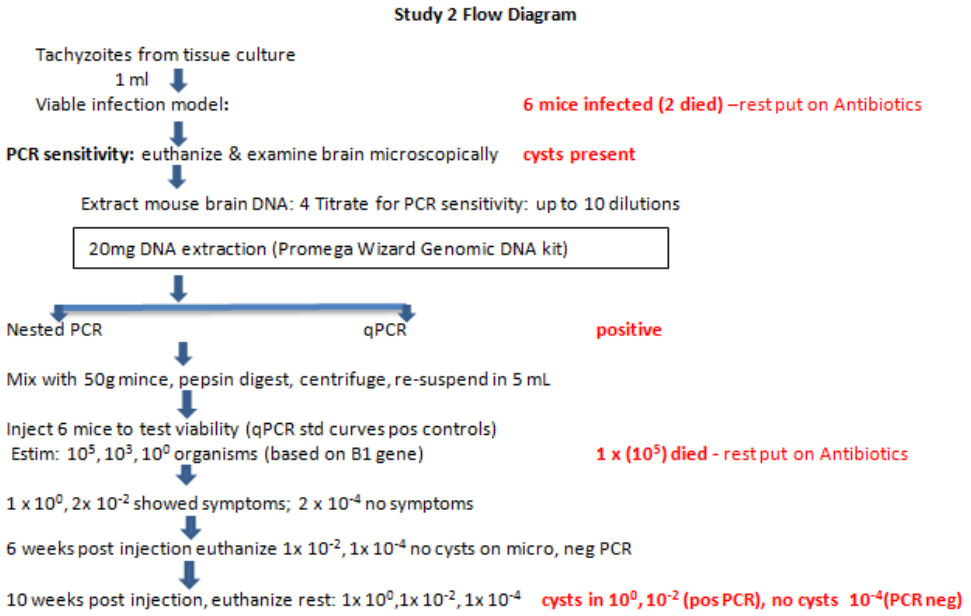

Figure 4. Summary flow diagram for Study 2.

Pig strain tachyzoites were grown in tissue culture (Vero cell line) and harvested by adding 5 mL sterile saline to the culture bottle, scraping off the tissue cells and agitating gently.

As the virulence in mice of this particular *Toxoplasma* strain was unknown, serial dilutions of the tachyzoite suspension (10^0, 10^{-1} and 10^{-2}) were immediately prepared and 1mL injected subcutaneously into 3 pairs of naïve Swiss Webster mice ($t10^0$, $t10^{-1}$ and $t10^{-2}$), respectively.

After 8 days there was a sudden overnight death in both a $t10^{-2}$ and a $t10^{-1}$ mouse, with the other mice exhibiting hunching and reluctance to move. Assuming this was caused by acute toxoplasmosis, all 4 surviving mice were immediately medicated with sulphadiazine (1mg/mL) in the drinking water for 1 week. Symptoms disappeared within a day and there were no more deaths.

After 12 weeks the single remaining $t10^{-2}$ mouse was euthanised and the brain examined by microscopy and both nested and qPCR. There were many tissue cysts visible in the brain smears.

The PCR was run on both undiluted (10^0) and tenfold diluted (10^{-1}) DNA extracted from the homogenised brain, to confirm the test was performing as expected and to provide a preliminary indication of the amount of *Toxoplasma* present in the mince spiking material (each *Toxoplasma* organism contains 35 copies of the B1 gene). The qPCR results are shown in Table 2. There was an estimated 2.63×10^5 *T. gondii* in 20mg of the brain.

Table 2. qPCR results for undiluted and tenfold diluted *Toxoplasma* tachyzoite infected mouse brain pre-spiking pork mince and for precipitate recovered from spiked mince post digestion

Sample		Ct reading	#B1 copies /uL DNA	Average #B1 copies/100µL DNA	Estimated *#T. gondii* organisms /20mg brain	*#T. gondii* in 300mg brain spiking 50g mince
20mg Mouse brain 10^0	Repeat 1	25.21	9.87×10^4	9.19×10^6	2.63×10^5	$^{+}3.94 \times 10^6$
	Repeat 2	25.45	8.51×10^4			

20mg Mouse brain 10^{-1}	Repeat 1	27.49	2.40×10^4	1.97×10^6	5.61×10^4	n/a
	Repeat 2	28.22	1.53×10^4			
			#B1 copies /uL DNA	Average #B1 copies/100uL DNA in 25µl re-suspension	Estimated *#T. gondii* organisms/25 µl re-suspension	*#T. gondii* in 1mL injected into 3 pairs mice*
Mince post digestion	Repeat 1	32.61	6.87×10^2	9.19×10^4	2.62×10^3	1.05×10^5
	Repeat 2	31.85	1.15×10^3			1.05×10^3
						1.05×10^1

+ only 10^0 dilution used to spike mince; * 3 dilutions; n/a = not applicable

The PCR/qPCR positive brain material was macerated and blended with 50 g pork mince which had previously been frozen to <-12°C for over 3 days to ensure the inactivation of any *T. gondii* already present from natural infection.

The mince was digested with pepsin, filtered, centrifuged and re-suspended in saline following the protocol of Dubey 1998. The re-suspended material was examined by nested PCR and qPCR and 3 concentrations were prepared (10^0, 10^{-2} and 10^{-4}) by serial dilution in sterile saline. The mince digest was positive for *T. gondii* by nested PCR and qPCR. One mL of each dilution was injected into one of 3 pairs of naïve Swiss Webster mice ($m10^0$, $m10^{-2}$ and $m10^{-4}$, respectively) to assess the impact of the recovery process on the viability of the *T. gondii* bradyzoites. The estimated number of *T. gondii* organisms injected per mouse was therefore either 1.05×10^5, 10^3 or 10^1 (Table 2). Details of estimation calculations can be found in Appendix 4.

After 12 days there was a sudden overnight death in one of the $m10^0$ mice and both its pair and one of the $m10^{-2}$ mice appeared ruffled and hunched. Neither of the 2 x $m10^{-4}$ mice exhibited symptoms. All mice were immediately medicated with sulphadiazine (1mg/ml) in the drinking water for 1 week. Symptoms disappeared within a day and there were no more deaths in the 5 surviving mice.

Eight weeks post inoculation, 1x $m10^{-2}$ and 1x $m10^{-4}$ mice were euthanised and the brains examined by microscopy and nested PCR. This was a preliminary examination to confirm the nonspecific mouse symptoms observed had been due to Toxoplasmosis (this was considered prudent before undertaking the considerable cost of purchasing additional qPCR kits). Cysts were obvious in the $m10^{-2}$ mouse brain but cysts were not observed in the $m10^{-4}$ mouse brain. Both brains tested negative by nested PCR. In an attempt to confirm that the PCR negatives were not simply due to a low cyst concentrations in the brain (a possible although unlikely scenario), the remaining brain material from the 2 mice was put through the acid digestion and centrifugation process to concentrate any cysts present. However this approach proved unsuccessful as both brains tested PCR negative and a significant inhibition was apparent most likely due to the high lipid content of the brain tissue. The conclusion is that acid digestion/centrifugation is inappropriate for use with brain material.

The 3 surviving mice, one each of $m10^0$, $m10^{-2}$ and $m10^{-4}$ dilutions (i.e. 10^5, 10^3 and 10^1 injected Toxoplasma organisms) were kept for another 4 weeks and then euthanised and the brains examined as previously. The delay was necessary for the supplier to import the required additional qPCR kits but had the advantage of allowing additional time for cyst development in the mice. On examination numerous cysts were observed in both the $m10^0$ and $m10^{-2}$ mice (the cysts in the $m10^{-2}$ mouse were smaller and fewer in number) but were not seen in the $m10^{-4}$ mouse. The DNA extracts from the brains were analysed by nested PCR and qPCR. The DNA extracts of $m10^0$ and $m10^{-2}$ brains were positive and the $m10^{-4}$ was negative by both methods. This is in line with findings by other researchers

that even the most sensitive molecular methods have a threshold of detection of 25 to 50 parasites /mL (Ivovic et al 2012). The qPCR results are shown in Table 3. The results indicate that the infective mouse dose for this post digestion *Toxoplasma* pig strain lay between 10^1 and 10^3 organisms.

Table 3. qPCR results for brains from mice inoculated with either undiluted, one hundred fold or ten thousand fold diluted pepsin digest, representing an estimated 10^5, 10^3 and 10^1 *T. gondii* organisms, respectively, per mouse.

