Final report

Investigation of the viability and national serological prevalence of Toxoplasma gondii in Australian sheep

Project code: V.MFS.0419

Prepared by: D Hamilton, K Hodgson, A Howard, J Jolley, K Mahbub, V Torok
South Australian Research & Development Institute

M McAllister
University of Adelaide

Date published: 28 February 2021

PUBLISHED BY
Meat and Livestock Australia Limited
PO Box 1961
NORTH SYDNEY NSW 2059

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

Abstract

Toxoplasma gondii is a significant zoonotic parasite (30-40% of Australians are seropositive) which can often be foodborne. The aim of this project was to fill data gaps regarding toxoplasmosis in sheep and goats to better determine the magnitude of the risk associated with the consumption of undercooked sheep meat. Project objectives included development of gold standard negative and positive controls to make existing blood tests more accurate; conducting a national sheep blood testing survey to estimate prevalence; isolating and genotyping *T. gondii* organisms from sheep to compare with overseas isolates and to confirm the organisms remain viable in meat and are therefore potentially infectious.

Paired mutton heart and blood samples were collected at slaughter. Hearts from animals seropositive for *T. gondii* were processed overnight and bio-assayed in mice to assess parasite viability (infectivity) and to facilitate isolation of the organism. Overall, a total of eighteen sheep *T. gondii* isolates were cultured and cryo-preserved during the project. A selection of isolated organisms were inoculated into seronegative sheep and goats which were then regularly monitored for sero-conversion. After three months, inoculated animals were euthanised, bled out and infection confirmed by mouse bio-assay. The gold standard positive and negative sera collected were used to calibrate the *T. gondii* test kit for a national serological sheep survey. That survey estimated the national seroprevalence in mutton to be 11.5%. All eighteen *T. gondii* sheep isolates (Tg-AS01 to 18) were genotyped, with one being fully genome sequenced. This demonstrated that all Australian isolates are very closely related, regardless of their geographic origin.

The project results will enable the industry to make a more informed assessment of the risk posed by toxoplasmosis.

Executive summary

Background

Worldwide, toxoplasmosis is a very common parasitic infection in warm-blooded animals including humans (30 – 40% of Australians have antibodies). *Toxoplasma gondii,* the causative parasite, has a complex life-cycle, with 3 main routes of infection: from ingestion of sporozoites originating in feline faeces; from ingestion of undercooked infected meat; from congenital infection in the womb. As with other domestic animals coming to slaughter, sheep and lamb may be infected with *Toxoplasma gondii*. Human toxoplasmosis can be asymptomatic (no clinical symptoms) or can have more severe consequences such as congenital neurological defects, eye disease, or potentially fatal encephalitis in immunocompromised individuals.

Contact with domestic cats is considered a major source of human toxoplasmosis; the proportion of cases caused by eating raw or undercooked meat is not known, however the consumption of undercooked sheep meat has been identified as a risk factor in epidemiological studies. From being a parasite of cats only worthy of consideration by women in early pregnancy, *Toxoplasma* has now become a food safety concern.

There is ongoing international interest in the significance of meat as a source of toxoplasmosis. For example, the WHO Global Burden of Foodborne Disease study and a recent publication on the risks to US consumers from US domestic lamb highlight the potential burden of disease. There is active consideration of *Toxoplasma* risks in meat by European researchers.

MLA has conducted work to address the knowledge gaps in *Toxoplasma* risks since 2007, through several research projects (A.MFS.0129, G.MFS.0292). *Toxoplasma* risks continue to be identified in MLA risk assessment projects (PRMS.038c, V.MFS.0410).

A 2008 MLA national baseline serological survey (A.MFS.0129) estimated the seroprevalence of *T. gondii* in lambs and sheep at 16% and 32%, respectively (as an all-of-life infection, older animals have higher infection rates). However, uncertainty existed regarding the wisdom of relying solely on serological testing. Further, there remained a lack of knowledge around the *T. gondii* genotypes present and whether they mirror those in human infections. This, combined with the scarcity of data regarding the viability (infectivity) of *Toxoplasma* present in Australian sheep, beef and goat meat, meant a proper assessment of the risk associated with the consumption of undercooked meat could not be made. This project was designed to address some of these concerns.

Objectives

- Establish gold standard positive and negative controls for sheep and goats to calibrate *T. gondii* serological tests.
    - This would provide confidence that serological testing was accurately identifying both infected and un-infected animals.
- Estimate a prevalence of *T. gondii* infection in sheep through a national survey.
    - Allowing an estimate of the extent of potential consumer exposure.
- Genotype and preserve Australian *T. gondii* isolates collected during the project.
    - Providing a valuable library of Australian strains for future research into risk mitigation e.g. rapid testing; vaccine development.

All objectives were successfully achieved.

Methodology

Stage 1 - pilot study to collect Australian isolates

- Kangaroo Island mutton were opportunistically sampled at slaughter, and paired blood and heart collected.
- Hearts from serologically positive animals were processed and bio-assayed in mice to isolate viable *T. gondii* present.
- Tissue from euthanised mice was inoculated into tissue culture and successful cultures cryo-preserved for use in Stages 2 and 4.
- Sheep and mouse tissues were examined for *T. gondii* DNA by PCR to confirm infection.

Stage 2 - calibration of serological test

- Seronegative sheep and goats were randomly allocated into treatment and control groups and inoculated with Australian *T. gondii* strains from Stage 1.
- Animals were regularly monitored for signs of seroconversion.
- After three months, animals were euthanised, exsanguinated and their tissues tested for *T. gondii* by qPCR, mouse bio-assay and direct culture.
- Gold standard positive and negative sera were stored for use, calibrating the *T. gondii* test kit used in Stage 3.

Stage 3 - national sheep seroprevalence survey

- After analysis, the serological test kit cut-off was set at 1:60.
- A total sample size of 350 mutton was allocated nationally in proportion to the states' sheep population.
- To maximise the geographic spread, sample numbers were generally limited to three or less per farm.
- Serologically positive samples were processed as in Stage 1, using acid-pepsin digestion, mouse bio-assay and cell culture. *T. gondii* isolates were similarly cryo-preserved.

Stage 4 – genotyping of isolates

- Whole genome sequencing and variant analysis was conducted on one of the 18 Australian sheep isolates (Tg-AS02). The genome was then compared to 20 publicly available whole genome *T. gondii* sequences, sourced from a variety of host species and countries.
- All 18 Australian sheep isolates (Tg-AS01 to 18) and an Australian dog isolate were typed using 15 microsatellite markers and 4 introns, and compared with a range of overseas *T. gondii* isolates.

Results/Key Findings

- Using serology, followed by acid-pepsin digestion and then followed by mouse bio-assay, a total of 18 *T. gondii* isolates were obtained from 18 seropositive sheep, demonstrating viability (potential infectivity) in meat.
- We were able to also culture the organism directly from acid digested sheep heart, omitting the usual pre-culture bio-assay step of first injecting the digest into mice to multiply the organism. This has the potential to greatly reduce the future cost, time and complexity of isolating *T. gondii*.
- Gold standard *T. gondii* positive and negative control sera were produced and used to enable successful calibration of the serological test kits.
- An Australian *T. gondii* isolate (Tg-Dog- a canine isolate provided by Prof. John Ellis at UTS) was found to initiate a strong immunological response without detectable infection during the gold standard test calibration component of this project, making it a potential vaccine

contender. Tg-Dog is a Type 11 isolate genetically very similar to the Australian isolates collected.

- The Stage 3 national survey of all six states indicated a national serological prevalence of 11.5% (46/401), with a range by state of 0 to 44%. This compares with the 31.9% prevalence estimated in 2009.
- The first Australian culture collection of 18 *T. gondii* isolates (TgAS01 - TgAS18) specific to livestock/sheep has been established and cryo-preserved for future research purposes. For example, there has already been a preliminary approach from a company to utilise the isolates to test the efficacy of a vaccine against Australian *T. gondii* strains.
- Whole genome sequencing of one Australian sheep isolate (Tg-AS02) revealed close genetic similarity to a sheep isolate (ME49) collected in 1965 in the USA.
- Microsatellite and intron analysis of all the Australian *T. gondii* strains (TgAS01 -18) isolated in this study indicate that all 18 isolates are genetically highly similar (clonal), regardless of geographic location, yet distinct from most global strains to which they were compared. In particular, they were not clustered with any of the three South American isolates examined (2 from Brazilian cats and 1 from a Uruguayan sheep). Recent studies have indicated that South American strains are less clonal and may be more virulent in immune competent humans (Carme et al 2009; Grigg et al 2015).

Benefits to industry

The results fill a number of existing data gaps with regard to toxoplasmosis in Australian sheep and highlight the importance of developing a risk management strategy to deal with this issue. The demonstration of *T. gondii* viability in sheep meat combined with an improved seroprevalence estimate, allows industry to more effectively assess consumer risk. The library of cryo-preserved Australian isolates will provide a useful resource for developing future mitigation capability.

Future research and recommendations

T. gondii was readily isolated from fresh sheep tissue, demonstrating the viability of the muscle cysts present and underlining the potential risk they represent to consumers. The hearts, however, were processed within one or two days of slaughter. It is uncertain to what extent normal sheep meat storage parameters reduces that viability. Freezing kills the organism but the effect of chilling over time is less understood. This is an investigation that could be readily and economically undertaken.

In the Stage 3 national survey, Tasmania had a very high prevalence of *Toxoplasma* (44%). While based on a small sample size (nine sheep for the whole state), Tasmania has been found to have one of the highest stray and feral cat *Toxoplasma* seroprevalences in the world (84%). Therefore, a larger Tasmanian survey should be undertaken to confirm/disprove this finding.

Although mature sheep are likely to have higher prevalence of infection with *T. gondii*, due to the life-time persistence of viable infection, it is lamb that is most likely to be eaten rare and therefore provide the greatest foodborne risk to consumers. A lamb seroprevalence survey should be conducted.

Table of contents

1. Background - Overview

Toxoplasma gondii is one of the most common protozoan parasites of humans worldwide. It was first discovered in a gundi (a hamster-like rodent) that died in the laboratory at the Tunis Pasteur Institute in the early 1900s. It was named after its shape (*toxo* -arc or bow and *plasma* -life) and the animal where it was first identified. In Australia, approximately 30-40% of the mainland human population have antibodies to the parasite, a seroprevalence comparable to other developed countries. *T. gondii* is considered a significant foodborne public health hazard, since one of the transmission routes to humans is via raw or undercooked meat from infected livestock.

Most human infections with *Toxoplasma* are asymptomatic, but severe symptoms and diseases can occur in developing foetuses (e.g. blindness, mental retardation), in the immunocompromised (e.g. AIDS patients) and the immunosuppressed (e.g. transplant patients). It can also cause ocular infections in immunocompetent individuals. Most infections in humans and other secondary hosts progress to a chronic stage with the formulation of tissue micro-cysts in the brain and other organs without major complications, where they may remain clinically dormant but viable for the life of the host. Any later suppression of the host's immune system, however, may result in a re-emergence of an active infection. Further, there is emerging evidence that implicates *Toxoplasma* in a suite of mental disorders (e.g. schizophrenia or risk-taking behaviour) occurring in otherwise healthy people.

The US Department of Agriculture estimates that the cost of *Toxoplasma* infection to the US was $US 3.4 billion in 2013, second only to *Salmonella* ($US 3.7 billion) in terms of foodborne disease.

In 2008, MLA conducted a national baseline survey (A.MFS.0129; Kiermeier et al., 2008) to estimate the seroprevalence of *T. gondii* in lambs and sheep. Matched blood and tongue samples were collected at abattoirs with the national seroprevalence for lambs and sheep being estimated at 16% and 32% respectively. However, uncertainty existed regarding the reliability of serological testing. Further, there is a lack of knowledge around the genotypes present and whether they mirror those in human infections. This, combined with the scarcity of data regarding the viability (infectivity) of *Toxoplasma* present in Australian sheep, beef and goat meat, meant a proper assessment of the risk associated with the consumption of undercooked meat could not be made.

In order to fill this significant data gap for risk managers in the red meat industry, this project was designed to develop gold standard *Toxoplasma gondii* positive and negative sheep sera in order to calibrate the serological test kit and improve confidence in its accuracy. A national survey was then conducted to more accurately estimate the seroprevalence in sheep and directly relate the serology to the presence of *Toxoplasma gondii* in the animals' tissue. Paired sheep blood and fresh (unfrozen) heart tissue were collected at slaughter. Blood samples were then serologically screened, with the hearts from positive animals being tested for the presence of *T. gondii* DNA, using molecular test protocols and techniques previously developed and verified by SARDI in a joint MLA/Australian Pork Limited project (APL Project 2011-1017.401 / MLA Project G.MFS.0292 ; Hamilton et al., 2014). Tissue positive for *T. gondii* DNA was bio-assayed in mice to demonstrate viability (infectivity) and to provide organisms for genotyping and the creation of a library of preserved Australian *Toxoplasma* genotypes for future research. This enabled an initial indication of the range of genotypes present in Australian sheep.

The project was designed with logical steps and go/no go stages and this report is structured accordingly.

- Stage 1 – Preliminary study to obtain Australian *T. gondii* isolates.
- Stage 2 – Calibration of serological test using Australian *T. gondii* isolates from Stage 1.
- Stage 3 – National survey of *T. gondii* in sheep using the calibrated serological test.
- Stage 4 – Genotyping of Australian *T. gondii* isolates.

2. Objectives

The project objectives were to:

- Establish gold standard positive and negative controls for sheep and goats to calibrate *Toxoplasma* serological tests.
- Estimate a prevalence of *T. gondii* infection in sheep through a national survey.
- Isolate, genotype and preserve the *T. gondii* isolates collected.

3. Stage 1 – Preliminary study to obtain Australian *T. gondii* isolates

3.1 Background

Commercial serological tests for *T. gondii* are primarily developed for human sera and can vary in their sensitivity and specificity. It is important to validate any serological test against known positive and negative sera from sheep and goats (Stage 2) – sera from these experimental animals was used to calibrate commercial serological assays for this and future studies in sheep and goats.

Prior to the commencement of this project, in the absence of existing Australian sheep isolates, the only option to generate "gold standard" animals would have been to use an imported laboratory strain of *T. gondii* isolated from a non-ruminant host. Inoculating a non-indigenous strain into live animals would have been an expensive and complicated exercise, necessitating approved quarantine facilities. Further, the generated sera would be to a non-Australian non-ruminant *T. gondii* isolate, and therefore potentially of limited relevance to the Australian meat industry.

Therefore, this small preliminary / pilot sampling of sheep (sourced from areas considered to have a higher *T. gondii* prevalence, for example, Kangaroo Island), was undertaken in order to source an Australian *T. gondii* isolate for injecting animals to generate positive and negative sera in Stage 2 and to trial our procedures to harvest isolates from naturally infected animals.

3.2 Methodology

In summary, paired mutton heart and blood samples were collected at slaughter. Processed heart tissue from serologically positive animals were then bio-assayed in mice to isolate *T. gondii*.

3.2.1 Animal Ethics

The mice used for Stage 1 were covered by the Animal Ethics application 0000021902: Production of control sera and tissues for *Toxoplasma gondii* and *Neospora caninum* in animals (S-2016-150) under the University of Adelaide.

3.2.2 Abattoir sample collection

Paired mutton samples (heart and blood) from ten Kangaroo Island sheep herds were opportunistically collected from abattoirs in South Australia. Mature sheep were sampled to increase the likelihood of animals being positive (as *Toxoplasma* is an all-of-life infection, older animals have a greater chance of being infected). Sheep hearts were selected from the offal tray on the slaughter floor and blood from each heart was poured into 8.5mL gold capped BD Vacutainer SST II Advance tubes. These tubes contain spray-coated silica particles to accelerate clotting and a gel layer to assist with serum separation. Exsanguinated hearts were then rinsed with warm water, placed into an individual labelled sample bag and transported on ice to the SARDI Food Sciences laboratories (Urrbrae, South Australia) along with the paired blood sample. Blood tubes were centrifuged at 3700 x g for 10 minutes at 4°C, allowing the gel layer to separate red blood cells from blood serum. Approximately 2mL of supernatant blood serum was decanted, aliquoted and stored at 4°C for immediate serological testing.

3.2.3 Abattoir sheep serological testing

The serology was performed using a commercial Modified Agglutination (MAT) Test kit, specifically the Toxo-Screen Direct Agglutination (DA) kit (BioMérieux, Marcy-l'Étoile France) following the manufacturer's 'quantitative test' instructions. This kit works on the principle that formalin-treated toxoplasma agglutinates when in the presence of diluted serum containing specific IgG antibodies. A positive reaction is indicated by agglutination of the *Toxoplasma* in a mat covering about half of the well base (may be an irregular shape), a negative reaction is indicated by sedimentation of the *Toxoplasma* in a button or ring and a borderline reaction (weak positive) is indicated by agglutination of the *Toxoplasma* in a mat covering less than half of the well base. Non-specific agglutination is suppressed by using a diluting buffer containing 2-mercaptoethanol, which denatures IgM. Results were interpreted using the following (from the manufacturer's instructions):

- Positive reaction: agglutination of *Toxoplasma* in a mat covering about half of the well base,
- Negative reaction: sedimentation of *Toxoplasma* in a button or ring.

Sheep serum was diluted 1:20 (100µL serum + 1.9mL phosphate buffered saline (abbreviated PBS)) in 4mL glass tubes and vortexed thoroughly. 50µL of 0.2 mol/L 2-mercaptoethanol (abbreviated 2ME) was dispensed into six successive wells of a 96 well micro titration plate. 25µL of the 1:20 diluted serum was dispensed in the 1st well and mixed with a pipette. 25µL of the first well was transferred to the second well and mixed, this was repeated up to the sixth well, creating a threefold dilution series of 1:60, 1:180, 1:540, 1:1620, 1:4860 and 1:14580. 50µL of *Toxoplasma* antigen (diluted 1:5 with BABS albumin buffer) was added to each well. All samples were tested in duplicate with positive, negative and antigen controls (provided in the Toxo-Screen MAT kit) included on each plate. The plate was gently shaken on the bench to homogenise the well solutions, covered with a self-adhesive sheet and left to stand for 18 hours at room temperature, away from vibrations and sources of drying. Results were then read and interpreted; the Toxo-Screen MAT kit specifies that a positive reaction at a 1:60 dilution, equivalent to a titre of 6 IU/mL, indicates a serologically positive result.

Paired heart samples from serologically positive sheep underwent acid-pepsin digestion before being subcutaneously injected into mice.

3.2.4 Abattoir sheep heart acid pepsin digestion

Hearts from seropositive animals were digested using a method based on the Dubey protocol (Dubey, 1998). Fat and connective tissue were excised from the muscle and discarded. The muscle was cut with sterile scalpels into 1–2 cm pieces. A 50g portion of each was homogenised for 15 seconds at low speed then blended with 50mL of sterile 0.9% saline at high speed for 30 seconds. The homogenate was transferred into a filter stomacher bag. The blender was rinsed several times with a total of 200mL of saline and added to the sample. Freshly prepared 250mL of acid pepsin solution pre-warmed to 37°C (20.8g pepsin; 1:2500, 10g sodium chloride, 14.0mL 32% hydrochloric acid and deionised water to make up to 1 L, pH 1.1-1.2) was added. The digest was incubated at 37°C with gentle shaking for 1 hr. The digests were filtered through the stomacher bag filter and 250mL of the liquid phase transferred into a 500mL wide mouth centrifuge bottles and centrifuged at 1200 × g for 10 min at room temperature.

The supernatants were discarded and the sediments re-suspended in 20mL of phosphate buffered saline (pH 7.4) and transferred to a 50mL centrifuge tube. The homogenates were neutralised to pH 7 with freshly prepared 1.2% sodium bicarbonate (pH 8.3) using pH indicator strips. The extracts were centrifuged at 1200 x g for 10 min, the supernatants discarded and re-suspended in 4mL of saline and 400µL of penicillin/streptomycin solution (10,000 units/mL penicillin and 10,000 µg/mL streptomycin).

For the bio-assay, the suspension was subcutaneously injected into mice (1mL per animal) on the same day. The remainder of each digest was stored at -20°C for DNA extraction and analysis by polymerase chain reaction (PCR) for detection of *T. gondii* genome.

3.2.5 Bio-assay mouse serology

At euthanasia, approximately 1mL of blood was collected via cardiac puncture from mice previously inoculated with sheep heart digest and stored in 2mL plastic Eppendorf tubes. Whole blood was centrifuged at 15500 x g for 10 minutes at 4°C, separating red blood cells from blood serum. The Toxo-Screen MAT quantitative test was again used to determine the IgG serological titre of all samples as described in Section 3.2.3.

3.2.6 Sheep and mouse DNA extraction and purification

The extraction and purification of the DNA of both the sheep heart digest and murine tissue was undertaken using the Wizard Genomic DNA Extraction Kit (Promega, Wisconsin USA) following the manufacturer's instructions. Nuclei lysis solution (600µL) was dispensed into 1.5mL tubes plus 17.5µL of Proteinase K (20mg/mL) then 25µL of heart digest or 25mg of murine tissue was added and incubated overnight at 55°C with gentle shaking. An extraction blank of 25µL purified water was included with each batch of extractions. Protein precipitation solution (200µL) was added to each sample, vortexed for 20 sec, chilled on ice for 5 min then centrifuged for 4 min at 16,000 × g. The supernatants were transferred to fresh 1.5mL tubes containing 600µL isopropanol at room temperature, mixed gently by inversion then centrifuged for 60 sec at 16,000 × g. The supernatants were discarded and the pellets gently washed with 600µL of 70% ethanol by inverting the tubes several times. The tubes were centrifuged for 60 sec at 16,000 × g and the ethanol removed by aspiration. The tubes were inverted on clean absorbent paper, air-dried for 15 min, then 100µL DNA Rehydration solution added and incubated at 65°C for 60 min. The DNA extracts were stored at −20°C for analysis by PCR.

3.2.7 Mammalian house-keeping gene PCR

For process control purposes, the efficiency of the DNA extraction process for each sample was confirmed by PCR detection of a mammalian house-keeping gene (Frericks and Esser, 2008). The primers used are detailed in Table 1. The 25µl reaction mixture consisted of 5µl of template DNA, 12.5µL of MyTaq™ Red master mix (Bioline Pty Ltd, NSW, Australia) and 0.4µM of each primer (Cxxc1-F and Cxxc1-R). The PCR was performed in the Axygen Maxygene thermocycler with cycling conditions of 94°C for 15 min, followed by 45 amplification cycles (94°C for 20 sec, 55°C for 15 sec, 72°C for 20 sec) then 72°C for 3 min and holding at 4°C.

Table 1. House-keeping gene primers

Target	Primer name	Primer sequence
Cxxc1	*Cxxc1-F*	CAG ACG TCT TTT GGG TCC A
	Cxxc1-R	AGA CCT CAT CAG CTG GCA C

Results of PCR amplification were checked visually under ultra-violet light following agarose gel electrophoresis with GelRed™ (Biotium Inc, California USA).

3.2.8 *T. gondii* 529-bp repeat element qPCR

The DNA extracts were analysed by qPCR for the 529-bp repeat element fragment from *T. gondii* using the primers and probes as outlined by Opsteegh and colleagues (Opsteegh et al., 2010). The primers and probe used are detailed in Table 2. The 25µl reaction mixture consisted of 5µl of template DNA, 12.5µL of Platinum® qPCR SuperMix-UDG (2×) master mix (Invitrogen), 0.7µM of each primer (Tox-9F and Tox-11R) and 0.1µM of Tox-TP1 probe. The quantitative (or real-time) PCR (qPCR) was performed and analysed using the QuantStudio™ 6 Flex Real-Time PCR system with cycling parameters: 50°C for 2 min, followed by 45 amplification cycles (95°C for 10 sec and 58°C for 20 sec) then 72°C for 20 sec.

Table 2. Primers and probe for Toxo529 qPCR

Target	Primer name		Sequence
529bp fragment	*Tox-9F*	Fw	AGGAGAGATATCAGGACTGTAG
	Tox-11R	Rev	GCGTCGTCTCGTCTAGATCG
	Tox-TP1	Probe	FAM-CCGGCTTGGCTGCTTTTCCT-BHQI

Each sample was analysed in triplicate with 1µL of control *T. gondii* ME49 DNA added to one aliquot as an amplification control. Inhibition of the qPCR was evident when the Ct of the sample with ME49 was >3 units of the ME49 positive control. Samples with a Ct (threshold cycle) value ≤ 35 were designated positive (detected), Ct ≥ 37 negative (not detected) and between 35 and 37, the result was positive (detected) dependent on a smooth exponential amplification curve.

3.2.9 Cell culture of sheep and mouse tissue

In addition to mouse inoculation, abattoir sheep heart digests were also inoculated onto a cell culture in an attempt to directly culture *Toxoplasma*. Harvested bio-assay mouse tissue was also directly inoculated onto cell culture after maceration to isolate *Toxoplasma*.

The host cell culture consisted of Hs27 human foreskin fibroblasts (ATCC® CRL-1634™) maintained in Dulbecco's modified Eagle's Medium (DMEM) with glutamine (Sigma Aldrich D5796) supplemented with 10% foetal calf serum (FCS) and 1% penicillin/streptomycin (Pen 10,000 U/mL and Strep 10,000 mg/mL (Sigma Aldrich P4333) incubated at 37°C with 5% CO_2. The medium was refreshed every 5-6 days or more often as required.

The Hs27 cells and *T. gondii* isolates were stored long-term in cryopreservation medium (50:50 DMEM and 15% DMSO) in liquid nitrogen.

Sheep heart digests were inoculated directly onto Hs27 cells. A 1mL aliquot of digest was pipetted onto the cells with 8mL of DMEM and incubated for 60 min at 37°C with 5% CO_2. The medium and homogenate were aspirated from the cells & replaced with 8mL of fresh warm complete DMEM. Flasks were incubated for up to 4 weeks at 37°C with 5% CO_2. Flasks were checked regularly by microscopy for evidence of *T. gondii* infection.

Mice exhibiting symptoms of *T. gondii* infection (ruffled, hunched, less active) were euthanised as soon as symptoms were observed. The remaining asymptomatic animals were euthanised 8 or more weeks post injection with the heart digest, to allow time for bradyzoite cysts to develop. The euthanised animals were immediately transferred to the SARDI Food Sciences laboratories. Murine tissues were removed aseptically. The liver, spleen and lung were taken from symptomatic animals with acute infection (to harvest tachyzoites). The brain was taken from asymptomatic animals with chronic infection (to harvest bradyzoites). Half of each organ was manually homogenized in 3 to 5 mL of warm complete DMEM. The remaining half was stored at −20°C for DNA extraction and analysis by PCR. The homogenate (3mL) was inoculated onto Hs27 cells in 25cm^2 flask after removing 3mL of the DMEM from the flask and incubated for 60 min at 37°C with 5% CO_2. The medium and homogenate were aspirated from the cells and replaced with 8mL of fresh warm complete DMEM. Flasks were incubated for up to 4 weeks at 37°C with 5% CO_2 with regular microscopic examination for *T. gondii*.

3.3 Results

3.3.1 Sample collection

Forty-seven paired mutton samples (heart and blood) from ten Kangaroo Island sheep herds were collected from abattoirs in South Australia on two separate occasions.

3.3.2 Sheep hearts

Thirty-one of the 47 (66%) Kangaroo Island sheep sampled were found to be serologically positive for *T. gondii*. Serological titres ranged from 6 to 1458 IU/mL. Due to limitations in the number of samples that could be digested at one time, 18 samples with the highest serological titres were digested and inoculated into mice (Table 3).

The *T. gondii* genome was detected in 13 of the 18 heart digests by qPCR (Table 4).

3.3.3 Mouse bio-assay

Eight of the 18 mice inoculated with heart digests from serologically positive abattoir sheep from Kangaroo Island developed clinical signs associated with an active *T. gondii* infection, between two and four weeks post inoculation. These clinical signs included weight loss, a hunched posture, a ruffled coat and a reduction in general activity. At eight weeks post inoculation, blood was collected from the remaining ten mice at euthanasia and serologically tested.

Despite the absence of clinical signs in over half the mice, 18/18 were found to be serologically positive, with titres ranging from 162 to 1458 IU/mL. The maximum serological titre of samples was not able to be determined as 1:14580 was the highest dilution used. Nine *T. gondii* isolates were successfully cultured from the 18 serologically positive mice, and named Tg-AS01, Tg-AS02…Tg-AS09 (i.e. *Toxoplasma gondii* - Australian sheep isolate 01, 02 etc.), sequentially in the order that they were first isolated (Table 3).