		Ct reading	#B1 copies /uL DNA	Average #B1 copies/100µL DNA	Estimated #*T. gondii* genome copies /100uL DNA*	Clinical signs /brain cysts observed
Mouse brain m10^0	Repeat 1	32.84	7.19×10^2	7.93×10^4	2.3×10^3	Yes/Yes
	Repeat 2	32.44	8.67×10^2			
Mouse brain m10^{-2}	Repeat 1	31.39	1.42×10^3	1.41×10^5	4.03×10^3	Yes/Yes
	Repeat 2	31.42	1.40×10^3			
Mouse brain m10^{-4}	Repeat 1		nd			No/No
	Repeat 2		nd			

nd = not detected * Each *T. gondii* organism contains 35 copies of B1 gene

5. Study 3. Recovery rate of *T. gondii* from spiked pork mince

Aim:

To estimate the recovery rate of *T. gondii* from spiked pork mince using the pepsin digestion/ centrifugation method to establish the sensitivity of the qPCR.

Methodology

A surviving tachyzoite infected mouse from Study 2 (inoculated 8 months earlier) was euthanised and the brain examined by microscopy. Typical *Toxoplasma* cysts were observed.

Serial dilutions of the brain material were prepared using PBS, resulting in 4 dilutions: 10^{0}, 10^{-1}, 10^{-2} and 10^{-3} and examined by qPCR for *Toxoplasma* DNA. Five x 50g pork mince samples were prepared and spiked with one of the four brain dilutions, with a fifth control mince spiked only with PBS.

The five mince samples were then processed by pepsin digestion/centrifugation, to recover the *Toxoplasma* DNA. More details can be found in Appendix 4.

Results and Discussion

The qPCR of the mouse brain spiking dilutions and the mince digestion recovery material qPCR were compared. The results are shown in Table 4.

Toxoplasma DNA was detected in both the 10^{0} (undiluted) and 10^{-1} (tenfold diluted) tachyzoite infected mouse brain spiking material, containing an estimated 4.6×10^{4} and 1.22×10^{2} organisms, respectively. The 10^{0} brain estimate compares very favourably with the 1.1×10^{5} recovered from the corresponding spiked mince, indicating that the majority of the *T. gondii* were recovered.

There are, however, discrepancies between the results for the diluted brain material and their corresponding spiked mince samples (e.g. compare 1.22×10^{-2} organisms added with the 2.3×10^{4} organisms estimated recovered from the corresponding spiked mince sample for the tenfold dilution).

This discrepancy and the failure to detect *Toxoplasma* in the more diluted brain samples compared with the mince (Table 4), can be explained by the use of a manual homogeniser that is unlikely to have dispersed the organisms evenly through the material, compounded by the very small sample of suspended brain material (25uL) that is examined by PCR. Compare this to the mechanical agitation followed by centrifugation and re-suspension process applied to the spiked mince. In addition, there was not enough of the qPCR kit left to do repeat samples on the brain material (this "additional" study was done partly so as not to waste what remained of the test kit). The qPCR kit includes an internal positive control that verifies the efficacy of the DNA extraction and the absence of inhibitors. In this Study 3 there appeared to be excess DNA template and/or inhibition in the DNA extracts that may also have contributed to the inconsistent values. One possible future option is to dilute the DNA extracts to mitigate this inhibition, thereby producing more definitive values.

Table 4. Comparison of estimates of the number of *Toxoplasma* organisms used to spike pork mince with the estimated number of organisms recovered by the pepsin digestion/ centrifugation method.

Sample		Ct reading	#B1 copies /uL DNA from brain in 1mL	#B1 copies/100µL DNA from brain	Estimated *#T. gondii* organisms /100ul DNA	*#T. gondii* in 1mL homogenised brain added to 50g mince
Mouse brain 10^{0}		30.11	4.00×10^{2}	4.00×10^{4}	1.15×10^{3}	4.6×10^{4}
Mouse brain 10^{-1}		40.19	1.07×10^{0}	1.07×10^{2}	3.06×10^{0}	1.22×10^{2}
Mouse brain 10^{-2}		0	nd			nd
Mouse brain 10^{-3}		0	nd			nd
			#B1 copies /uL DNA	Average #B1 copies/100uL DNA in 25µl re-suspension	Estimated *#T. gondii* / 25 µl re-suspension	*#T. gondii* recovered from mince
Mince control	No repeat	nd				nd
10^{0} Mince post digestion	Repeat 1	32.15	1.21×10^{2}	1.21×10^{4}	3.46×10^{2}	1.1×10^{5}
	Repeat 2	nd	nd			
10^{-1} Mince post digestion	Repeat 1	34.48	3.07×10^{1}	3.06×10^{3}	8.74×10^{1}	2.3×10^{4}
	Repeat 2	34.49	3.05×10^{1}			
10^{-2} Mince post digestion	Repeat 1	36.43	9.77×10^{0}	4.98×10^{2}	1.42×10^{1}	4.3×10^{3}
	Repeat 2	43.01	2.05×10^{-1}			
10^{-3} Mince post digestion	Repeat 1	nd	nd			nd
	Repeat 2	nd	nd			

nd = not detected

6. Study 4. Pilot testing of stored sheep tongues using pepsin digestion and qPCR to assess prevalence

Aim: To determine the *T. gondii* prevalence in a sub-sample of 648 sheep tongues using the molecular approach refined by this collaborative APL/MLA funded project.

In 2008, sheep tongues were collected by SARDI at abattoirs throughout Australia as part of an MLA project. The intention was to examine this tissue at a later stage when both funding and an appropriate molecular approach were determined.

Following validation and refinement of the molecular approach in this collaborative project, a sub-sample of 24 tongues from serologically positive sheep were tested by both the nested and qPCR. For comparison, testing was conducted both on a traditional 25mg direct tongue sample and a larger tongue material processed by pepsin digestion/centrifugation.

Results

None of the samples were conclusively positive for *T. gondii* DNA by any of the molecular techniques utilised. Given the success of the techniques in the other studies in this project on fresher material, it was surmised that the 6 year storage of the tongues, under at times less than ideal conditions (i.e. insufficient minus 80°C storage available), had resulted in degradation of the *T. gondii* DNA. Details of Study 4 can be found in Appendix 5.

The conclusion was that further testing of the sheep tongues in storage would be a waste of limited resources and fresh material would need to be collected to ensure reliable results.

7. Key outcomes

- Collaborations were established with not one but two (2) Australian based Toxoplasma experts- Professor John Ellis (University of Technology Sydney) and Dr Milton McAllister (University of Adelaide, School of Animal and Veterinary Sciences). The collaboration with Dr McAllister provided a valuable additional resource with the advantage of local accessibility. Dr McAllister's laboratory is developing its own Toxoplasma qPCR which could prove useful for further work.
- As outlined in Section 2.2 Molecular Methods, a refined molecular test was established to detect Toxoplasma in meat.
    - ❖ DNA extraction protocols were compared and demonstrated to be effective in providing sufficient *T. gondii* DNA for testing by PCR (section 2.2.1). The extracted DNA can also be used for future genotyping by a specialised multiplex nested PCR if required.
    - ❖ A nested PCR to detect infection was established and validated and a mouse housekeeping gene was incorporated to act as a positive control (section 2.2.2). This provided confirmation that the PCR was working appropriately and confidence that a negative result was due to an absence of Toxoplasma DNA.
    - ❖ It was demonstrated that both the nested PCR and the qPCR were effective in detecting Toxoplasma DNA (section 2.2.4).
    - ❖ The qPCR was compared to the nested PCR (section 2.2.4) and although more expensive, was found to be more sensitive (10 x), less time consuming (approx. 1/3rd

the time) and was able to more accurately quantify the amount of DNA present (and by extension the number of Toxoplasma organisms- Appendix 3).