Table 3: Serological results for Stage 1 – preliminary study to obtain Australian Toxoplasma gondii isolates (Tg-AS01-Tg-AS09).

Sheep ID	**Farm**	**Sheep *T. gondii* titre (IU/mL)**	**Heart digest inoculated into mice**	**Mouse *T. gondii* titre (IU/mL)**	***T. gondii* isolated from mice**
FS17-338	A	162	Yes	1458	ND*
FS17-341	B	18	-	-	-
FS17-342	B	54	Yes	1458	ND
FS17-343	B	1458	Yes	1458	**Tg-AS09**
FS17-344	B	54	Yes	162	ND
FS17-345	C	1458	Yes	486	ND
FS17-346	C	54	Yes	486	ND
FS17-347	C	6	-	-	-
FS17-359	D	1458	Yes	486	ND
FS17-361	D	6	-	-	-
FS17-362	D	1458	Yes	1458	ND
FS17-363	D	6	-	-	-
FS17-368	E	1458	Yes	1458	**Tg-AS06**
FS17-369	E	6	-	-	-
FS17-373	E	1458	Yes	1458	**Tg-AS05**
FS17-374	F	1458	Yes	1458	**Tg-AS08**
FS17-375	F	1458	Yes	1458	**Tg-AS01**
FS17-376	G	18	-	-	-
FS17-377	H	1458	Yes	1458	**Tg-AS07**
FS17-378	I	8	Yes	1458	**Tg-AS02**
FS17-379	J	1458	-	-	-
FS17-380	J	1458	Yes	1458	**Tg-AS03**
FS17-381	J	486	Yes	1458	**Tg-AS04**
FS17-382	J	6	-	-	-
FS17-383	J	6	-	-	-
FS17-384	J	1458	Yes	486	ND
FS17-385	J	1458	Yes	1458	ND

*ND = not detected

Of the eight mice exhibiting symptoms, the livers, lungs, spleens, and in some cases, the brains were collected. The remaining asymptomatic mice were euthanised a minimum of eight weeks post infection. In some instances, the tissues were pooled for inoculation onto the Hs27 cells. All mouse tissues were individually analysed by qPCR for detection of the 529-bp repeat element fragment of the *T. gondii* genome (Table 4).

In a preliminary trial, two sheep heart digests were concurrently inoculated directly onto Hs27 cells, specifically FS217-0359 and FS17-0378, without pre-multiplication in mice. One heart sample (FS17-0378) had *T. gondii* isolated from both mouse bio-assay and direct culture. This isolate was designated

Tg-AS02. The other heart sample (FS217-0359) had no *T. gondii* isolated from either method. Note, the normal *T. gondii* isolation sequence is heart digestion → mouse injection (bio-assay) → mouse tissue inoculation of tissue culture to grow tachyzoites. Direct culture omits the mouse injection (bio-assay) step i.e. heart digest → tissue culture inoculation to grow tachyzoites. This approach has the potential to greatly reduce the time, cost and complexity of isolating viable *T. gondii*.

The *T. gondii* 529-bp repeat element genome fragment was detected in at least one of the tissues from 13/18 mice (eight mice exhibiting symptoms and five asymptomatic mice).

In total, nine *T. gondii* isolates were successfully cultured from the murine samples, designated Tg-AS01 to Tg-AS09 and cryopreserved for further investigation. Infection of the tissue culture cells with *T. gondii* was evident through lysis of the Hs27 cells, disruption of the cell monolayer and release of typical curved motile tachyzoites, the rapidly growing life stage of the parasite (Figure 1).

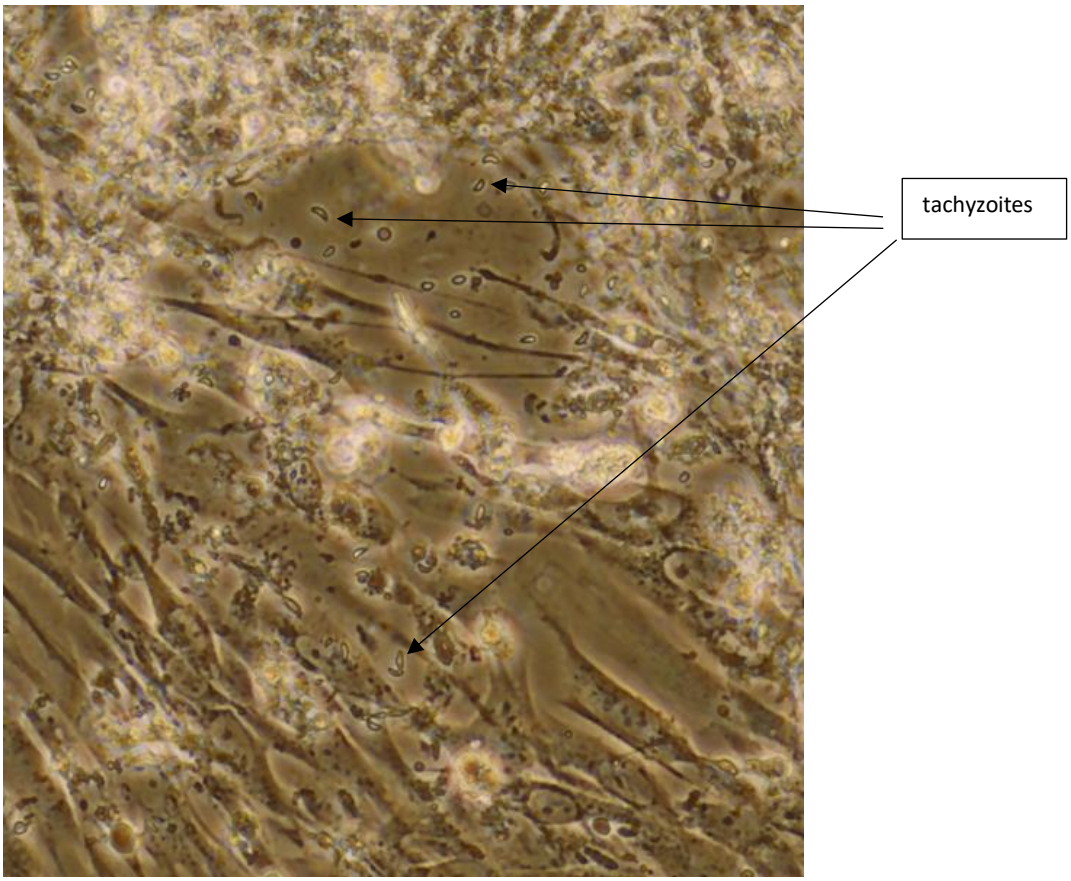

Figure 1. Hs27 cells infected with T. gondii

Table 4: qPCR results for Stage 1 – preliminary study to obtain Australian Toxoplasma gondii isolates (Tg-AS01-Tg-AS09).

Heart sample Lab ID	Heart digest/ inoculation date	Heart digest *T. gondii* qPCR	Murine symptoms	Euthanasia date	Tissue sample	Murine tissue qPCR	Tissue inoculated onto Hs27 cells	Tachyzoites observed date	Isolate ID
FS17-0338	23.06.17	Detected	none	08.09.17	brain	ND*	brain	-	
FS17-0342	23.06.17	ND	none	08.09.17	brain	ND	brain	-	
FS17-0343	23.06.17	Detected	none	08.09.17	brain	Detected	brain	06.10.17	**Tg-AS09**
FS17-0344	23.06.17	ND	none	08.09.17	brain	ND	brain	-	
FS17-0345	23.06.17	ND	none	08.09.17	brain	ND	brain	-	
FS17-0346	23.06.17	ND	none	08.09.17	brain	ND	brain	-	
FS17-0359	07.07.17	Inhibition	none	29.08.17	liver	Detected	pooled	-	
					spleen	ND			
					lung	Detected			
					brain	Detected		-	
							heart digest	-	
FS17-0368	07.07.17	Detected	07.08.17	07.08.17	liver	ND	pooled	05.09.17	**Tg-AS06**
					spleen	Detected			
					lung	Detected	lung	-	
					brain	Detected	-		
FS17-0373	07.07.17	Detected	07.08.17	07.08.17	liver	Detected	pooled	28.08.17	**Tg-AS05**
					spleen	Detected			
					lung	Detected	-	-	
					brain	Detected	-		
FS17-0374	07.07.17	Detected	none	29.08.17	liver	Detected	pooled	03.10.17	**Tg-AS08**
					spleen	Detected			
					lung	Detected		-	
					brain	Detected	brain	03.10.17	
FS17-0377	07.07.17	Detected	07.08.17	07.08.17	liver	Detected	pooled	29.08.17	**Tg-AS07**
					spleen	Detected			

					lung	Detected	lung	28.08.17	
					brain	Detected	-	-	
FS17-0378	07.07.17	Detected	07.08.17	07.08.17	liver	Detected	pooled	06.09.17	
					spleen	Detected			
					lung	Detected	Detected		
					brain	Detected	-	-	
							heart digest		**Tg-AS02**
FS17-0362	10.07.17	Detected	01.08.17	01.08.17	liver	ND	liver	-	
					spleen	Inhibition	spleen	-	
					lung	Detected	lung	-	
FS17-0375	10.07.17	Detected	23.07.17	23.07.17	liver	ND	liver	16.08.17	
					spleen	Detected	spleen	16.08.17	
					lung	Detected	lung	09.08.17	**Tg-AS01**
FS17-0380	10.07.17	Detected	01.08.17	01.08.17	liver	Detected	liver	28.08.17	
					spleen	Inhibition	spleen	21.08.17	
					lung	Detected	lung	21.08.17	**Tg-AS03**
FS17-0381	10.07.17	Detected	01.08.17	01.08.17	liver	Detected	liver	28.08.17	**Tg-AS04**
					spleen	Inhibition	spleen	21.08.17	
					lung	Detected	lung	21.08.17	
FS17-0384	10.07.17	Detected	none	29.08.17	liver	ND	pooled	-	
					spleen	Detected			
					lung	Detected			
					brain	Detected	brain	-	
FS17-0385	10.07.17	Detected	none	29.08.17	liver	Detected	pooled	-	
					spleen	Inhibition			
					lung	Detected			
					brain	ND	brain	-	

*ND = not detected

3.3.4 Key results

- *T. gondii* isolates were successfully isolated from 9 of 18 Australian sheep hearts examined by mouse bio-assay (named Tg-AS01, Tg-AS02, Tg-AS03, Tg-AS04, Tg-AS05, Tg-AS06, Tg-AS07, Tg-AS08 and Tg-AS09).
- *T. gondii* (Tg-AS02) was successfully isolated from one of two sheep heart samples (FS17-0378) by direct culture.
- Neither mouse bio-assay nor direct culture could isolate *T. gondii* from sample FS17-0359.
- The preliminary comparable 50% success rate of both methods led to the decision to run a mouse bio-assay/direct culture comparison trial in future isolation attempts.

4. Stage 2 – Calibration of serological test

4.1 Background

Antibodies are the major component of humoral immunity that has evolved to combat disease-causing organisms. The serum level (i.e. titre) of a particular antibody against an organism can be high, low or negative, depending upon the organism, the duration of infection, whether the animal has rid itself of the organisms and other factors.

Antibody titres for *Toxoplasma* can be detected by serology tests that use *Toxoplasma* organisms as antigen. *Toxoplasma*-infected animals develop antibody titres against the parasite that endure for life, because although most animals will overcome and control the initial acute infection, *Toxoplasma* organisms are never completely removed from the body. Instead, the parasite switches from a rapidly growing form (tachyzoite) and becomes a slow growing form (bradyzoite) that permanently encysts in muscles, brain and heart which remain potentially infectious to people who consume rare meat. The continuous persistence of subclinical infection in an animal ensures that the immune system remains stimulated to produce antibodies. Since *Toxoplasma* antibody titres endure for life, it is possible to classify animals as being infected or not infected on the basis of serology. Animals with an antibody titre are classified as “seropositive” and animals without an antibody titre are classified as “seronegative”, which theoretically equates with being infected or being uninfected.

All serology tests are subject to errors. One type of error is to falsely classify an animal as being uninfected, when in fact, it is infected - a false-negative result. Another type of error is to falsely classify an animal as being infected, when in fact, it is uninfected – a false-positive result. Since there may be minor cross-reactions between antibodies against one organisms and a different organisms, and for other technical reasons, it is possible for a low antibody titre to be detected in an animal that is truly uninfected. To avoid false-positive results, it is necessary to establish a minimum antibody level below which the result can be confidently classified as negative. This minimum titre is called the “cut-off”. Setting the cut-off too low will result in too many false-positive results, but setting the cut-off too high will result in too many false-negative results. It can be very difficult to accurately establish the best cut-off, because it is often impossible to know with absolute certainty which animals are truly uninfected and which animals are truly infected.

The objective of Stage 2 was to create “gold standard” serum specimens which have been proven to be from truly uninfected or truly infected sheep and goats, so as to ensure that *Toxoplasma* serology testing methods can be properly assessed for accuracy. In particular, the goal was to ensure that false-

positive reactions can be avoided so that future testing of Australian small ruminants would not exaggerate the prevalence of *Toxoplasma* infection of lamb, sheep and goats.

1.1 Methodology

1.1.1 Animal ethics

The Stage 2 sheep and goat trial was carried out under the approved Animal Ethics application 0000021902: Production of control sera and tissues for *Toxoplasma gondii* and *Neospora caninum* in animals (S-2016-150) under the University of Adelaide. The mice used under this aspect of the project were covered by the same approval.

In summary, serologically negative sheep and goats were divided randomly into treatment and control groups and inoculated with either a known Australian *T. gondii* isolate or a placebo. They were then regularly serologically tested for an indication of seroconversion. After three months, animals were euthanised, exsanguinated and their tissues tested by qPCR, mouse bio-assay and direct cell culture.

1.1.2 Animal selection and husbandry

Twenty presumed seronegative sheep and ten presumed seronegative goats were purchased, tested and demonstrated to be serologically negative for *T. gondii*.

Ten sheep and five goats were randomly assigned to be inoculated with graded doses (1,000 or 100,000 tachyzoites) of one of four Australian sheep *T. gondii* isolates from Stage 1 (Tg-AS01, Tg-AS02, Tg-AS04, Tg-AS09) or an Australian dog isolate, supplied by Prof. John Ellis from UTS (1,000 or 60,000 tachyzoites of Tg-Dog) (Table 5). The sheep isolates (all from Kangaroo Island sheep) were selected simply on the basis that they had replicated sufficiently in tissue culture to be utilised; the dog isolate was the only pre-existing/available Australian *T. gondii* isolate (Al-Qassab et al., 2009). Although many *T. gondii* infection experiments in the literature use millions of organisms per animal, we wished to avoid inducing an excessive antibody response from artificially high infection doses. Therefore, low (1,000 organisms) and moderate (100,000 organisms) dosages were chosen.

The remaining ten lambs and five goats were kept as non-inoculated, negative control animals. The animals were housed indoors on the University of Adelaide's Roseworthy campus, provided with tap water and fed only processed feedstuffs, so as to avoid potential contamination with *T. gondii* oocysts.

Animals were housed for a total of three months. Animals (infected and uninfected) were bled prior to inoculation with *T. gondii* and again at eight time points; days 0, 7, 14, 21, 28, 49, 70 and at slaughter between days 82-93. At each time point, whole blood (50mL for sheep and 20mL for goats) was collected from the jugular vein, stored in 50mL conical centrifuge tubes and left standing at room temperature for several hours to allow the blood to clot. Tubes were centrifuged at 250-350 x g for 10 minutes at 4°C. The supernatant blood serum was decanted, aliquoted and stored at -80°C for future serological testing. Following the final blood collection point at slaughter, all animals were euthanised via lethal pentobarbital injection and tissues were collected from the brain, heart, tongue, diaphragm, psoas back muscle and rectus femoris thigh muscle

1.1.3 Sheep and goat serological testing

The *Toxoplasma* Modified Agglutination Test (MAT) produced by BioMérieux was selected as the serological test for validation and optimisation for use with sheep and goats. The manufacturer's instructions indicate the same cut-off titre (40) for all animals and people, which is quite low and is

probably designed to minimise false-negative reactions in people. The aim was to determine whether a higher cut-off titre could be justified for sheep and goats, so as to avoid false-positive reactions.

To develop gold standard sera, it was necessary to confirm the infected or uninfected status of animals (sheep and goats) using a number of supporting tests. The Toxo-Screen MAT screening test was used to determine the *T. gondii* serological status of sheep and goats prior to commencement of the trial. Serum was tested at three dilutions; 1:20, 1:40 and 1:2000. Samples were classified as serologically positive if a positive reaction was observed at the 1:20 dilution. This low cut-off titre (20, instead of the manufacturer's recommended 40) was used, so as to minimise the risk of a false-negative result. Animals with a titre of 1:20 or above were excluded from the trial.

The Toxo-Screen MAT screening test was used to determine the *T. gondii* serological status of gold-standard sheep and goats. All samples (inoculated and non-inoculated) were tested blind, with serum initially diluted 1:20 (100µL serum + 1.9mL PBS) and 1:2000 (50µL of 1:20 dilution + 2.450mL PBS) before 25µL of each were dispensed into adjacent wells of a 96 well micro titration plate. 25µL of 0.2 mol/L 2ME was added to each well, diluting the serum 1:40 and 1:4000. 50µL of toxoplasma antigen (diluted 1:5 with BABS albumin buffer) was then added to each well. All samples were tested in duplicate with positive, negative and antigen controls (provided in the Toxo-Screen MAT kit) included on each plate. The plate was gently shaken on bench to homogenise the well solutions, covered with a self-adhesive sheet and left to stand for 18 hours at room temperature, away from vibrations and sources of drying. Results were then read and interpreted. Serologically positive samples were re-tested using the Toxo-Screen MAT quantitative test at a range of dilutions (from 1:60 up to 1:1,180,980) to determine the maximum IgG serological titre for each sheep/goat at each blood collection interval.

1.1.4 Sheep and goat acid pepsin digestion

At the conclusion of the gold standard trial, heart tissues from the sheep and goats, including negative control animals, underwent acid pepsin digestion and a portion of their brains macerated. Tissue digests or brain material were subcutaneously injected into three bio-assay mice per trial animal (two with heart digest; one with brain tissue) to confirm active *T. gondii* infection.

The diaphragm, tongue, psoas back muscle and rectus femoris thigh muscle from selected test animals were also digested and analysed by qPCR for confirmation of results from the heart and brain tissue. The selected animals were those that were serologically positive but where *T. gondii* DNA was detected in neither the heart nor brain – specifically, one of five control and one of five inoculated goats and three of ten control and three of ten inoculated sheep.

The same methodology was carried out as described in Section 3.2.4 – Abattoir sheep heart acid pepsin digestion.

1.1.5 Bio-assay mouse serology

Isolation of live *T. gondii* organisms into mice was attempted from the brain and heart of all sheep and goats.

At euthanasia, approximately 1mL of blood was collected via cardiac puncture from mice inoculated with heart digest containing *T. gondii* and stored in 2mL plastic Eppendorf tubes. Whole blood was centrifuged at 15500 x g for 10 min at 4°C, separating red blood cells from blood serum. The Toxo-Screen MAT quantitative test was again used to determine the IgG serological titre of all samples.

Murine blood serum was diluted 1:20 and 1:2000 with PBS in 4mL glass tubes and vortexed thoroughly. 50µL of 0.2 mol/L 2ME was dispensed into the first well and 25µL 2ME was dispensed into the second and third successive wells of a 96 well micro titration plate. 25µL the 1:20 diluted serum was dispensed in the first well and mixed with a pipette, creating a 1:60 plate dilution. This process was repeated with the 1:20 and 1:2000 serum dispensed into wells two and three, creating a 1:40 and 1:4000 plate dilutions respectively. *Toxoplasma* antigen (diluted 1:5 with BABS albumin buffer) was then added to each well. All samples were tested in duplicate with positive, negative and antigen controls (provided in the Toxo-Screen MAT kit) included on each plate. The plate was gently shaken on the bench to homogenise the well solutions, covered with a self-adhesive sheet and left to stand for 18 hours at room temperature, away from vibrations and sources of drying. After this, results were read and interpreted; samples were classified as serologically positive if a positive reaction was observed at the 1:40 dilution.

The brain of each mouse was also harvested for cell culture, DNA extraction and analysis by qPCR.

1.1.6 Sheep and goat DNA extraction and purification

An aliquot of each digest was stored at -20°C for DNA extraction, purification and analysis by qPCR. The same methodology was carried out as described in Section 3.2.6 – Sheep and mouse Sheep and mouse DNA extraction and purif.

1.1.7 *Toxoplasma gondii* 529-bp repeat element qPCR

Detection of organisms in tissues of sheep and goats was attempted in the brain, heart and diaphragm using qPCR. Detection of *T. gondii* DNA was also carried out on murine tissue. The same methodology was carried out as described in Section 3.2.8 - *T.* gondii 529-bp repeat element.

1.1.8 Cell culture of sheep, goat and mouse tissue

The same methodology was carried out as described in Section 3.2.9 - Cell culture of sheep and mouse tissue.

1.1.9 Validation of serological test

Each sheep and goat was classified as “infected” if, in addition to developing a seropositive MAT titre using the manufacturer’s recommended titre cut-off of 1:40, one or more of the other test results was also positive (isolation of organisms, qPCR positive result in tissues, seroconversion of inoculated mice, qPCR positive tissue result in inoculated mice). Each sheep and goat was classified as “uninfected” if, in addition to remaining negative using *Toxoplasma* MAT serology, all other test results were also negative. Results between inoculated test animals and non-inoculated negative control animals were compared using the Fisher Exact test.

1.2 Results

1.2.1 Gold-standard sheep and goat trial

At one week post inoculation (Day 7), one out of five of the *T. gondii* inoculated goats (goat inoculated with 100,000 tachyzoites of Tg-AS09) and three out of ten of the *T. gondii* inoculated sheep (inoculated with either 100,000 tachyzoites of Tg-AS09, 100,000 tachyzoites of Tg-AS09 or 60,000 tachyzoites of Tg-Dog) were found to be serologically positive.

By two weeks post inoculation (Day 14), all sheep and goats inoculated with *T. gondii* were found to be serologically positive whilst all control, non-inoculated sheep and goats remained serologically negative. This result was again observed at three, four, seven, eleven- and twelve-weeks post inoculation. A higher initial dose rate appeared to stimulate a stronger initial immunological response but the peak serological titre achieved appeared unrelated to the initial dose.

This result validated the Toxo-Screen MAT test for the detection of *T. gondii* in sheep and goats.

All serologically positive samples were re-tested using the Toxo-Screen MAT quantitative test at a range of dilutions (from 1:60 up to 1:1,180,980) to determine the maximum IgG serological titre for each lamb/goat at each blood collection interval. Maximum serological titres for 14/15 of the gold-standard positive animals peaked at between 1458-13,122 IU/mL. One goat (60,000 Tg-Dog) was found to have a maximum serological titre of 39,366 IU/mL at 11 weeks post inoculation. All sheep/goat titres peaked at between 3-11 weeks post inoculation (Table 5). The complete set of serological results are contained in Appendix 1.

Table 5: Sheep, goat and bio-assay mouse serological results from inoculation sheep and goat trial

Sheep/goat ID	***T. gondii* strain**	**Dose (tachyzoites)**	**Sheep/goat *T. gondii* screen result**	**Sheep/goat peak *T. gondii* titre**	**Murine *T. gondii* screen result**
G1	Tg-Dog	60,000	Seropositive	39366 IU/mL (at 11 weeks)	Seronegative
G3	Tg-AS01	100,000	Seropositive	4374 IU/mL (at 4 weeks)	2/3 seropositive
G26	Tg-AS02	100,000	Seropositive	13122 IU/mL (at 11 weeks)	2/3 seropositive
G27	Tg-AS04	100,000	Seropositive	13122 IU/mL (at 4 weeks)	1/3 seropositive
G17	Tg-AS09	100,000	Seropositive	13122 IU/mL (at 7 weeks)	2/3 seropositive
G7	Control	-	Seronegative	-	Seronegative
G16	Control	-	Seronegative	-	Seronegative
G20	Control	-	Seronegative	-	Seronegative
G21	Control	-	Seronegative	-	Seronegative
G24	Control	-	Seronegative	-	Seronegative
S37	Tg-Dog	60,000	Seropositive	4374 IU/mL (at 7 weeks)	Seronegative
S32	Tg-Dog	1,000	Seropositive	1458 IU/mL (at 4 weeks)	Seronegative
S33	Tg-AS01	100,000	Seropositive	4374 IU/mL (at 7 weeks)	Seronegative
S41	Tg-AS01	1,000	Seropositive	13122 IU/mL (at 7 weeks)	Seronegative
S31	Tg-AS02	100,000	Seropositive	13122 IU/mL (at 7 weeks)	2/3 seropositive
S34	Tg-AS02	1,000	Seropositive	1458 IU/mL (at 4 weeks)	Seronegative
S47	Tg-AS04	100,000	Seropositive	1458 IU/mL (at 3 weeks)	1/3 seropositive
S36	Tg-AS04	1,000	Seropositive	1458 IU/mL (at 3 weeks)	Seronegative
S39	Tg-AS09	100,000	Seropositive	4374 IU/mL (at 7 weeks)	2/3 seropositive
S42	Tg-AS09	1,000	Seropositive	13122 IU/mL (at 7 weeks)	Seronegative
S35	Control	-	Seronegative	-	Seronegative
S38	Control	-	Seronegative	-	Seronegative
S40	Control	-	Seronegative	-	Seronegative
S43	Control	-	Seronegative	-	Seronegative
S44	Control	-	Seronegative	-	Seronegative
S45	Control	-	Seronegative	-	Seronegative
S46	Control	-	Seronegative	-	Seronegative
S48	Control	-	Seronegative	-	Seronegative
S49	Control	-	Seronegative	-	Seronegative
S50	Control	-	Seronegative	-	Seronegative

In addition, as all gold-standard serologically positive sheep and goats produced a positive result and all serologically negative sheep and goats produced a negative result at the 1:60 serum dilution, this was determined to be an appropriate cut-off dilution to determine the serological *T. gondii* status of sheep in Stage 3, the national survey of *T. gondii* in sheep, to minimise false positives.

Using qPCR, *T. gondii* genome was detected in the heart digests of four out of five inoculated goats (all injected with a high dose (100,000 organisms) of tachyzoites) and six out of ten inoculated sheep (four injected with a high dose (100,000 organisms) of tachyzoites). No *T. gondii* genome was detected in the control animals or the diaphragm, tongue, psoas back muscle and rectus femoris thigh muscle of the selected animals. Tg-Dog injections at both low and moderate doses did not lead to the detection of *T. gondii* DNA in any sheep or goats (Table 6).

Table 6: qPCR results from sheep and goats in gold-standard sheep and goat trial.

Sheep/goat ID	*T. gondii* strain	Dose	*T. gondii* DNA					
			Brain	Heart	Diaphragm	Tongue	Psoas	Femoris
G1	Tg-Dog	60,000	ND*	ND	ND	ND	ND	ND
G3	Tg-AS01	100,000	ND	Detected				
G17	Tg-AS09	100,000	ND	Detected				
G26	Tg-AS02	100,000	ND	Detected				
G27	Tg-AS04	100,000	ND	Detected				
G7	Control	-	ND	ND	ND	ND	ND	ND
G16	Control	-	ND	ND				
G20	Control	-	ND	ND				
G21	Control	-	ND	ND				
G24	Control	-	ND	ND				
S31	Tg-AS02	100,000	ND	Detected				
S32	Tg-Dog	1,000	ND	ND	ND	ND	ND	ND
S33	Tg-AS01	100,000	ND	Detected				
S34	Tg-AS02	1,000	ND	ND	ND	ND	ND	ND
S36	Tg-AS04	1,000	ND	Detected				
S37	Tg-Dog	60,000	ND	ND	ND	ND	ND	ND
S39	Tg-AS09	100,000	ND	Detected				
S41	Tg-AS01	1,000	ND	ND				
S42	Tg-AS09	1,000	ND	Detected				
S47	Tg-AS04	100,000	ND	Detected				
S35	Control	-	ND	ND	ND	ND	ND	ND
S38	Control	-	ND	ND				
S40	Control	-	ND	ND				
S43	Control	-	ND	ND				
S44	Control	-	ND	ND	ND	ND	ND	ND
S45	Control	-	ND	ND				
S46	Control	-	ND	ND				
S48	Control	-	ND	ND				
S49	Control	-	ND	ND				
S50	Control	-	ND	ND	ND	ND	ND	ND

*ND = not detected; Blank cells indicate not tested

To investigate the validity of direct culture of heart digest or brain onto cell culture, heart digests and brains from ten seropositive and five seronegative sheep were inoculated onto Hs27 cells, as described in the methods, and incubated at 37°C with 5% CO_2.