- The Dubey digestion/centrifugation technique was validated in Study 2 (section 2.4) to improve sensitivity and the recovery rate investigated in Study 3 (section 2.5). It was further demonstrated in Study 4 (section 2.6) that there was less inhibition seen with the qPCR of the digested product than with the qPCR of the straight sample. It must be remembered, however, that the spiked mince simply had *T. gondii* organisms added while naturally infected meat would contain the organisms within cysts within the muscle structure itself which may reduce the recovery rate.
- An effective mouse bioassay was developed which enabled:
  - ❖ the production of known positive Toxoplasma material that was used to validate both the nested and qPCR (Study 2-section 2.4).
  - ❖ the sensitivity of PCRs to be established by direct comparison. Serially diluted PCR positive brain material was compared to its ability to cause infection (Study 2-section 2.4 Table 3)
  - ❖ confirmation that digestion/centrifugation did not appear to inhibit *T. gondii* viability by demonstrating that spiked, PCR positive digested mince still caused infection (Study 2-section 2.4)
  - ❖ the future verification of the infectivity of meat samples of concern found to be PCR positive (if required to facilitate further studies e.g. UCFM)
  - ❖ the capability to multiply toxoplasma strains of interest to obtain DNA for genotyping if needed in future work (e.g. to compare meat isolated strains with those from human infection).
- A sub-sample of stored sheep tongues from serologically positive animals was tested by both nested and qPCR and by direct sampling and following pepsin digestion/centrifugation (Study 4-section 2.6). No *T. gondii* DNA was detected, presumably due to DNA degradation during the 6 year storage period.
- The Toxoplasma methods developed have been formally written up as five SARDI standard test methods and laboratory procedures, covering Toxoplasma DNA extraction (FS1.04), Toxoplasma isolation from muscle using digestion/centrifugation (FS14.01), Toxoplasma nested PCR (FS14.03), Toxoplasma qPCR (FS14.04) and Toxoplasma bioassay in mice (FS14.05). The SARDI procedures can be found in Appendix 6.

8. Issues/Recommendations

Issue

- This project established and validated the of pepsin digestion/centrifugation process used in combination with a qPCR for use in Toxoplasma prevalence studies in livestock at slaughter. It also established the DNA extraction protocol that can be used to provide DNA for genotyping.

Recommendation

- That the qPCR be used to conduct a baseline survey of *T. gondii* in sow hearts collected at slaughter, to compare with previous serological results
- That extracted *T. gondii* DNA be stored for future genotyping

Issue

- *T. gondii* cysts do not occur in high concentrations in pig meat but, none-the-less, represent a significant zoonosis. This project demonstrated that the Dubey pepsin digestion/centrifugation method was effective in concentrating and recovering *T. gondii* diffused throughout a meat sample that could then tested by PCR to confirm infection and/or bioassay to confirm infectivity.

Recommendation

- That the pepsin digestion/centrifugation process be utilised in in future meat surveys to maximise the probability of detection.

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Appendix I

Detailed Methods for DNA extraction, Acid Digestion, PCR and Mouse bioassay

1. DNA extraction

DNA is extracted from brain and tissue using the Promega Wizard® Genomic DNA purification kit. The kit was supplied with two alternative protocols (i.e. Mouse Tail Protocol; Mouse Liver & Brain Protocol), the main difference being (as the names imply) the type of tissue you wish to test.

We compared these two protocols on both mouse brain and sheep tongues, to identify the most effective for our purposes.

A. Mouse Tail Protocol (using brain "suspension" from injected mouse)

1. Mouse brain (1/2) is macerated gently in 500µL saline.
2. Add 17.5µL 20 mg/mL proteinase K to 600µL chilled Nuclei lysis solution.
3. Add 25µL of the macerated brain.
4. Incubate overnight @ 55°C with gentle shaking.
5. Add 200µL Protein precipitation solution at room temperature and vortex for 20 seconds.
6. Centrifuge for 4 min @ 16000 × g.
7. Carefully remove the supernatant into an eppendorf tube containing 600µL of room temperature iso-propanol.
8. Invert the tube gently until white threads are visible (not usually observed with these samples).
9. Centrifuge for 60sec @ 16000 × g.
10. Carefully discard the supernatant and add 600µL of room temperature 70% ethanol.
11. Invert several time then discard the ethanol.
12. Invert the tube onto absorbent paper then air dry.
13. Add 100µL DNA Rehydration solution.
14. Incubate @ 65°C for 60min or overnight @ 4°C.
15. Store the DNA @ -20°C for analysis.

B. Mouse Liver & Brain Protocol (using brain from injected mouse)

1. Mouse brain 20mg is gently homogenised in 600µL chilled Nuclei lysis solution.
2. Incubate @ 65°C for 30min.
3. Add 17.5µL of 20 mg/mL proteinase K.
4. Incubate overnight @ 55°C with gentle shaking.
5. Continue as above section A. 5.

C. Mouse Tail Protocol (using pepsin digested sheep tongue)

1. Add 17.5µL of 20 mg/mL proteinase K to 600µL chilled Nuclei lysis solution.
2. Add 25µL of the acid pepsin digested tongue.
3. Incubate overnight @ 55°C with gentle shaking.

4. Continue as above section A. 5.

D. Mouse Tail Protocol (using sheep tongue)

1. Sheep tongue 20mg (from 3 regions of the tongue) is gently homogenised in 600µL chilled Nuclei lysis solution.
2. Incubate @ 65°C for 30min.
3. Add17.5µL of 20 mg/mL proteinase K.
4. Incubate overnight @ 55°C with gentle shaking.
5. Continue as above section A. 5.

2. PCR sensitivity

The DNA extracted from tissue is analysed by polymerase chain reaction (PCR) to detect the presence of *Toxoplasma gondii*. Note that PCR does not indicate infectivity. A positive PCR result indicates the presence of DNA from the parasite only. There may be no viable organisms present. Both methods enable the detection of the genome sequence encoding the B1 gene specific to the genus *Toxoplasma.*

Two protocols have been compared:

A. Nested PCR as outlined in Grigg & Boothroyd (2001):

1. The reaction mix for each primer set consists of 2 × MyTaq™ Red (Bioline BIO-2504), 10 pmol of each forward and reverse primer and 1 µL of DNA template made up to a total volume of 25 µL.
2. The cycling conditions are 35 cycles of [94°C for 30 sec, 60°C for 30 sec, 72°C for 30 sec] followed by extension @ 72°C for 10 min.
3. The product from the first PCR reaction is diluted 1/10 in TE buffer and used as the template for reaction 2.
4. The final product is run on 1.5% agarose gel with gel-red @ 100V for 75 min. The positive control is ME49 purified DNA and the negative is Vero DNA.

	Primer name	**Sequence**	**Reference**
Oligo primer set 1	Forward (F_{ext})	5'-TGT TCT GTC CTA TCG CAA CG-3'	Grigg & Boothroyd 2001
	Reverse (R_{ext})	5'-ACG GAT GCA GTT CCT TTC TG-3'	
Oligo primer set 2	Forward (F_{int})	5'-TCT TCC CAG ACG TGG ATT TC-3'	Grigg & Boothroyd 2001
	Reverse (R_{int})	5'-CTC GAC AAT ACG CTG CTT GA-3'	

B. Real time PCR using TaqMan® probes - TaqVet™ *Toxoplasma gondii* kit TXP manufactured by Laboratoire Service International (LSI), Lissieu, France.

The real time PCR protocol is as recommended by the manufacturer:

1. The reaction mix consists of 20µL <<Mix Toxo>> mastermix and 5µL of DNA template.
2. The cycling conditions are one cycle of 50°C for 2 min, one cycle of 95°C for 10 min and 45 cycles of [95°C for 15 sec, 60°C for 1 min].
3. The real time PCR is carried out in the Qiagen Rotorgene.

C. Murine house keeping gene PCR for CpG binding protein & mitochondrial ribosomal protein L48:

1. The reaction mix for each gene consists of 2 × MyTaq™ Red (Bioline BIO-2504), 10 pmol of each forward and reverse primer and 2 µL of DNA template made up to a total volume of 25 µL.
2. The cycling conditions are one cycle of 94°C for 15 min, 45 cycles of [94°C for 20 sec, 55°C for 15 sec, 72°C for 20 sec] followed by extension @ 72°C for 3 min.
3. The final product is run on 1.5% agarose gel with gel-red @ 100V for 90 min.

	Primer name	**Sequence**	**Reference**
Oligo primer set Cxxc1	Cxxc1-f	5'-CAG ACG TCT TTT GGG TCC A-3'	Frericks & Esser 2008
	Cxxc1-r	5'-AGA CCT CAT CAG CTG GCA C-3'	
Oligo primer set Mrpl48	Mrpl48-f	5'-AGC CAT TAA TGT GGG GAC A-3'	Frericks & Esser 2008
	Mrpl48-r	5'-AGG ACC ATT TTG TTG CCT T-3'	

3. Acid pepsin digestion method

This protocol is based on the method published by Dubey (1997).

The Dubey method for acid pepsin digestion to isolate *Toxoplasma gondii* from infected tissue (sheep tongue)

1. Weigh the tongue (gm).
2. Chop finely with scalpels.
3. Set aside 3 small portions for direct DNA extraction using the Promega Wizard® Genomic DNA purification kit.
4. Homogenise the remainder with saline (1:50 w/v in total) using the Waring blender**.**
5. Transfer the homogenate to a stomacher bag with filter.
6. Warm to 37°C.
7. Add an equal volume of warmed acid/pepsin solution (pepsin 2.6 gm, NaCl 5.0 gm, HCl 7.0 mL, deionised water to 500 mL: pH 1.10 – 1.20)
8. Incubate @ 37°C for 60 min, rotating @ 100 rpm.
9. Transfer the filtrate to 500 mL centrifuge tubes.

10. Centrifuge @ 1200 × g for10 min.

11. Discard the supernatant into a container with disinfectant (Virkon).

12. Resuspend the sediment in 20 mL PBS and transfer to a 50 mL centrifuge tube.

13. Add few drops of phenol red & neutralise with 1.2% Na bicarbonate (colour change from yellow → orange).

14. Centrifuge @1200 × g for 10 min.

15. Resuspend in 5 ml saline (with 1000 U of penicillin & 100 µg streptomycin per mL if to be inoculated into mice).

16. Store @ 4°C for DNA extraction and/or inoculation into mice for infectivity studies.

4. Mouse bioassay

Mice were injected subcutaneously with either 1 mL of

- tachyzoite cell culture material (to produce infective brain material spike mince)
- re-suspended material recovered from spiked mince digestion (to determine if bradyzoites remained viable during the Dubey process)

Mice were injected under fluorothane gaseous anaesthesia for ease of handling and to minimise the discomfort of injecting a relatively large volume of material.

Initially BalbC mice were used and treated with Dexamethasone (5 ug/mL) in the drinking water for 1 week to inhibit their immune system to facilitate the infection process. The mouse type was later changed to Swiss Webster, a strain reported to be more susceptible to *Toxoplasma gondii*.

During the acute infection stage, mice showing strong symptoms were treated with sulphadiazine (1mg/mL) in the drinking water for 1 week.

Mice were euthanised under fluorothane anaesthesia by exsanguination from the heart with a 3mL syringe.

The head was removed using surgical scissors between the skull and the occipital process. The skin was peeled forward towards the nose leaving enough skin attached to hold the head firmly. Artery forceps were then inserted into the foramen magnum and the top of the skull broken away to expose the brain which was then excised into a petri dish and used for microscopy, PCR and mince spiking.

Appendix 2

2.3: Study 1. Recovery of Toxoplasma gondii from spiked pork mince detailed methodology

Aim: Spike pork mince with *Toxoplasma* infected mouse brains and recover using the Dubey pepsin digestion/ centrifugation method. Confirm recovery by PCR and assess the sensitivity of the PCR and the impact of the process on *Toxoplasma* viability using mouse bioassay.

Toxoplasma gondii strains ME49 and Dog were grown by mouse bioassay by University of Technology Sydney (UTS). Infected mouse heads were then provided to SARDI Food Safety & Innovation, Adelaide South Australia. The brains were removed and a portion of the cerebrum (enough to fill a 22 mm^2 coverslip) was crushed between a glass slide and a cover slip. The smear was then examined for cysts at 100× or 400× magnification. Half of each brain was then placed into a sterile eppendorf tube and sterile physiological saline solution added to 500 µL. The mixture was then homogenized using a sterile pipette tip and a 50 µL aliquot placed in a fresh sterile eppendorf tube.

Study 1a Pepsin digestion of spiked mince with PCR as per Burg et al 1989)

The total DNA was extracted from the 50µL aliquot using a Wizard Genomic DNA Extraction Kit (Promega, Wisconsin USA) as per manufacturer's instructions. The DNA pellet was then resuspended in TE Buffer (50 µL), 1 µL of which was used as template DNA for a PCR as per Burg *et al*. (1989). Serial decimal dilutions of known concentration of ME49 DNA were used as a comparison. Briefly, each reaction mix consisted of 1 x Immomix Red, forward and reverse primer (100 µM each, forward primer 5'-GGA ACT GCA TCC GTT CAT GAG-3', reverse primer 5'-TCT TTA AAG CGT TCG TGG TC-3'), made up to 25µL including 1 µL DNA template. Cycle conditions were 94°C for 3 min followed by 40 cycles of 94°C for 30 s, 59°C for 30 s, 72°C for 30 s with final extension step of 72°C for 10 min (less cycles may be used for brain tissue samples with a high concentration of cysts as determined by light microscopy). Reactions were run using a Maxygene Gradient Thermal Cycler (Axygen, Union City California). All reactions were visualized by gel electrophoresis. Electrophoretic separation of DNA fragments was carried out at room temperature using 1% (w/v) agarose gels prepared in 1 × Tris-acetate-EDTA (TAE) buffer (50 × TAE Buffer: 242 g Tris base, 57.1 mL Acetic acid, 100 mL 0.5M EDTA, ddH2O to 1 litre and adjusted pH to 8.5) containing GelRed DNA stain (1x) (Biotium, Hayward California). A Hyperladder IV DNA ladder (Bioline, London England) was used as molecular weight markers for DNA fragments separated in agarose gels. Gels were run at 100 V in 1 × TAE buffer. Gels were visualized by UV transillumination and photographed using an E-Box VX2 (Vilber Lourmat, France). Concentration of *T. gondii* in the brain sample was then determined by comparing band brightness from the sample to that obtained from serial decimal dilutions of ME49 *T. gondii* DNA of known concentration.

Homogenised mouse brain tissue was then diluted to a final concentration of ~10,000 bradyzoites per µL. ~1, ~10, ~100 and ~1,000 bradyzoites were then added to 50 g of pork mince that had been prepared from a single pork loin that had been frozen for 3 days to kill any existing *T. gondii* bradyzoites. The inoculated mince samples were then digested as per Dubey *et al*., 1998 with minor alterations. Briefly, connective tissue, fat, epithelium (eg. from tongue) was trimmed from muscle tissues using a disposable razor. The muscle tissue was then ground in a hand turned meat grinder. The ground meat (50 g) was then placed into a sterile stomacher bag and physiological saline (250 mL) added and the suspension stomached for 1 min. The homogenate was warmed to 37°C and fresh, acid pepsin solution [pepsin (2.6 g), NaCl (5 g), HCl (7 mL), distilled water to 500 mL, pH 1.1 – 1.2] (250 mL) pre-warmed to 37°C added. The mixture was then incubated at 37°C for 1 hr with constant shaking. The homogenate was then filtered through two layers of gauze and centrifuged at 1,200 × g for 10 min. The supernatant was aspirated, phosphate buffered saline (PBS) (20 mL) added and the pellet resuspended. The homogenate was neutralised with sodium bicarbonate (1.2 % w/v. pH 8.3) with neutral red used as a pH indicator. The homogenate was centrifuged at 1,200 × g and the pellet resuspended in saline (5 mL) containing Penicillin (1,000 U/mL) and Streptomycin (1,000 µg/mL).

Study 1b Bio-assay and nested PCR (Grigg & Boothroyd 2001)

Total DNA was extracted from 1 mL of tissue homogenate using the Wizard Genomic DNA Extraction Kit (Promega, Wisconsin USA) as per manufacturer's instructions. The DNA pellet was

then resuspended in TE Buffer (50 µL), 1 µL of which was used as template DNA for PCR as per Grigg & Boothroyd (2001) to determine the presence or absence of *T. gondii* DNA in the sample. Briefly, a nested PCR was conducted with 2 primer sets (Oligonucleotide Primer set 1: Forward (F_{ext}): 5'-TGT TCT GTC CTA TCG CAA CG-3', Reverse (R_{ext}): 5'-ACG GAT GCA GTT CCT TTC TG-3'; Oligonucleotide Primer set 2: Forward (F_{int}): 5'-TCT TCC CAG ACG TGG ATT-3' TC, Reverse (R_{int}): 5'-CTC GAC AAT ACG CTG CTT GA-3'). The reaction mix for each step consisted of 1 × PCR Buffer, 0.1 mM each dNTP, 10 pmol forward and reverse oligonucleotide primer, 1.5 U *Taq* DNA polymerase with final reaction volume of 25 µL. The supernatant from the first reaction was diluted 1:10 in 1 × TE buffer and 1 µL used as a template for the second reaction. The reaction cycle for each reaction consisted of 35 cycles of 94°C for 30 s, 60°C for 30 s, 72°C for 30s with a final extension step of 72°C for 10 min.

Tissue homogenate (1 mL) was also subcutaneously injected into a single BALB/c mouse using a 4 cm long 23 gauge needle. Homogenate from each meat sample with no *T. gondii* bradyzoites added was injected into three mice as a control. Mice were observed for clinical symptoms for up to 2 months. Mice were then sacrificed and the brains harvested. An initial screen for the presence or absence of cysts was conducted as above followed by DNA extraction as previously described. The prepared DNA was then used as template for PCR as per Grigg & Boothroyd (2001) as previously described.