T. gondii was cultured directly from heart digests in four out of ten inoculated sheep samples – S31, S39, S42 and S47 (three high dose and one low dose animal), but none of the control sheep. No *T. gondii* was successfully cultured from direct inoculation of the sheep brain tissue (Table 7).

Table 7: Direct cell culture results from sheep hearts and brains in gold-standard sheep and goat trial.

Sheep ID	Inoculation	Brain	Heart digest
S31	Tg-AS02 100,000	ND*	Detected
S32	Tg-Dog 1,000	ND	ND
S33	Tg-AS01 100,000	ND	ND
S34	Tg-AS02 1,000	ND	ND
S36	Tg-AS04 1,000	ND	ND
S37	Tg-Dog 60,000	ND	ND
S39	Tg-AS09 100,000	ND	Detected
S41	Tg-AS01 1,000	ND	ND
S42	Tg-AS09 1,000	ND	Detected
S47	Tg-AS04 100,000	ND	Detected
S35	Control	ND	ND
S38	Control	ND	ND
S43	Control	ND	ND
S46	Control	ND	ND
S48	Control	ND	ND

*ND = not detected

1.2.2 Mouse bio-assay

For each gold-standard seropositive and seronegative sheep and goat, two mice were inoculated with heart digest and one with brain material (mice n=90). None of the 90 mice developed clinical signs associated with an active *T. gondii* infection. However, a few male mice had to be separated into individual housing cages due to concerns over aggressive behaviour which resulted in minor wounds. At eight weeks post inoculation, blood serum was collected from mice at euthanasia and serologically tested.

Inoculation of heart digests from three out of ten gold-standard seropositive sheep and four out of five gold-standard seropositive goats resulted in at least one serologically positive mouse, whilst none of the heart digests from the remaining gold-standard seropositive and all seronegative sheep and goats resulted in serologically positive mice (Table 5). None of the mice injected with brain material were seropositive. Maximum serological titres were not established as the Toxo-Screen MAT screening test provides a serological result at only two dilutions, 1:40 and 1:4000, indicating either a positive or negative result.

In addition to the serological testing of the murine blood serum, the brain of each mouse was harvested at euthanasia and the DNA extracted and tested by qPCR to detect the presence of *T. gondii* DNA.

- *T. gondii* DNA was only detected in the brains of mice injected with the **heart** digest of goats (seven out of ten) and sheep (five out of ten) inoculated with a high dose (100,000 organisms) of tachyzoites (Table 8).
- *T. gondii* DNA was not detected in the brains of mice injected with the brain or heart of control goats or sheep.
- *T. gondii* DNA was not detected in the brains of mice injected with the brain of inoculated goats or sheep.

- *T. gondii* DNA was not detected in the brains of mice injected with the heart digest of goats and sheep inoculated with Tg-Dog tachyzoites.

Table 8: qPCR results from murine brain in gold-standard sheep and goat trial.

Sheep/goat ID	Mouse	*T. gondii* Inoculum	Tissue Inoculum	*T. gondii* DNA
G1	1	Tg-Dog 60,000	Brain	ND*
	2		Heart digest	ND
	3		Heart digest	ND
G3	1	Tg-AS01 100,000	Brain	ND
	2		Heart digest	Detected
	3		Heart digest	Detected
G17	1	Tg-AS09 100,000	Brain	ND
	2		Heart digest	Detected
	3		Heart digest	Detected
G26	1	Tg-AS02 100,000	Brain	ND
	2		Heart digest	Detected
	3		Heart digest	Detected
G27	1	Tg-AS04 100,000	Brain	ND
	2		Heart digest	ND
	3		Heart digest	Detected
G7	1	Control	Brain	ND
	2		Heart digest	ND
	3		Heart digest	ND
G16	1	Control	Brain	ND
	2		Heart digest	ND
	3		Heart digest	ND
G20	1	Control	Brain	ND
	2		Heart digest	ND
	3		Heart digest	ND
G21	1	Control	Brain	ND
	2		Heart digest	ND
	3		Heart digest	ND
G24	1	Control	Brain	ND
	2		Heart digest	ND
	3		Heart digest	ND
S31	1	Tg-AS02 100,000	Brain	ND
	2		Heart digest	Detected
	3		Heart digest	Detected
S32	1	Tg-Dog 1,000	Brain	ND
	2		Heart digest	ND
	3		Heart digest	ND
S33	1	Tg-AS01 100,000	Brain	ND
	2		Heart digest	ND
	3		Heart digest	ND
S34	1	Tg-AS02 1,000	Brain	ND
	2		Heart digest	ND
	3		Heart digest	ND
S36	1	Tg-AS04 1,000	Brain	ND
	2		Heart digest	ND

	3		Heart digest	ND
S37	1	Tg-Dog 60,000	Brain	ND
	2		Heart digest	ND
	3		Heart digest	ND
S39	1	Tg-AS09 100,000	Brain	ND
	2		Heart digest	Detected
	3		Heart digest	Detected
S41	1	Tg-AS01 1,000	Brain	ND
	2		Heart digest	ND
	3		Heart digest	ND
S42	1	Tg-AS09 1,000	Brain	ND
	2		Heart digest	ND
	3		Heart digest	ND
S47	1	Tg-AS04 100,000	Brain	ND
	2		Heart digest	Detected
	3		Heart digest	ND
S35	1	Control	Brain	ND
	2		Heart digest	ND
	3		Heart digest	ND
S38	1	Control	Brain	ND
	2		Heart digest	ND
	3		Heart digest	ND
S40	1	Control	Brain	ND
	2		Heart digest	ND
	3		Heart digest	ND
S43	1	Control	Brain	ND
	2		Heart digest	ND
	3		Heart digest	ND
S44	1	Control	Brain	ND
	2		Heart digest	ND
	3		Heart digest	ND
S45	1	Control	Brain	ND
	2		Heart digest	ND
	3		Heart digest	ND
S46	1	Control	Brain	ND
	2		Heart digest	ND
	3		Heart digest	ND
S48	1	Control	Brain	ND
	2		Heart digest	ND
	3		Heart digest	ND
S49	1	Control	Brain	ND
	2		Heart digest	ND
	3		Heart digest	ND
S50	1	Control	Brain	ND
	2		Heart digest	ND
	3		Heart digest	ND

*ND = not detected

To investigate the validity of direct culture of murine brain tissue onto cell culture, the pooled brains of each group of three mice were inoculated onto Hs27 cells, as described in the methods, and

incubated at 37°C with 5% CO_2. *T. gondii* was cultured from four out of five seropositive goats, three out of ten seropositive sheep and no control animals (Table 9).

Table 9: Direct cell culture results from murine brain tissue in gold-standard sheep and goat trial.

Sheep/goat ID	Inoculation	*T. gondii* culture
G1	Tg-Dog 60,000	ND*
G3	Tg-AS01 100,000	Detected
G17	Tg-AS09 100,000	Detected
G26	Tg-AS02 100,000	Detected
G27	Tg-AS04 100,000	Detected
G7	Control	ND
G16	Control	ND
G20	Control	ND
G21	Control	ND
G24	Control	ND
S31	Tg-AS02 100,000	Detected
S32	Tg-Dog 1,000	ND
S33	Tg-AS01 100,000	ND
S34	Tg-AS02 1,000	ND
S36	Tg-AS04 1,000	ND
S37	Tg-Dog 60,000	ND
S39	Tg-AS09 100,000	Detected
S41	Tg-AS01 1,000	ND
S42	Tg-AS09 1,000	ND
S47	Tg-AS04 100,000	Detected
S35	Control	ND
S38	Control	ND
S43	Control	ND
S46	Control	ND
S48	Control	ND

*ND = not detected

1.2.3 Key results

- Using the classification defined in Section 1.1.9, 11 out of 15 sheep and goats inoculated with *T. gondii* were classified as positive or infected. All 15 non-inoculated sheep and goats (controls) were classified as negative or uninfected. There is a statistically significant difference in the prevalence (Fisher Exact Test, P-value < 0.001).
    - This is a good result for the validation of the gold-standard positive and negative specimens. Validation of negative specimens is rare, so this is an especially valuable achievement.
    - The two sheep and the single goat inoculated with the Tg-Dog isolate were classified as negative or uninfected, despite developing strong antibody titres following infection. This result implies that this particular strain of *T. gondii* may be naturally attenuated and unable to establish permanent viable infections. Thus, Tg-Dog should be considered as a strong potential candidate vaccine strain to prevent abortion and/or to prevent chronic infection of small ruminants.

- All inoculated sheep and goats became seropositive, far exceeding the manufacturer's recommended cut-off titre of 40 and all non-inoculated animals remained seronegative.
    - This is a clear result (P-value < 0.001), tested using a blinded protocol to avoid bias.
    - The appropriate cut-off dilution to determine the serological *T. gondii* status of Australian sheep was determined to be the 1:60 serum dilution.
        - This cut-off was applied during the Stage 3 national sheep survey.

2. Stage 3 – National survey of *T. gondii* in sheep

2.1 Background

The commercial *Toxoplasma* serological test validated for Australian sheep and goats in Stage 2 was utilised in Stage 3 to update a national sheep prevalence baseline study, previously conducted for MLA in 2008. The aim was to provide a more accurate prevalence estimate and demonstrate the viability or otherwise of *T. gondii* present in sheep meat by bio-assay. This would help assess the risk associated with the consumption of undercooked sheep meat and provide a library of genotypes present in Australia for future investigation.

2.2 Methodology

In summary, paired mutton heart and blood samples were collected nationally at slaughter, in proportion to state sheep population. Kangaroo Island sheep were excluded from the national survey, as they had already been sampled in the Stage 1 pilot study. Processed heart tissue from serologically positive animals were then examined by qPCR and bio-assayed in mice to confirm viability and isolate *T. gondii*.

2.2.1 Animal ethics

The mice used for the mouse bio-assay component of the national sheep survey were covered by two Animal Ethics applications under the University of Adelaide: 0000021902: Production of control sera and tissues for *Toxoplasma gondii* and *Neospora caninum* in animals (S-2016-150) and 34071: Assessing the consumer risk from *Toxoplasma gondii* in meat products.

2.2.2 Sample allocation and collection

Paired sheep blood and heart samples were opportunistically collected from export abattoirs around Australia in order to represent the geographical spread of sheep production in Australia. As *T. gondii* is an all-of-life infection, mature sheep or mutton (as opposed to lambs) have a higher expected prevalence of infection. Although lamb potentially presents the greatest consumer risk (due to the likelihood of it being consumed rare), the genotypes can be expected to be the same as in mature sheep.

The original plan was to survey both sheep and goats, but Stage 2 demonstrated that sheep and goats were susceptible to the same strains so a decision was made to focus resources on sampling sheep.

A sample size of 350 was calculated based on prevalence estimates from the previous baseline survey (A.MFS.0129) and to give a margin of error of ±5% and a confidence level of 95%. The number of samples was allocated on a proportional basis to reflect the contribution of each state to the national

sheep kill (Table 10) – the average annual kill figures (over a five year period, 1 July 2013 to 30 June 2017) were sourced from the Australian Bureau of Statistics (ABS).

Table 10: Number of sheep slaughtered and allocation of samples by state (2013-2017, ABS).

	NSW	VIC	QLD	SA	WA	TAS	Total
Sheep kill	2,015,700	3,195,300	379,800	1,266,000	1,323,800	211,400	8,392000
Proportion of total sheep kill	24%	38%	4.5%	15%	16%	2.5%	100%
Number of samples	84	133	16	53	55	9	350

During the sampling process, sheep hearts were selected from the offal tray on the slaughter floor and blood from each heart was poured into 8.5mL gold capped BD Vacutainer SST II Advance tubes. Exsanguinated hearts were then placed into labelled sample bags and transported on ice along with matching blood tubes to the SARDI Food Science laboratories (Urrbrae, South Australia). Blood tubes were centrifuged at 3700 x g for 10 min at 4°C, allowing the gel layer to separate red blood cells from blood serum. Approximately 2mL of supernatant blood serum was decanted, aliquoted and stored at 4°C for immediate serological testing.

2.2.3 Serological testing

Serology was performed using the Toxo-Screen MAT quantitative test. Rather than using the threefold dilution series of 1:60, 1:180, 1:540, 1:1620, 1:4860 and 1:14580 outlined in the manufacturer's instructions, serum was instead diluted 1:20, 1:200 and 1:500 in glass tubes before being dispensed into the plate wells containing 0.2 mol/L 2ME. The final plate dilutions for all national prevalence survey samples were 1:60, 1:400 and 1:1000. This change increased the throughput of the quantitative test protocol through the preservation of kit reagents whilst still allowing researchers to identifying 'strong' serological positives for acid pepsin digestion and subsequent mouse bio-assay and direct cell culture attempts.

Sheep serum samples and control sera were diluted 1:20 (100µL serum + 1.9mL PBS), 1:200 (200µL of 1:20 serum + 1.8mL PBS) and 1:500 (100µL of 1:20 serum + 2.4mL PBS) in 4mL glass tubes and vortexed thoroughly. 50µL of 0.2 mol/L 2ME was dispensed into the first well and 25µL dispensed into the second and third successive wells of a 96 well micro titration plate. 25µL of the 1:20 diluted serum was dispensed in the first well and mixed with a pipette, creating a 1:60 plate dilution. This process was repeated as the 1:200 and 1:500 diluted serum were dispensed into the second and third successive wells, creating 1:400 and 1:1000 plate dilutions respectively. 50µL of *Toxoplasma* antigen (diluted 1:5 with BABS albumin buffer) was then added to each well. All samples were tested in duplicate with positive, negative and antigen controls (provided in the Toxo-Screen MAT kit) included on each plate. The plate was gently shaken on the bench to homogenise the well solutions, covered with a self-adhesive sheet and left to stand for 18 hours at room temperature, away from vibrations and sources of drying. Results were then read and interpreted; samples were classified as serologically positive if a positive reaction was observed at the 1:60 dilution. This cut-off serum dilution of 1:60 was previously validated by the results of the gold standard sheep and goat trial (Stage 2).

Paired heart samples from selected serologically positive sheep underwent acid-pepsin digestion and were subcutaneously injected into mice or directly inoculated onto cell culture.

2.2.4 Acid pepsin digestion

The same methodology was carried out as described in Section 3.2.44 - Abattoir sheep heart acid pepsin digestion.