References

Burg, J., Grover, C., Pouletty, P., Boothroyd J. (1989). Direct and Sensitive Detection of a Pathogenic Protozoan, *Toxoplasma gondii*, by Polymerase Chain Reaction. Journal of Clinical Microbiology **27 (8):** 1787 - 1792

Dubey, J. (1998). Refinement of Pepsin Digestion Method for Isolation of *Toxoplasma gondii* from Infected Tissues. Veterinary Parasitology **74**: 75-77

Grigg, M., Boothroyd, J. (2001). Rapid Identification of Virulent Type I Strains of the Protozoan Pathogen *Toxoplasma gondii* by PCR-Restriction Fragment Length Polymorphism Analysis of the *B1* Gene. Journal of Clinical Microbiology **39 (1):** 398-400

Appendix 3

Use of the qPCR to estimate of the number of *T. gondii* organisms recovered from spiked mince and injected into 3 pairs of mice in Study 2.

Toxoplasma gondii infection & viability

1. *T. gondii* infected mouse brain gently homogenised in 1 mL PBS.
2. The brain was mixed through 50 gm pork mince.
3. *T. gondii* re-isolated from the mince using the Dubey (1998) acid-pepsin digestion method & re-suspended in 5 mL sterile saline + Pen/strep.
4. An aliquot was taken for DNA extraction using the Promega Wizard® Genomic Purification kit.
5. 25 µL of digest → 100 µL DNA rehydration solution.
6. The DNA was analysed for *T. gondii* by nested PCR & qPCR.
7. The digest was +ve for *T. gondii* DNA using both methods.

Estimated Quantification:

The TaqVet™ kit also detects the sequence encoding the B1 gene specific to the genus *Toxoplasma.* The kit includes the ready-to-use mastermix with one set of primers for the B1 gene and another primer set for detection of an internal positive control (IPC). The IPC is an endogenous gene present in organs and enables verification of the DNA extraction efficiency and the presence or absence of inhibitors in the same sample tube or well. If the Ct for a sample is negative (Ct>45) inhibition of the PCR reaction is likely. The test should be repeated with a 1/10 dilution of the DNA. The inhibitors should be reduced in concentration producing a valid Ct for the IPC (Ct<45) however if not the DNA extraction should be repeated on a diluted sample.

The qPCR quantification is significantly more accurate than the nested PCR as a positive control plasmid encoding one copy of the B1 gene is included in the kit and enables construction of a standard curve for sample quantification. Fig 1 shows the amplification curves of the IPC dilution series, ME49 dilution series and DNA extracted from the brain of one mouse exhibiting cysts after infection with the Dubey pig strain and Figure 2 the constructed Standard Curve.

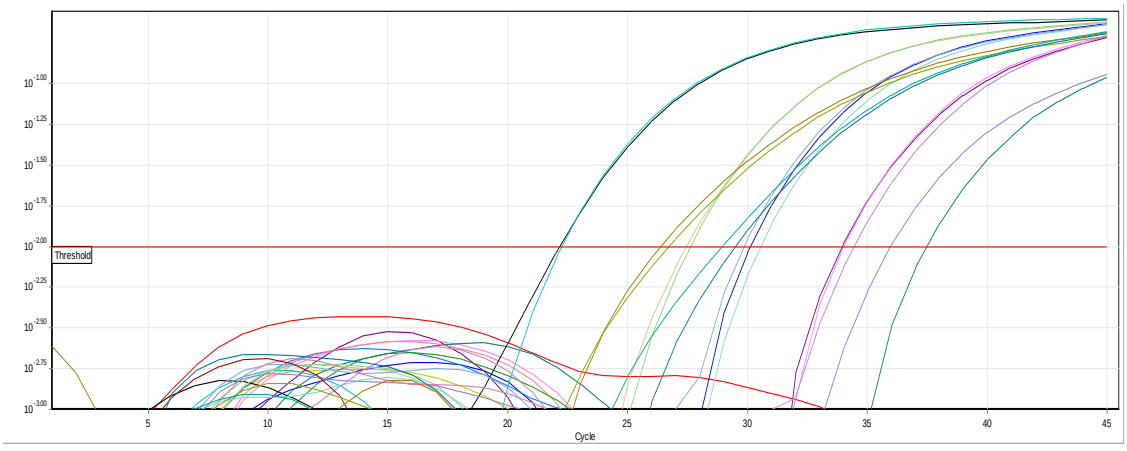

Fig 1. Real-time qPCR TaqVet *Toxoplasma gondii*

Appendix 3

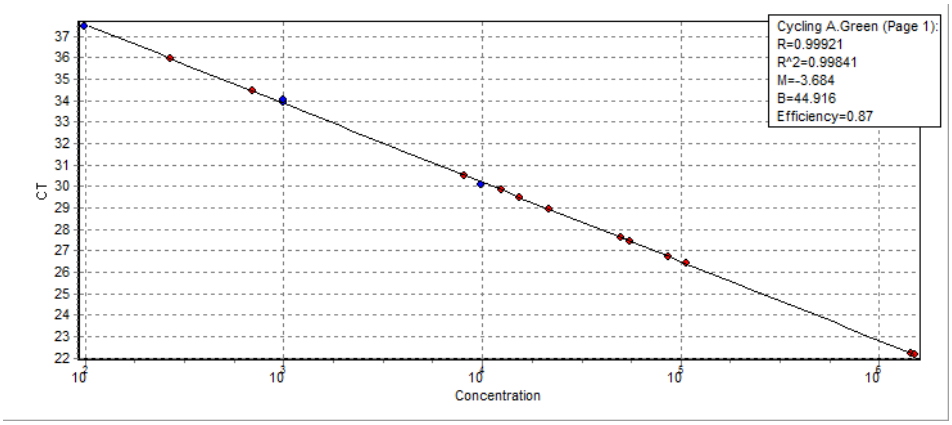

Fig 2. Standard curve with the plasmid positive control dilutions (blue) and samples (red)

Table 1. Data from qPCR Qiagen Rotorgene Q series TaqVet for B1 gene outlining the calculated concentration in copies/µL of the ME49 DNA and mouse brain (pig strain),

No.	Name	Type	Ct	Av Ct	Given Conc (copies/ul)	Calc Conc (copies/ul)	Av Conc
1	NC	Unknown					
2	mQ blank	Unknown					
3	EPC Toxo	Standard	30.1		9.90E+03	1.05E+04	1.05E+04
4	1/10	Standard	33.95	33.98	9.90E+02	9.48E+02	9.30E+02
5	"	Standard	34.01		9.90E+02	9.11E+02	
6	1/100	Standard		37.46	9.90E+01		1.05E+02
7	"	Standard	37.46		9.90E+01	1.05E+02	
8	1/1000	Standard			9.90E+00		
9	"	Standard			9.90E+00		
10	Vero DNA	Unknown					
11	ME49 10^{-2}	Unknown	22.15	22.19		1.51E+06	1.48E+06
12	"	Unknown	22.23			1.44E+06	
13	10^{-3}	Unknown	27.44	27.52		5.55E+04	5.29E+04
14	"	Unknown	27.59			5.03E+04	
15	10^{-4}	Unknown	30.51	30.17		8.12E+03	1.03E+04
16	"	Unknown	29.83			1.25E+04	
17	10^{-5}	Unknown	35.97	35.21		2.68E+02	4.84E+02
18	"	Unknown	34.44			6.99E+02	
19	10^{-6}	Unknown					
20	"	Unknown					
21	Mouse 5.05.14	Unknown	26.39	26.56		1.07E+05	9.67E+04

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22	"	Unknown	26.73			8.64E+04	
23	10^{-1}	Unknown	28.94	29.21		2.17E+04	1.86E+04
24	"	Unknown	29.48			1.55E+04	

Appendix 3

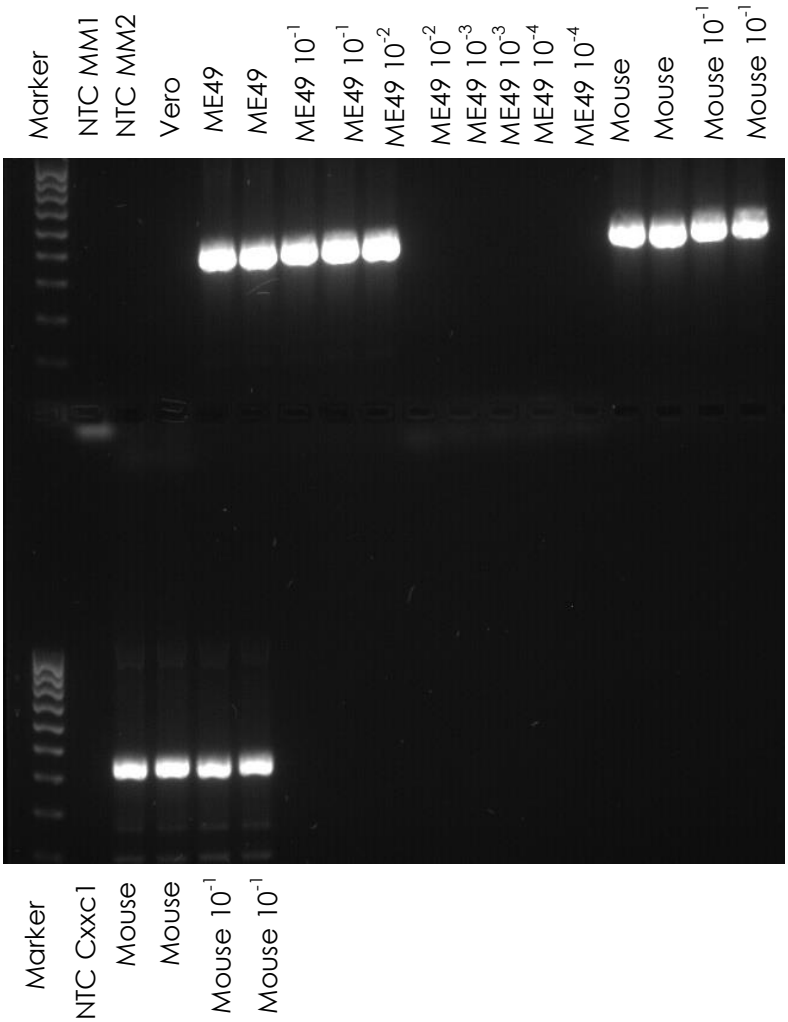

Fig 3. Gel of nested PCR products showing B1gene (top lanes) & Cxxc1 (bottom lanes) bands

Top; Lane1 100-1000 bp marker, Lane 2 No template control, Lane 3 Vero –ve control, Lanes 5&6 ME49 10^{-2}, Lanes 7&8 ME49 10^{-3}, Lanes 9&10 ME49 10^{-4}, Lanes 10&12 ME49 10^{-5}, Lanes 13&14 ME49 10^{-6}, Lanes 15&16 Murine brain undiluted, Lanes 17&18 Murine brain 10^{-1}

Bottom; Lane 1 100-1000 bp marker, Lane 2 No template control, Lanes 3&4 Murine brain undiluted, Lanes 5&6 Murine brain 10^{-1}

Study 2. Calculation of number of *T. gondii* organisms recovered by digestion of pork mince and injected into mice

- LSI *T. gondii* qPCR kit external +ve control is quantified as 9.9 × 106 copies B1 gene/mL – used to construct a standard curve to estimate numbers.

- Result for the digest = 9.19×10^2 copies/µL DNA
  = $9.19 \times 10^2 \times 10^2$ in 100 µL DNA
  = 9.19×10^4 copies from 25 µL digest
- *T. gondii* is reported to carry 35 copies of the B1 gene/genome:
  = $9.19 \times 10^4 \div 35$ estimated *T. gondii* in 25 µL digest
  = 2.6×10^3 *T. gondii* /25 µL digest

Appendix 3

$= 1.05 \times 10^5$ *T. gondii* /mL digest

- Mice pairs were injected with 1 mL diluted digest, containing the following estimated numbers of *T. gondii* organisms :

  1 mL $10^0 \rightarrow 1.05 \times 10^5$ *T. gondii* organisms

  1 mL $10^{-2} \rightarrow 1.05 \times 10^3$ *T. gondii* organisms

  1 mL $10^{-4} \rightarrow 10.5$ *T. gondii* organisms

NB. These are likely to be over-estimates of the actual infective *T. gondi* as they are based on genome copies present & don't account for non-viable parasites & will include all DNA even some partially degraded. However there would also be losses each step of the digestion & DNA extraction.

Appendix 4

2.5: Study 3 – investigation of recovery rate of *Toxoplasma* spiking material from pork mince using digestion method:

The brain of one mouse infected with *T. gondii* tachyzoites from Study 2 was examined microscopically. Typical *Toxoplasma* cysts were observed. The brain was homogenised in 500 µL PBS using a disposable manual homogeniser. A 25 µL aliquot was taken for qPCR using the LSI TaqVet™ *Toxoplasma gondii* kit detecting the 35 repeat B1 gene.

The remainder was diluted & homogenised in PBS to a final volume of 1200 µL then serially diluted ten-fold to 10^{-3}. Aliquots of each dilution of each were taken for DNA extraction, purification and qPCR determination of the initial dose.

Pork mince was weighed into 5 × 50 gm lots in stomacher bags:

1. Pork mince
2. Pork mince + 1 mL 10^{0} brain homogenate
3. Pork mince + 1 mL 10^{-1} brain homogenate
4. Pork mince + 1 mL 10^{-2} brain homogenate
5. Pork mince + 1 mL 10^{-3} brain homogenate

A modified version of the Dubey acid/pepsin digestion method for isolation of *T. gondii* was undertaken. Specifically, 250 mL saline was added to each sample, stomached for 2 min then warmed to 37°C. Then 250 mL of warmed acid/pepsin (pH 1.1-1.2) was added. The mixture was incubated @ 37°C for 60 min shaking @ 100 rpm.

The liquid was decanted and centrifuged @ 1200 × g for 10 min. The supernatant was discarded & the pellet resuspended in 20 mL saline. A few drops of phenol red indicator were added & the pH adjusted to 7 using sodium bicarbonate. This was centrifuged @ 1200 × g for 10 min & the pellet resuspended in 5 mL PBS.

The DNA of a 25 µL aliquot of each suspension extracted and purified using the Promega Wizard® Genomic DNA Purification kit using a modified version of the manufacturer's instructions for animal tissue. The DNA was rehydrated in 100 µL volumes.

Results:

T. gondii B1 gene was detected by qPCR in the original suspended mouse brain and the 10^{-1} dilution.

There were no detectable levels of the *T. gondii* B1 gene in the untreated pork mince.

The *T. gondii* B1 gene was detected in pork mince spiked with brain suspension at levels of 10^{0}, 10^{-1} and 10^{-2}.

The LSI TaqVet™ *Toxoplasma gondii* kit includes a plasmid positive control that can be used for quantification of *T. gondii*. The kit also has an internal positive control (IPC) for determination of the efficiency of the DNA isolation and the absence of PCR inhibitors in the sample.

The IPC results indicated significant inhibition of the qPCR. This could lead to false negatives. Dilution of the DNA in the qPCR can be used to limit the effect of the inhibitors but also dilutes the number of *T. gondii* genome copies.

The results are valid for the samples positive for *T. gondii* but the quantification is not accurate.

T. gondii was successfully recovered from the mince spiked with homogenised brain undiluted, diluted 1/10 and 1/100 but not 1/1000. The actual % recovery could not be determined from these data.

The disposable manual homogenisers used to prepare the brain do not produce completely dispersed samples. In addition, *T. gondii* cysts may not be distributed evenly through the tissue. This also likely

contributed to some of the variation in the qPCR data that was derived from 25 µL aliquots of the homogenate. Ideally a mechanical homogeniser would produce a more even suspension.

Further investigations:

The procedure described could be repeated using the bead homogeniser at the Uni of Adelaide Roseworthy campus, School of Animal & Veterinary Science, Dr M. McAllister's laboratory. The issue of uneven suspension of the cysts could be reduced however this would not address qPCR inhibition by samples.

An investigation into the use of an alternative qPCR target could be valuable; the 200- to 300- fold repetitive 529 bp DNA fragment reported by Homan et al 2000. This target has been identified to be more sensitive than the B1 target used in previous studies. The quantification would require a dilution series of *T. gondii* genomic DNA to produce a standard curve. This method is currently being developed in Dr McAllister's laboratory.

Appendix 5

Study 4. Pilot testing of stored sheep tongues using pepsin digestion and qPCR to assess prevalence

In 2008, an MLA funded survey of sheep and lambs was conducted at abattoirs in the 5 mainland states. In total paired useable serum samples and tongues were collected from 249 lambs and 388 sheep (Project A.MFS.0129).