2.2.5 Mouse bio-assay

At euthanasia, approximately 1mL of blood was collected via cardiac puncture from all mice inoculated with heart digest and stored in 2mL plastic Eppendorf tubes. Whole blood was centrifuged at 15500 x g for 10 minutes at 4°C, separating red blood cells from blood serum. The Toxo-Screen MAT screening test was used to determine whether IgG antibodies specific to *T. gondii* were present within the serum. Murine blood serum was diluted 1:20 and 1:2000 with PBS in 4mL glass tubes and vortexed thoroughly. 25µL of 0.2 mol/L 2ME was dispensed into the first two rows of wells of a 96 well micro titration plate. 25µL the 1:20 diluted serum was dispensed in the first well and mixed with a pipette, creating a 1:40 plate dilution. This process was repeated with the 1:2000 serum, creating a 1:4000 plate dilution. *Toxoplasma* antigen (diluted 1:5 with BABS albumin buffer) was added to each well. All samples were tested in duplicate with positive, negative and antigen controls (provided in the Toxo-Screen MAT kit) included on each plate. The plate was gently shaken on the bench to homogenise the well solutions, covered with a self-adhesive sheet and left to stand for 18 hours at room temperature, away from vibrations and sources of drying. After this, results were read and interpreted – samples were classified as serologically positive if a positive reaction was observed at the 1:40 dilution.

2.2.6 DNA extraction and purification

The same methodology was carried out as described in Section 3.2.6 – Sheep and mouse Sheep and mouse DNA extraction and purif.

2.2.7 Mammalian house-keeping gene PCR

The same methodology was carried out as described in Section 3.2.7 - Mammalian house-keeping gene PCR.

2.2.8 *T. gondii* 529-bp repeat element qPCR

The same methodology was carried out as described in Section 3.2.8 - *T.* gondii 529-bp repeat element.

2.2.9 Cell culture

The same methodology was carried out as described in Section 3.2.9 - Cell culture of sheep and mouse tissue.

2.3 Results

2.3.1 Sample collection

A total of 401 paired mutton samples (blood and heart) were collected from several export abattoirs located in South Australia, Victoria, New South Wales, Queensland and Western Australia (note, Tasmanian sheep were sourced from a Victorian abattoir). Figure 2 shows the geographical distribution from which the sampled sheep originated. For the majority of samples, it was possible to use National Vendor Declarations (NVDs) to identify the exact geographical location from which the

sheep that were sampled originated. However, for some samples (in particular, sheep from Western Australian and Tasmania), sheep were processed in mixed lots and it was not possible to know the exact geographical origin of the herds.

Sampling was complicated by a shortage of mutton leading to erratic slaughter and mixed farm lots in 2019/20 – abattoir lots from saleyards could contain groups of 2 or 3 sheep from 40 or 50 farms. In addition, 2020 COVID-19 restrictions limited both state and abattoir access.

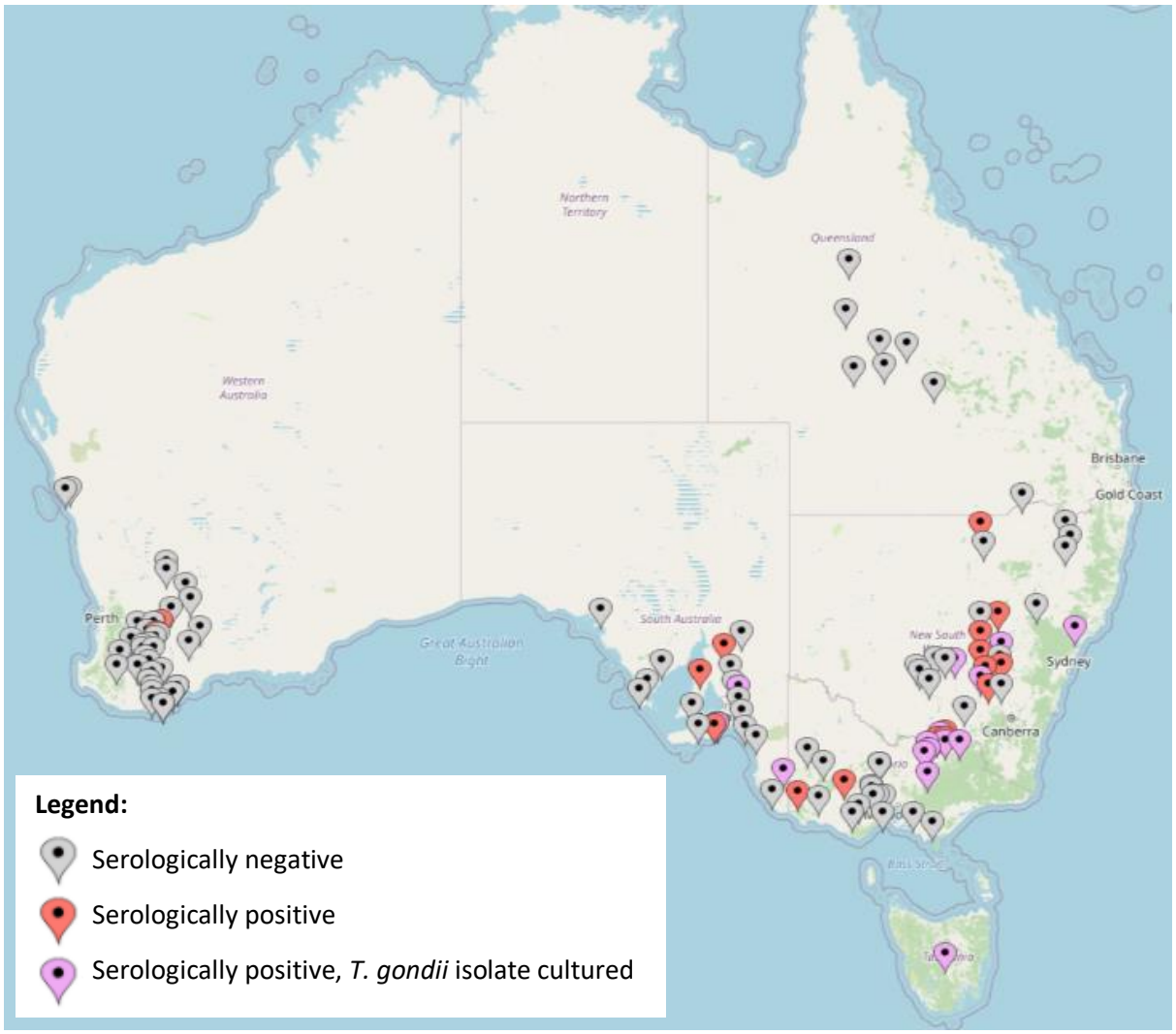

Figure 2: Map showing the geographical distribution of sheep sampled in the national T. gondii prevalence survey. The colour of the markers reflects the T. gondii status of each group of samples from that location; grey indicates serologically negative samples, red and pink indicate serologically positive samples, (pink indicates those serologically positive samples from which T. gondii isolates were cultured).

2.3.2 Sheep hearts

Forty-six out of the 401 sheep samples collected were found to be serologically positive, giving an estimated Australian sheep *T. gondii* prevalence of 11.5%. Table 11 shows a detailed summary of the state-by-state distribution of samples and the *T. gondii* prevalence for all sheep samples.

Table 11: Serological results for Stage 3 – state-by-state distribution of sheep samples and T. gondii prevalence.

State	Number of serological positives /total number sampled	*T. gondii* serological prevalence	Number of *T. gondii* isolates cultured
New South Wales	21/89	23.6%	1
South Australia	11/68	16.2%	4
Victoria	7/139	5.0%	2
Western Australia	3/71	4.2%	0
Tasmania	4/9	44.4%	2
Queensland	0/25	0.0%	0
Total	**46/401**	**11.5%**	**9**

Tasmania was observed to have the highest *T. gondii* prevalence (44.4%). However, to put this result in context, only a small number of Tasmanian samples were collected (n=9) as a mixed saleyard lot at a Victorian abattoir and the associated NVDs were not able to be retrieved. COVID-19 restrictions meant we were unable to collect additional samples in Tasmania. Therefore, the very high prevalence may be an artefact of sample size or sampling bias. It is possible for example, that the serologically positive sheep may have originated from a single Tasmanian property and that the samples do not represent the broader Tasmanian sheep population. On the other hand, anecdotally Tasmania, like Kangaroo Island, is considered to be a *Toxoplasma* "hotspot". Tasmania has one of the highest recorded rates of cat-carried *T. gondii* infection in the world, according to a CSIRO study in 2014. This study found that over 84% of stray and feral cats carried the parasite. (Fancourt and Jackson, 2014)

New South Wales (23.6%) and South Australia (16.2%) were shown to have a higher *T. gondii* prevalence in comparison to the national figure of 11.5%. Conversely, *T. gondii* in Western Australia (4.2%) and Victoria (5%) were found to be lower than the national level. No samples from Queensland were found to be serologically positive for *T. gondii*. Care should be taken in interpreting these apparent state differences, however, due to the small sample sizes.

Due to limitations in the number of samples that could be digested at one time, samples with high titres, indicated by positive reactions at 1:400 and/or 1:1000, were prioritised for acid pepsin digestion and inoculation into mice. This was done in order to maximise the chance of culturing viable *T. gondii*. Where possible, serologically positive samples from a wide range of geographical locations were also prioritised so that *T. gondii* isolates could be captured from different regions throughout Australia (Figure 2).

Paired heart samples from 33 of the 46 serologically positive sheep samples underwent acid pepsin digestion. Twenty-nine of the 33 serologically positive heart digests were inoculated directly into cell culture and injected into bio-assay mice. Two out of the 33 were injected only into mice while the last two digests were inoculated only into cell culture due to project time constraints (Table 13).

Each of the 33 heart digests were analysed for the presence of *T. gondii* DNA using qPCR and *T. gondii* DNA was detected in 9 of the 33 heart digests. *T. gondii* was also successfully cultured directly from seven of the 9 heart digests.

Although low qPCR CT values are associated with high numbers of the *T. gondii* 529-bp repeat element, and therefore indicative of a higher concentration of organisms in the heart sample, the inherent possibility of sampling error (for example, qPCR using only 25uL from 500mL of digest) makes

estimating the actual number of organisms present problematic. None-the-less, it is possible to rank the samples by CT value and to compare with the serological response.

2.3.3 Mouse bio-assay

None of the 31 mice inoculated with serologically positive sheep heart digests from the national survey developed clinical signs associated with an active *T. gondii* infection. At eight weeks post inoculation, blood serum was collected from mice at euthanasia and serologically tested. Seven out of the 31 mice were found to be serologically positive for *T. gondii* (Table 12). Using qPCR, *T. gondii* DNA was detected in seven of the 31 murine brains, one of which was seronegative (Tables 12 & 13).

In total, nine *T. gondii* isolates were successfully cultured from the national survey, designated Tg-AS10 to Tg-AS18 (Tables 12 and 13). Each isolate was purified and stored under liquid nitrogen as described in the methods.

Table 12: Serological and cell culture results for serologically positive sheep in national survey.

Sample ID	**State**	**Sheep *T. gondii* titre (IU/mL)**	**Mouse *T. gondii* serology result**	**Isolate ID**	**Successful culture method/s**
FS19-068	SA	400	Seropositive	**Tg-AS10**	Direct culture and mouse bio-assay
FS19-071	SA	100	Seronegative	ND*	
FS19-103	SA	100	Seronegative	**Tg-AS13**	Mouse bio-assay
FS19-116	NSW	6	Seronegative	ND	
FS19-140	VIC	6	Seropositive	**Tg-AS14**	Mouse bio-assay
FS19-144	SA	6	Seronegative	ND	
FS19-149	NSW	100	Seropositive	**Tg-AS12**	Direct culture and mouse bio-assay
FS19-150	NSW	6	Seronegative	ND	
FS19-156	NSW	100	Seronegative	ND	
FS19-159	NSW	100	Seronegative	**Tg-AS11**	Direct culture
FS19-172	NSW	6	Seronegative	ND	
FS19-176	NSW	40	Seronegative	ND	
FS19-180	NSW	40	Seronegative	ND	
FS19-183	NSW	6	Seropositive	ND	
FS19-185	SA	100	Seronegative	**Tg-AS16**	Direct culture and mouse bio-assay
FS19-190	SA	100	Seropositive	**Tg-AS15**	Direct culture and mouse bio-assay
FS19-195	NSW	6	Seronegative	ND	
FS19-196	NSW	6	Seronegative	ND	
FS19-198	NSW	6	Seronegative	ND	
FS19-199	NSW	6	Seronegative	ND	
FS19-204	NSW	40	Seronegative	ND	
FS19-206	NSW	40	Seronegative	ND	
FS19-213	NSW	100	Seronegative	ND	
FS19-239	NSW	100	Seronegative	ND	
FS19-272	VIC	100	Seronegative	ND	
FS19-273	VIC	100	Seronegative	ND	
FS19-278	TAS	100	Seronegative	ND	
FS19-280	TAS	100	Seropositive	**Tg-AS17**	Direct culture and mouse bio-assay
FS19-284	TAS	100	Seropositive	**Tg-AS18**	Direct culture and mouse bio-assay
FS19-286	TAS	100	Seronegative	ND	
FS20-095	WA	6	Seronegative	ND	
FS20-372	WA	400	Mouse bio-assay not used	ND	
FS20-375	WA	400	Mouse bio-assay not used	ND	

*ND = not detected

Table 13: qPCR and cell culture results for national sheep survey.