The serum samples were tested for *T. gondii* antibodies using the Eiken Indirect Latex Agglutination Test (ILAT) and the sero-prevalence calculated. The matching tongue samples were stored frozen awaiting a decision by MLA to test using appropriate molecular techniques.

Following the satisfactory refining of molecular techniques in this APL/MLA collaborative project, 24 tongue samples from 24/126 serologically positive sheep (with a range of titres) were tested, by both nested PCR and qPCR. Further, both direct sampled tongue tissue (25mg) and tongue tissue (10 to 20 g) undergoing pepsin digestion/centrifugation were sampled. Detailed methods can be found in Appendix 1.

All tests were negative, with only trace levels (inconclusive) of DNA detected in 2 of the digested samples (in one replicate test) using qPCR. All other tests were negative for *T. gondii* DNA (Table 1). There was considerable inhibition evident in the qPCR on the direct tongue material, indicating that the digestion method is the more appropriate approach. The reduced inhibition may be due to pepsin destruction of inhibiting factors or simply a dilution effect (digested precipitate product is re-suspended in 5mL of saline).

It was concluded that the probable reason for the discrepancy between the serological and PCR results was the 6 year storage of the tongues under less than ideal conditions (including moving premises and a shortage of sufficient minus 80°C storage) that resulted in the degradation of the *T. gondii* DNA present. It was further concluded that continued testing of the stored tongues would be a waste of resources and fresh material would need to be acquired to produce reliable results.

Table 1. PCR results for serologically positive sheep tongues.

Type	State	Sero-titre	Pepsin digest			Tissue - direct		
			Nested PCR	qPCR	qPCR inhibition	Nested PCR	qPCR	qPCR inhibition
Sheep	NSW	128	-	-	-	-	-	-
Sheep	NSW	128	-	-	-	-	-	+
Sheep	NSW	256	-	-	-	-	-	+
Sheep	-	64	-	-	-	-	-	+
Sheep	-	1024	-	-	-	-	-	+
Sheep	Vic	64	-	-	-	-	-	+
Sheep	-	64	-	-	-	-	-	-
Sheep	-	512	-	-	-	-	-	+
Sheep	-	128	-	trace level	-	-	-	+
Sheep	-	512	-	-	-	-	-	-
Sheep	Vic	1024	-	-	-	-	-	+
Sheep	-	128	-	-	-	-	-	+

Appendix 5

Sheep	Vic	1024	-	trace level	-	-	-	+
Sheep	WA	64	-	-	-	-	-	+
Sheep	WA	128	-	-	-	-	-	+
Sheep	WA	128	-	-	-	-	-	-
Sheep	WA	128	-	-	-	-	-	+
Sheep	WA	128	-	-	-	-	-	+
Sheep	WA	128	-	-	-	-	-	+
Sheep	WA	256	-	-	-	-	-	+
Sheep	-	256	-	trace level	-	-	-	+
Sheep	WA	512	-	-	-	-	-	+
Sheep	WA	512	-	-	-	-	-	+
Sheep	WA	128	-	-	-	-	-	-

Appendix 6

SARDI FOOD SAFETY RESEARCH

METHOD: FS1.04 – **DNA extraction & purification from animal tissue & acid/pepsin digested tissue**

PREPARED BY: K Hodgson DATE: 27/11/2014

Purpose

This method is for the extraction & purification of DNA from tissue and the acid/pepsin digest of tissue.

Reference

Wizard® Genomic DNA Purification kit technical manual

Media and Reagents

Wizard® Genomic DNA Purification kit Promega

Proteinase K

Isopropanol

Ethanol

Procedure

Lysis:

Tissue:

1. Chill 600µL Nuclei lysis solution per sample on ice.
2. Add 20 mg tissue to 600µL Nuclei lysis solution & homogenise gently.
3. Incubate 15-30min @ 65°C.
4. Add 17.5µL of Proteinase K (20 mg/mL).
5. Incubate O/N @ 55°C with gentle shaking.

Tissue digest:

1. Aliquot 600µL chilled Nuclei lysis solution per sample into sterile eppendorf.
2. Add 17.5µL of 20mg/mL Proteinase K.
3. Add 25µL of tissue digest.
4. Incubate O/N @ 55°C with gentle shaking.

Then

DNA purification:

1. Add 200µL Protein precipitation solution to each tube & vortex 20sec.
2. Chill on ice 5min.
3. Centrifuge for 4min @ 16000 × g.
4. Transfer S/N carefully to fresh eppendorf with 600µL isopropanol @ RT.
5. Mix by inversion till white threads visible.
6. Centrifuge 60sec @ 16000 × g → small white pellet.
7. Carefully decant S/N.
8. Add 600µL RT 70% ethanol & gently invert several times to wash pellet.
9. Centrifuge 60sec @ 16000 × g @ RT.

10. Carefully aspirate off the ethanol.
11. Invert on clean absorbent paper & air-dry for 15 min
12. Add 100µL DNA Rehydration solution.
13. Incubate @ 65°C for 60 min or O/N @ 4°C.
14. Store @ -4°C for immediate use or < -20°C for long term storage.

Appendix 6

SARDI FOOD SAFETY RESEARCH

METHOD: FS14.01 – ***Toxoplasma gondii* – digestion of animal tissues and concentration by centrifugation to allow detection of the organism.**

PREPARED BY: G Holds DATE: 11/12/2014

Purpose

This method describes the digestion of animal tissues and the subsequent concentration of *Toxoplasma gondii*. The resulting suspension may be used for mouse bioassay or PCR analysis.

Reference

Dubey J.P. Toxopasmosis in Animals and Humans, 2010. CRC Press.

Media and Reagents

Saline Solution (9.0g/L)

Acid Pepsin Solution (made fresh)

Phosphate Buffered Saline (pH 7.2)

Sodium bicarbonate Solution (1.2%, pH 8,3)

Phenol Red Indicator

Penicillin Solution (1000units per mL)

Streptomycin Solution (100µg per mL)

Procedure

1. Matrix digestion and *T. gondii* concentration

1.1 Remove connective tissue, fat, epithelium from muscular tissues using nonporous, hard plastic cutting boards and scalpels for solid portions of tissue. Cut muscle into small (1-2cm) pieces.

1.2 Grind muscle (50g) in a blender for 15 seconds at low speed without saline. Add 125mL of saline and blend at high speed for 30 seconds.

1.3 Pour the homogenate into a filter stomacher bag. Rinse the blender with a further 125mL of saline and add this rinse to the bag.

1.4 Add 250mL of freshly prepared pre-warmed (37°C) acid pepsin solution and incubate at 37°C with shaking for 1 hour.

1.5 Mix well and filter the digest utilising the filter stomacher bag or gauze if necessary and pour off 250mL the liquid phase into a 500mL wide mouth centrifuge bottle and centrifuge at 1200g for 10 minutes.

1.6 Pour off the supernatant. Resuspend the sediment with 20mL of phosphate buffered saline using disposable plastic pipettes and transfer the homogenate to a 50mL centrifuge tube.

1.7 Add a few drops of phenol red indicator or use pH indicator strips and neutralise the homogenate with freshly prepared 1.2% sodium bicarbonate (pH 8.3). Colour change to orange (pH=7.0±0.2). Gently mix the sample.

1.8 Centrifuge the sample at 1200g for 10min.

1.9 Pour off the supernatant and add 5-10mL of saline.

1.10 If the digest is to be injected into mice, add 1000units of penicillin and 100µg of streptomycin per mL.

Appendix 6

SARDI FOOD SAFETY RESEARCH

METHOD: FS14.03 – **Nested PCR for detection of the *Toxoplasma gondii* B1 gene & PCR for murine housekeeping genes**

PREPARED BY: K Hodgson DATE: 27/11/2014

Purpose

This method is for the detection of the *Toxoplasma gondii* B1 gene by nested PCR and murine housekeeping genes by PCR.

Reference

Frericks, M. and C. Esser (2008). "A toolbox of novel murine house-keeping genes identified by meta-analysis of large scale gene expression profiles." Biochimica et Biophysica Acta - Gene Regulatory Mechanisms **1779(12)**: 830-837.

Grigg, M. E. and J. C. Boothroyd (2001). "Rapid identification of virulent type I strains of the protozoan pathogen toxoplasma gondii by PCR-restriction fragment length polymorphism analysis at the B1 gene." Journal of Clinical Microbiology **39(1)**: 398-400.