	Heart digest			Murine brains		
Sample ID	***T. gondii* DNA**	**Cell culture tachyzoites observed**	**Isolate ID**	***T. gondii* DNA**	**Cell culture tachyzoites observed**	**Isolate ID**
FS19-0068	Detected	yes	**Tg-AS10**	Detected	yes	
FS19-0071	ND	not inoculated		ND	no	
FS19-0103	ND	no		ND	yes	**Tg-AS13**
FS19-0116	ND	no		ND	no	
FS19-0140	ND	no		Detected	yes	**Tg-AS14**
FS19-0144	ND	no		ND	no	
FS19-0149	Detected	yes	**Tg-AS12**	Detected	yes	
FS19-0150	ND	not inoculated		ND	no	
FS19-0156	ND	no		ND	no	
FS19-0159	Detected	yes	**Tg-AS11**	ND	no	
FS19-0172	ND	no		ND	no	
FS19-0176	ND	no		ND	no	
FS19-0180	ND	no		ND	no	
FS19-0183	ND	no		ND	no	
FS19-0185	Detected	yes	**Tg-AS16**	Detected	yes	
FS19-0190	Detected	yes	**Tg-AS15**	Detected	yes	
FS19-0195	ND	no		ND	no	
FS19-0196	ND	no		ND	no	
FS19-0198	ND	no		ND	no	
FS19-0199	ND	no		ND	no	
FS19-0204	ND	no		ND	no	
FS19-0206	ND	no		ND	no	
FS19-0213	ND	no		ND	no	
FS19-0239	Detected	no		ND	no	
FS19-0272	ND	no		ND	no	
FS19-0273	ND	no		ND	no	
FS19-0278	ND	no		ND	no	
FS19-0280	Detected	yes	**Tg-AS18**	Detected	yes	
FS19-0284	Detected	yes	**Tg-AS17**	Detected	yes	
FS19-0286	Detected	no		ND	no	
FS20-0095	ND	no		ND	no	
FS20-0372	ND	no		not inoculated	-	
FS20-0375	ND	no		not inoculated	-	

*ND = not detected

T. gondii was successfully cultured through inoculation of Hs27 cells with the brain tissue of eight mice, including two serologically negative mice – Tg-AS13 and Tg-AS16 (Table 12). DNA extracts from the two serologically negative mice were analysed by qPCR, one mouse was found to be positive and one was negative. It is possible that inoculation of these two mice with *T. gondii* resulted in a *T. gondii* infection, hence, the cell culture results, without producing an IgG antibody response sufficient enough to be detected by the Toxo-Screen MAT screening test.

2.3.4 Key results

- The estimated Australian sheep *T. gondii* serological prevalence is 11.5% (46 positive samples out of 401).
- Tasmania appears to have a very high prevalence, albeit based on a small sample size.
- Nine additional *T. gondii* isolates were successfully cultured from the national survey, designated Tg-AS10 to Tg-AS18.

3. Stage 4 - Genotyping of isolates

3.1 Background

The development of simple, sensitive and rapid methods for the detection and identification of *T. gondii* has been important for diagnosis and epidemiological studies of this zoonotic parasite. Over the past 20 years, molecular methods based on a variety of genetic markers have been developed, each with their own advantages and limitations. Until now, Australian studies of *T. gondii* have focussed primarily on detection and estimates of prevalence, with little genetic characterisation undertaken. Genetic characterisation, however, is important in generating additional information for epidemiological, population and phylogenetic studies of this pathogen of growing public health concern.

T. gondii has effectively established infection cycles in wild and domestic warm-blooded animals and humans, with some estimates putting the global human infection rate at 30 to 40%. A large part of its success is its ability to propagate both sexually (in the feline gut) and clonally (intermediate host to intermediate host). Genetic recombination during sexual reproduction may lead to highly successful lines that then spread clonally e.g. through cannibalism, expanding the parasite's host range (Grigg and Sundar, 2009).

Historically, using multi-locus enzyme electrophoresis, *T. gondii* was divided into four lineages (I, II, III and atypical). Advances in molecular methods have enabled further differentiation into 16 haplogroups, representing six clades (Lorenzi et al., 2016). *T. gondii* has also shown geographic segregation of major haplogroups. Phylogenetic analysis (study of evolutionary development of a species) provides a framework for considering genetic diversity and for grouping strains based on shared ancestries. Although highly abundant clonal genotypes continue to dominate in North America and Europe, there is growing evidence that the extent and structure of diversity is markedly different elsewhere, especially in South America. The population structure of *T. gondii* is of considerable importance for understanding epidemiological patterns, as well as, heterogeneity in disease manifestations or for developing new strategies for vaccination, treatment, or diagnosis (Ajzenberg et al., 2010). In the domestic cycle of *T. gondii* (i.e. between cats, humans, and peri-domestic and meat-producing animals, such as pigs and sheep), a highly clonal population structure is reported including lineages I, II and III. However, where livestock breeding and rearing is less advanced (such as Brazil and Africa) and in the wild cycle of *T. gondii* (i.e. between wild cats and diverse wild intermediate hosts), atypical and recombinant genotypes dominate.

Through this project we have established the first Australian culture collection of *T. gondii* specific to sheep. However, without some further genomic characterisation, this collection and resource would remain an unknown entity. Such information will be valuable in better understanding the risk posed by foodborne consumption of sheep meat, the relationship to clinical isolates and as a resource for

potential vaccine development. The mechanisms behind the virulence and pathogenesis, as well as, other biological aspects of *T. gondii* can be understood better through whole genome sequencing (WGS) and comparisons to other major lineages. However, such analysis is costly and requires complex bioinformatic analytical capability. Approximately 62 *T. gondii* strains, including type I (GT1 and RH strains), type II (ME49 strain) and type III (VEG strain), as well as recombinant strains, have been completely sequenced (Lau et al., 2016). A clearer picture of genome differences between the major *T. gondii* lineages will contribute towards better understanding on the different virulence attributed to these lineages.

We have chosen to undertake WGS of one Australian *T. gondii* isolate to determine how similar it is to other global animal and clinical isolates. We have also characterised all Australian sheep *T. gondii* isolates using lower resolution, but discriminatory molecular methods, namely microsatellite and intron analysis, which are rapid and cheap to implement.

Microsatellite sequences are short tandem repeats of two to six nucleotides. They are known to be hypervariable making them informative neutral markers, suited for individual identification of isolates (Weiss and Kim, 2007). They are rapidly evolving sequences, but in *T. gondii* some microsatellite markers appear to evolve slowly, as demonstrated by the relatively low number of alleles detected and their stability in a large number of isolates of the three main lineages (type I, II and II). The data obtained with microsatellite markers correlate well with the patterns observed via multilocus PCR-RFLP on single copy genes (Ajzenberg et al., 2002, 2004). However, microsatellites are more polymorphic and give greater resolution in genetic population studies. To date, 15 microsatellites have been described for genotyping *T. gondii* isolates (Ajzenberg et al., 2010). They are located either in the introns (intragenic region; non-coding region of an RNA transcript, or the DNA encoding it) of known genes or in expressed sequence tags (EST; a short sub-sequence of a coding DNA sequence). Microsatellite polymorphism can be evaluated by conventional PCR and allele sizing though the use of fluorescence dye labelled primers and an automated DNA sequencer. A multiplex PCR for 15 different microsatellites allows multilocus analysis of *T. gondii* isolates from a single PCR amplification (Ajzenberg et al., 2010, Su et al., 2012). We have used this method to type all our isolates.

Although, microsatellite analysis detects genetic variation, they do not reveal all polymorphisms present in a given locus and may misclassify other variants due to homoplasy (a trait which has been gained or lost independently, in separate lineages over the course of evolution) (Khan et al., 2007). Recently, direct sequencing of introns from housekeeping genes has provided a more accurate picture of *T. gondii* divergence. Intron sequencing has shown previously unseen diversity in South American *T. gondii* isolates Introns are selectively neutral and, therefore, well suited for phylogenetic comparisons (Khan et al., 2006). In this study we also used low cost Sanger sequencing of four intron regions (uracil phosphoribosyl transferase [UPRT] intron 1, UPRT intron 7, elongation factor [EF] intron 1, and hypothetical protein [HP] intron 2) from three genes to investigate the phylogenetic relationships among our Australian *T. gondii* sheep isolates and others representative of a broad geographical, host range and genomic diversity.

3.2 Methodology

3.2.1 *T. gondii* nucleic acid extraction from cell culture

Eighteen Australian *T. gondii* isolates (Tg-AS01 to Tg-AS18), as well as, ME49, Tg-Dog and Dubey, were established and passaged in cell culture (Hs27 host cell line) as previously described (see Section 3.2.9). The ME49 and Dubey *T. gondii* isolates were obtained from Milton McAllister (University of Adelaide). The Tg-Dog isolate was obtained John Ellis (University of Technology Sydney). ME49 is available from ATCC (ATCC 50611) and is a type II isolate. The Dubey isolate was collected from a pig in the USA. Tg-Dog (TgDgAu1) is an Australian isolate collected from the brain of a congenitally infected canine pup and characterised by PCR-RFLP genotyping as type II (Al-Qassab et al., 2009). For each isolate of *T. gondii*, tachyzoites (haploid stage) were purified from a single culture flask of heavily infected cells by recovering all cells and media from the flask, centrifugation at 1,300 x *g* for 10 min, removal of the supernatant and resuspension of the resulting pellet in 200 µL PBS. Total nucleic acid was then extracted using the Qiagen DNeasy® Blood and Tissue Kit according to the manufacturer's animal blood protocol modified for cultured cells. Presence of *T. gondii* DNA was confirmed by qPCR (see Section 3.2.8).

3.2.2 Whole genome sequencing

Whole genome sequencing (WGS) of Tg-AS02 and variant analysis was done by the Australian Genome Research Facility (AGRF, Brisbane Node). An Illumina genomic DNA shotgun library was prepared with a bead size selection protocol. Sequencing was done using the Illumina NexSeq500 platform. The Australian *T. gondii* Tg-AS02 genome was compared to 20 WGS of *T. gondii*, available in public genome databases, to identify phylogenetic clustering based on single nucleotide polymorphisms (SNPs) analysis (Lorenzi et al., 2016). We chose the 20 *T. gondii* isolates for phylogenetic analysis based on a variety of criteria: varying host and geographic location; clinical human versus opportunistic animal isolations; and representative of a variety of haplogroups and clades. The *T. gondii* isolates chosen for phylogenetic comparison are listed in Table 14.

Table 14: WGS of T. gondii available in public databases representing a variety of host and genomic characteristics*.

Strains	Host	Geographic	Year isolated	Haplogroups	Clade
GT1 (type I)	Goat	USA-MD	1980	1	A
RH (type I)	Human adolescent, encephalitis	France	1939	1	A
ME49 (type II)	Sheep	USA-CA	1965	2	D
SOU	Human AIDS	USA	1985	2	C
VEG (type III)	Human (AIDS)	USA-CA	1988	3	C
TgShUS28	Sheep	USA	2008	3	C
M7741	Sheep	USA	1958	3	C
MAS	Human (congenital)	France (Nice)	1991	4	B
RUB	Human adult	French Guiana	1991	5	F
FOU	Human (transplant)	France (Brest)	1992	6	A
TgCtBr9	Cat	Brazil	2006	6	A
CAST	Human (AIDS)	USA-CA	1988	7	A
TgCtBr5	Cat	Brazil	2006	8	B
P89	Pig	USA-IA	1991	9	C
VAND	Human adult	French Guiana	1997	10	F
COUG	Cougar	Canada-BC	1996	11	D
ARI	Human (transplant)	USA	1992	12	D
TgCtPRC2	Cat	China	2007	13	D
TgA105004	Chicken	Makokou, Gabon, Africa	2007	14	A
CASTELLS	Sheep	Uruguay	1993	16	E

*Modified from Lorenzi et al., 2016.

3.2.3 Microsatellite analysis

The Australian *T. gondii* isolates were typed using 15 microsatellite markers distributed on 10 of 14 chromosomes, as described previously (Ajzenberg et al., 2010). Multiplex PCR was done with 15 microsatellite primer pairs (Table 15). The forward primer for each microsatellite was labelled with a fluorescence dye (6-FAM, HEX or NED) to enable allele size calling. An equimolar primer stock was prepared in 500 µL, comprising 1 µM of each primer. The 25 µL multiplex PCR reaction contained 12.5 µL of 2 x QIAGEN Multiplex PCR Master Mix, 0.1 µM of each primer and 5 µL DNA. PCR amplification conditions were initial denaturation at 95°C for 15 minutes, followed by 35 cycles of 94°C for 30 sec, 61°C for 3 min, 72°C for 30 sec, with a final extension at 60°C for 30 min. Following PCR amplification, the purity of product was visualised by electrophoresis in a 1% agarose gel. If evidence of strong primer dimer was visually observed on the gel then the PCR product was diluted 1:500. Genotyping fragment separation was done by AGRF (Adelaide node) on an automatic sequencer (ABI PRISM 3130xl, Applied Biosystems) with an internal LIZ 500 sizing standard. The sizes, in base pairs, of the microsatellite alleles were estimated using Peak Sanner™ software (version 1.0; Thermo Fisher Scientific). Australian *T. gondii* isolates were compared with strains identified in Table 14; microsatellite data from Su et al., 2012.