Media and Reagents

MyTaq™ Red Mix Bioline Cat No BIO-25043 or BIO-25044

Primers as per procedure

TE buffer

RNase & DNase free water

Agarose

GelRed

Hyperline IV Bioline 100-1000 Kb ladder or similar

TAE buffer

Procedure

1. The primers are as outlined:

Target		Primer name	Primer sequence	Reference
B1 gene	Fw	F_{ext}	5'-TGTTCTGTCCTATCGCAACG-3'	Grigg & Boothroyd 2001
	Rev	R_{ext}	5'-ACGGATGCAGTTCCTTTCTG-3'	
B1 gene	Fw	F_{int}	5'-TCTTCCCAGACGTGGATTTC-3'	Grigg & Boothroyd 2001
	Rev	R_{int}	5'-CTCACAATACGCTGCTTGA-3'	
Cxxc1	Fw	Cxxc1f	5'-CAGACGTCTTTTGGGTCCA-3'	Frericks& Esser 2008
	Rev	Cxxc1r	5'-AGACCTCATCAGCTGGCAC-3'	
Mrpl48	Fw	Mrpl48f	5'-AGCCATTAATGTGGGGACA-3'	Frericks& Esser 2008

Rev	Mrpl48r	5'-AGGACCATTTTGTTGCCTT-3'

2. The mastermixes must be prepared in the PCR mastermix work station.
3. Always include a no template control of DNase RNase free water or TE buffer (NTC) in each reaction.
4.

***Toxoplasma gondii B1* gene**

B1 Mastermix 1	Required	Vol required
		µL per sample
2 x MyTaq red		12.5
Forward F_{ext} (10 µM)	10 pmol	1
Reverse R_{ext} (10 µM)	10 pmol	1
Extra mQ water		9.5
DNA template		1
Final volume µL		25

Cycling conditions:

94°C 30 sec
60°C 30 sec } 35 cycles
72°C 30 sec
72°C 10 min
Hold @ 4°C

5. Dilute the PCR product from reaction 1 in TE buffer and use as the DNA template in reaction 2

B1 Mastermix 2	Required	Vol required
		µL per sample
2 x MyTaq red		12.5
Forward F_{int} (10 µM)	10 pmol	1
Reverse R_{int} (10 µM)	10 pmol	1
Extra mQ water		9.5
DNA template		1
Final volume µL		25

Cycling conditions:

94°C 30 sec
60°C 30 sec } 35 cycles
72°C 30 sec
72°C 10 min

Appendix 6

Hold @ 4°C

Murine housekeeping genes:

The detection of these genes is used to verify the efficiency of DNA extraction from murine samples and the absence of PCR inhibitors.

6. Mastermixes:

Mastermix Cxxc1	**Required**	**Vol required**
		µL
2 x MyTaq red		12.5
Cxxc1-f (10 µM)	10 pmol	1
Cxxc1-r (10 µM)	10 pmol	1
Extra mQ water		8.5
DNA template		2
Final volume µL		25

OR

Mastermix Mrpl48	**Required**	**Vol required**
		µL
2 x MyTaq red		12.5
Mrpl48-f (10 µM)	10 pmol	1
Mrpl48-r (10 µM)	10 pmol	1
Extra mQ water		8.5
DNA template		2
Final volume µL		25

Cycling conditions:

94°C 15 min

then

94°C 20 sec
55°C 15 sec } 45 cycles
72°C 20 sec

72°C 3 min

Hold @ 4°C

7. Prepare an agarose gel cast and comb of appropriate size.
8. Prepare a 1.5% agarose gel (e.g. 1.5 gm, 100 mL TAE buffer) by melting in the microwave.
9. Allow to cool but not set & add 10 µL GelRed mixing gently.
10. Pour into the agarose gel cast & set.
11. Remove the comb.
12. Aliquot 2 µL of the DNA ladder & 2-5 µL DNA products into the wells
13. Run the gel @ 100 V for 90 min.

Appendix 6

14. Visualise the bands in the Gel-Doc & save as required.

SARDI FOOD SAFETY RESEARCH

METHOD: FS14.04 – **Quantitative PCR for the *Toxoplasma gondii* B1 gene**

PREPARED BY: K Hodgson DATE: 27/11/2014

Purpose

This method is for the detection of the *B1* gene of *Toxoplasma gondii* by quantitative PCR (qPCR).

Reference

LSI VetMAX™ *Toxoplasma gondii* TXP50 kit protocol

Media and Reagents

LSI VetMAX™ *Toxoplasma gondii* TXP50

Ultrapure water

Procedure

1. Agitate < Mix Toxo> briefly & centrifuge.
2. Aliquot 20µL per sample into Rotorgene tubes.
3. Thaw samples & external positive control Toxo (EPC).
4. Add 5µL EPC or sample to each tube except tube 1 (NC).
5. Dilute EPC in TE buffer 5µL → 50µL to 10^{-3} if running a standard curve.
6. Run the real time thermocycler as per the TaqVet Toxo cycle

Step 1:	50°C for 2 min	
Step 2:	95°C for 10 min	
then		
Step 3:	95°C for 15 sec	45 times
	60°C for 1 min	

7. Validation of the test:
   - 7.1. EPC Ct
     - 7.1.1. EPC = Ct_{QC} refer to Quality control certificate for the EPC batch
     - 7.1.2. IPC = > 45
   - 7.2. Negative control sample Ct
     - 7.2.1. EPC = > 45
     - 7.2.2. IPC = > 45
   - 7.3. Negative control (NC) Ct
     - 7.3.1. EPC = > 45
     - 7.3.2. IPC = > 45
   - 7.4.

Interpretation	<Detector> Toxo	<Detector> IPC
Positive *T. gondii*	Ct < 45	Ct < 45 or Ct > 45
Negative *T. gondii*	Ct > 45	Ct < 45
Non-validated	Ct > 45	Ct > 45

Appendix 6

Samples are not validated if the IPC has Ct > 45. The DNA can be diluted 1/10 in DNase free water and retested.

Appendix 6

SARDI FOOD SAFETY RESEARCH

METHOD: FS14.05 – ***Toxoplasma gondii*** **isolation for detection by mouse bioassay and microscopy and/or PCR**

PREPARED BY: Dr D R Hamilton DATE: 11/12/2014

Purpose

This method is for detection of *Toxoplasma gondii* from tissues of infected animal tissues by mouse bioassay and microscopy and/or PCR.

Reference

Dubey J.P. Toxopasmosis in Animals and Humans, 2010. CRC Press.

Media and Reagents

Saline Solution (9.0g/L)

Penicillin Solution (1000units per mL)

Streptomycin Solution (100µg per mL)

Procedure

1 Inoculation and bioassay.

1.1 Weigh and record mice weights. Use Swiss-Webster mice 20-25g if available as they appear more sensitive to toxoplasma infection.

1.2 Anaesthetise mice using fluorothane box.

1.3 If possible collect blood and freeze serum for post inoculation comparison if feasible.

1.4 Inoculate 1mL of tissue homogenate subcutaneously at the back of the neck, using a 4cm 21 – 23 gauge needle.

1.5 Gently massage the injection site to minimise swelling and discomfort on regaining consciousness.

1.6 Examine all inoculated mice daily for early non-specific signs of *T. gondii* infection (hunching, ruffled appearance, weight loss, inactivity).

1.7 If signs appear (usually 7-10 days post inoculation) immediately put sulphadiazine in the drinking water (1mg/mL) for one week. This will alleviate acute infection and should prevent deaths, but still allow brain cysts to form.

1.8 If acute signs are severe and mice are not drinking, immediately treat by restraining mouse and placing drops of medicated water directly into mouth by use of a 1mL syringe.

1.9 Monitor twice daily and repeat if no improvement after 12 hours.

1.10 Symptoms should disappear within 24-36 hours.

1.11 Allow a minimum of 6 weeks (but preferably 10weeks) for cyst development as they *T. gondii* may not be detectable by microscopy nor PCR at 6 weeks.

1.12 Euthanize mice by fluorothane anaesthesia followed by exsanguination directly from the heart using a 21 g needle. The serum can be frozen and used for serology using a toxoplasma ELISA, and compared with the pre-inoculation serum, in the event the PCR is proves negative.

1.13 Check that mouse has stopped breathing, heart has stopped and eyes have glazed.

1.14 Incise skin around neck with scissors and remove head by cutting between the first cervical vertebra and the skull.

1.15 Peel skin forward from skull to provide a firm grip with left hand and using small artery forceps in the right insert into foramen magna, grip dorsal skull plate and gently break it away in sections exposing the brain.

1.16 Tease complete brain into a sterile petri dish; moisten with a drop of sterile saline and cover.

1.17 Using a sterile scalpel separate the brain into two hemispheres. Put one half into a tube and refrigerate for DNA extraction. The other hemisphere can be used to make smears for microscopy.