Table 15: Microsatellite primer sequences used in T. gondii typing

Marker	Chromosome	Repeat Motif	Size Range	Primers
TUB2	IX	$[TG/AC]_n$	287-291	F: 6-FAM-GTCCGGGTGTTCCTACAAAA; R: TTGGCCAAAGACGAAGTTGT
W35	II	$[TG/AC]_n$, $TG/AC]_n$	242-248	F: HEX-GGTTCACTGGATCTTCTCCAA; R: AATGAACGTCGCTTGTTTCC
TgM-A	X	$[TG/AC]_n$	203-211	F: HEX-GGCGTCGACATGAGTTTCTC; R: TGGGCATGTAAATGTAGAGATG
B18	VIIa	$[TG/AC]_n$	156-170	F: 6-FAM-TGGTCTTCACCCTTTCATCC; R: AGGGATAAGTTTCTTCACAACGA
B17	XII	$[TC/AG]_n$	334-366	F: HEX-AACAGACACCCGATGCCTAC; R: GGCAACAGGAGGTAGAGGAG
M33	IV	$[TC/AG]_n$	165-173	F: 6-FAM- TACGCTTCGCATTGTACCAG; R: TCTTTTCTCCCCTTCGCTCT
IV.1	IV	$[TG/AC]_n$	272-282	F: HEX-GAAGTTCGGCCTGTTCCTC; R: TCTGCCTGGAAAAGGAAAGA
XI.1	XI	$[TG/AC]_n$	354-362	F: 6-FAM-GCGTGTGACGAGTTCTGAAA; R: AAGTCCCCTGAAAAGCCAAT
M48	Ia	$[TA/AT]_n$	209-243	F:6-FAM-AACATGTCGCGTAAGATTCG; R: CTCTTCACTGAGCGCCTTTC 3
M102	VIIa	$[TA/AT]_n$	164-196	F: NED-CAGTCCAGGCATACCTCACC; R: CAATCCCAAAATCCCAAACC
N60	Ib	$[TA/AT]_n$	132-157	F: NED-GAATCGTCGAGGTGCTATCC; R: AACGGTTGACCTGTGGCGAGT
N82	XII	$[TA/AT]_n$	105-145	F: HEX-TGCGTGCTTGTCAGAGTTC; R: GCGTCCTTGACATGCACAT
AA	VIII	$[TA/AT]_n$	251-332	F: NED-GATGTCCGGTCAATTTTGCT; R: GACGGGAAGGACAGAAACAC
N61	VIIb	$[TA/AT]_n$	79-123	F: 6-FAM-ATCGGCGGTGGTTGTAGAT; R: CCTGATGTTGATGTAAGGATGC
N83	X	$[TA/AT]_n$	306-338	F: 6-FAM-ATGGGTGAACAGCGTAGACA; R: GCAGGACGAAGAGGATGAGA

3.2.4 Intron analysis

The Australian *T. gondii* isolates were also genotyped by sequencing for introns UPRT1, UPRT7 EF1 and HP2 (Su et al., 2012). PCR and sequencing primers are shown in Table 16. Each intron region was amplified in a single reaction for each *T. gondii* isolate. Conventional PCR was done in a volume of 25 µL containing 12.5 µL of 2 x QIAGEN Multiplex PCR master mix, 0.25 µM of each forward and reverse primer and 5 µL of target DNA (from a 1:50 dilution). The PCR conditions were initial denaturation at 94°C for 4 min, followed by 35 cycles of 94° for 30 sec, 55°C for 60 sec, 72°C for 30 sec, and final extension at 72°C for 10 min. The PCR products were purified by Isolate II PCR and Gel Kit (Bioline) according to the manufacturer's PCR clean up protocol. Purified PCR product was visualised by gel electrophoresis. Purified PCR products were mixed with the appropriate sequencing primer and sent for Sanger sequencing (Applied Biosystems 3730) at AGRF, Adelaide node. Following sequencing, the nucleotide sequence for each intron was edited to remove primer sequences and then stitched together to form a continuous composite sequence including all four introns (1772-1774 bp). Our intron sequences and those available from the NCBI nucleotide sequence database for other *T. gondii*

(Table 14) were aligned using DNAMAN X (Lynnon Biosoft) with default settings. Aligned sequences were used in phylogenetic analysis either as nucleotide sequences or as SNPs. A total of 61 SNPs were identified across the four introns. Isolates where poor sequencing meant all 4 introns sequences could not be generated (i.e. Tg-AS07 and Tg-AS10), were excluded from the analysis.

Table 16: Intron PCR and sequencing primers

Locus	Chromosome	PCR primers	Sequencing primers	size (bp)
UPRT Intron 1	XI	F: CCCGATATTCGACAAACGAC; R: GAGCCGTCTGCTTCATGAGC	UPRT-SqF1: TCAACCGAAGTTTGCTTTCC	397
UPRT Intron 7	XI	F: TGGTCGTCGTCACTTGTTA; R: GCAGCCTCACAACTAAAACT	UPRT-7-SeqF: TCTTGTTTGCTTTCCTCGGC	459
EF Intron 1	X	F: AAATGCACCCTTTTCTTAAA; R: CACATGAAGGTACACCAAAA	EF1-Seq1: AAATTGTCCCGCCATCAG	447
HP Intron 2	IV	F: GACAGAAACACGCAGAGAAT; R: TAATCTTTGTTCCCATGCTT	HP-Seq1: ATAATACAGTCAGTTCCCTCGAT	470

3.2.5 Phylogenetic analysis of microsatellite and intron data

Microsatellite allele variants identified in base pairs were converted into binary data (0 vs 1). Intron SNPs were also converted into binary data. Microsatellite and intron data were phylogenetically analysed either individually or combined. These binary data were analysed using a Bray-Curtis similarity matrix and CLUSTER, with group averages and the SIMPROF test ($P < 0.05$), in PRIMER-E v6 (PRIMER-E Ltd, Plymouth, UK). CLUSTER provides hierarchical agglomerative linkage clustering. SIMPROF is a similarity profile permutation test providing statistical significance of structure within samples, which are *a priori* unstructured. Tests are performed at every node of a completed dendrogram starting from the left of the dendrogram, permitting interpretation of divisions to the right of each node only if the SIMPROF test shows evidence of multivariate structure within that group. Test results are displayed by a colour convention on the dendrogram; samples connected by red lines are not significantly differentiated by SIMPROF, only the structure shown by black lines in a dendrogram should be interpreted. The intron multiple sequence alignment generated in DNAMAN X and the combined microsatellite and intron binary data described above were also analysed using SplitsTree v4.4 to compute an unrooted phylogenetic network using the neighbor-net method with 1,000 bootstrap replicates. Neighbor-net is an algorithm for constructing phylogenetic networks which are based on the neighbor joining algorithm. Like neighbor joining, the method takes a distance matrix as input, and works by agglomerating clusters. However, the neighbour-net algorithm can lead to collections of clusters which overlap and do not form a hierarchy. CLUSTER and Neighbor-net are different ways of investigating sample relationships using phylogenetical analysis.

3.3 Results

3.3.1 Whole genome sequencing of Tg-AS02

The 150 bp paired end WGS data generated from sequencing Tg-AS02 is outlined in Table 17.

Table 17: Tg-AS02 150 bp paired end whole genome sequencing Illumina data

Lane	Sample Name	Paired Reads	Data Yield (bp)
1	Tg-AS02	33,146,635	10.01 Gb
2	Tg-AS02	31,514,973	9.52 Gb
3	Tg-AS02	35,946,117	10.86 Gb
4	Tg-AS02	29,309,443	8.85 Gb
Total		129,917,168	39.23 Gb

The assembly statistics of Tg-AS02 are shown in Table 18. The genome size of *T. gondii* Tg-AS02 is 69.96Mb, which is consistent with other *T. gondii* genomes (63.95-69.35Mb; Lau et al., 2016).

Table 18: T. gondii Tg-AS02 WGS assembly statistics

Metrics	Sequence length (bp)
Total assembly	69,962,385
Number if contigs*	13,732
Average contig length	5,094.84
Longest contig	944,770
N50†	268,445
NG50‡	268,445

*A contig is a set of overlapping DNA segments that together represent a consensus region of DNA.

†N50 is a measure to describe the quality of assembled genomes that are fragmented in contigs of different length. The N50 is defined as the minimum contig length needed to cover 50% of the genome

‡The NG50 statistic is the same as N50 except that it is 50% of the known or estimated genome size that must be of the NG50 length or longer. In the typical case that the assembly size is not more than the genome size, the NG50 statistic will not be more than the N50 statistic.

Figure 3 shows the phylogenetic tree for Tg-AS02 and the 20 *T. gondii* isolates selected in Table 14.

Based on whole genome SNPs, Tg-AS02 clusters most closely with ME49 (type II) and other clade D isolates. Interestingly ME49 was also originally isolated from sheep, but in the USA in 1965. Grouping of isolates into various clusters are indicated by coloured circles. The grouping identified is consistent with that described by Lorenzi et al. (2016) giving confidence in our phylogenetic analysis and identified relationship of Tg-AS02.

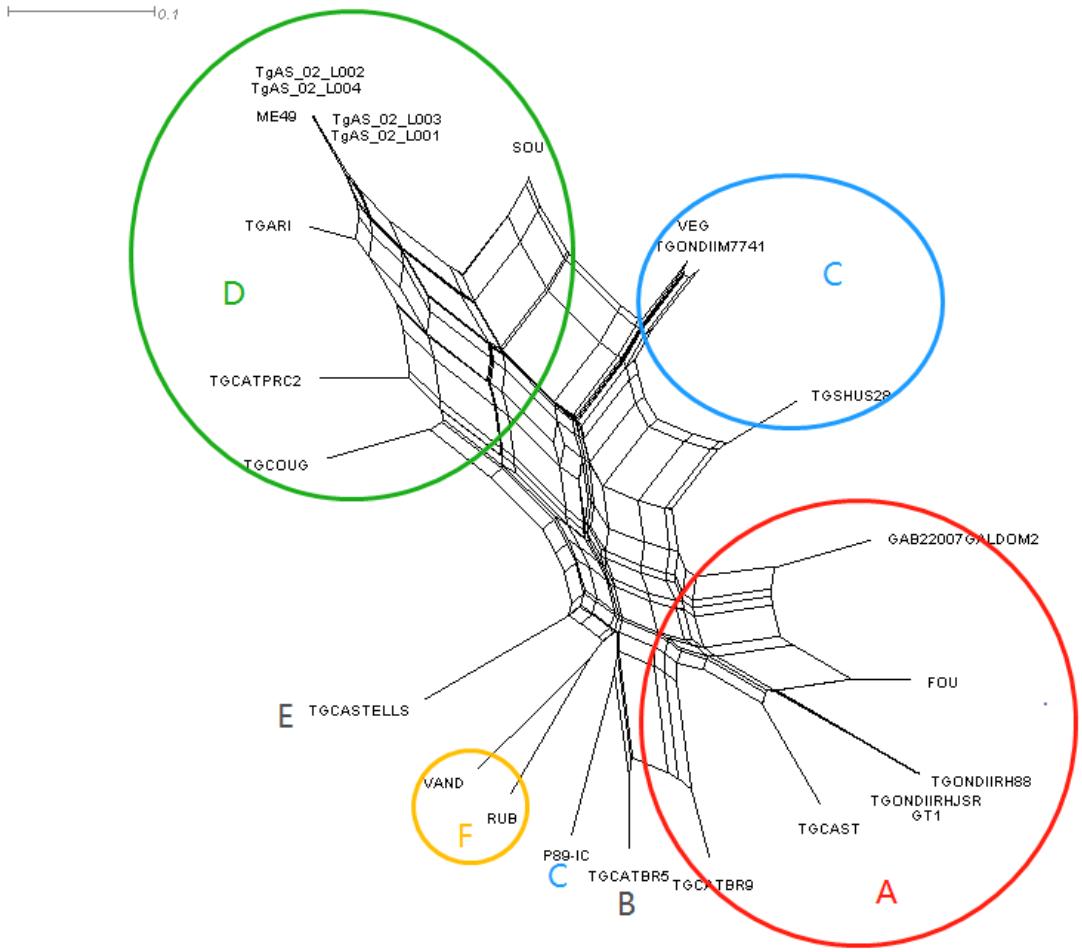

Figure 3: Population genetic structure of T. gondii including generated Tg-AS02 and the 20 other T. gondii strains listed in Table 14. Neighbor-net analysis based on genome-wide SNPs from 21 isolates of T. gondii.

3.3.2 Microsatellite analysis

The microsatellite results for our *T. gondii* isolates are shown in Table 18, along with previously reported data for other isolates of interest. Phylogenetic analysis of this data is shown in Figure 4 with isolates being identified by country of origin and clade, as reported by Lorenzi et al., 2016. All the Australian *T. gondii* isolates (sheep and dog) cluster together indicating that they are similar. Included in this Australian cluster is ME49 (type II).

Table 18: Microsatellite variants of Toxoplasma gondii isolates.

MS Markers	*Toxoplasma gondii* isolates																																				
	Current study																					Su et al., 2012																
	TgAS01	TgAS02	TgAS03	TgAS04	TgAS05	TgAS06	TgAS07	TgAS08	TgAS09	TgAS10	TgAS11	TgAS12	TgAS13	TgAS14	TgAS15	TgAS16	TgAS17	TgAS18	Dog	Dubey	ME49 (II)	GT1 (I)	M7741 (III)	VEG (III)	CASTELLS	MAS	RUB	FOU	TgCatBr09	CAST	TgCatBr05	P89	VAND	ARI	TgA15004	SOU	TgShUS28
TUB2	289	289	289	289	289	290	289	289	289	290	289	289	290	289	290	288	290	288	290	290	290	291	289	289	287	291	289	291	291	291	291	291	291	289	291	289	291
W35	245	245	245	245	245	245	245	245	245	245	245	246	244	245	245	244	245	244	245	245	245	248	242	242	242	242	242	248	242	242	242	242	242	242	242	242	248
TgM-A	207	207	207	207	207	208	208	207	207	207	207	207	208	208	208	206	207	206	208	208	208	209	205	205	207	205	205	205	205	205	205	205	205	203	209	207	205	209
B18	158	158	158	158	158	158	158	158	158	158	158	158	158	158	158	158	158	158	158	158	158	160	160	160	158	162	170	160	160	158	160	160	162	158	160	158	160
B17	336	335	335	335	335	336	335	335	335	335	336	334	335	335	336	334	336	334	336	336	336	342	336	336	358	362	360	342	362	342	362	348	344	336	342	336	336
M33	169	169	168	169	168	169	168	169	169	169	168	168	168	168	169	168	169	168	169	168	168	169	165	165	169	169	167	165	165	167	165	165	167	169	165	165	165
IV.1	276	276	276	276	276	276	276	276	276	276	276	276	276	276	276	275	276	275	276	276	276	274	278	278	274	272	274	274	278	276	278	278	276	274	278	278	278
XI.1	356	356	356	356	356	356	356	356	356	356	356	356	356	356	356	356	356	356	356	356	356	358	356	356	356	358	356	354	354	356	356	356	356	362	354	356	354
M48	217	215	215	215	215	215	217	215	217	215	215	215	214	215	215	214	217	216	217	215	217	209	215	213	239	221	223	227	227	211	237	213	217	215	223	225	213
M102	180	176	176	185	176	182	184	181	185	184	181	177	176	176	184	184	185	180	177	176	176	168	190	188	164	166	190	166	174	168	174	190	170	170	166	174	166
N60	147	147	151	144	151	145	147	145	145	147	141	149	151	151	145	145	145	145	147	147	147	145	147	153	138	142	142	147	140	147	140	142	142	147	142	142	147
N82	112	112	114	110	114	115	113	112	112	112	112	116	112	115	112	112	113	112	112	112	112	119	111	111	109	111	109	111	111	119	111	111	113	131	111	111	111
AA	271	271	265	276	265	276	276	276	276	278	271	265	265	265	267	280	276	280	269	263	269	265	267	267	283	332	259	281	269	279	265	261	277	295	277	259	277
N61	92	92	98	94	98	95	90	92	90	92	98	96	98	98	92	91	90	92	92	96	92	87	91	89	87	95	85	89	89	87	89	87	91	89	97	89	87
N83	309	309	309	311	309	311	311	311	309	311	311	309	309	309	311	311	309	311	309	309	309	306	312	312	324	338	312	306	308	306	314	314	308	316	310	312	304

* The size of each microsatellite allele is shown in base pairs.

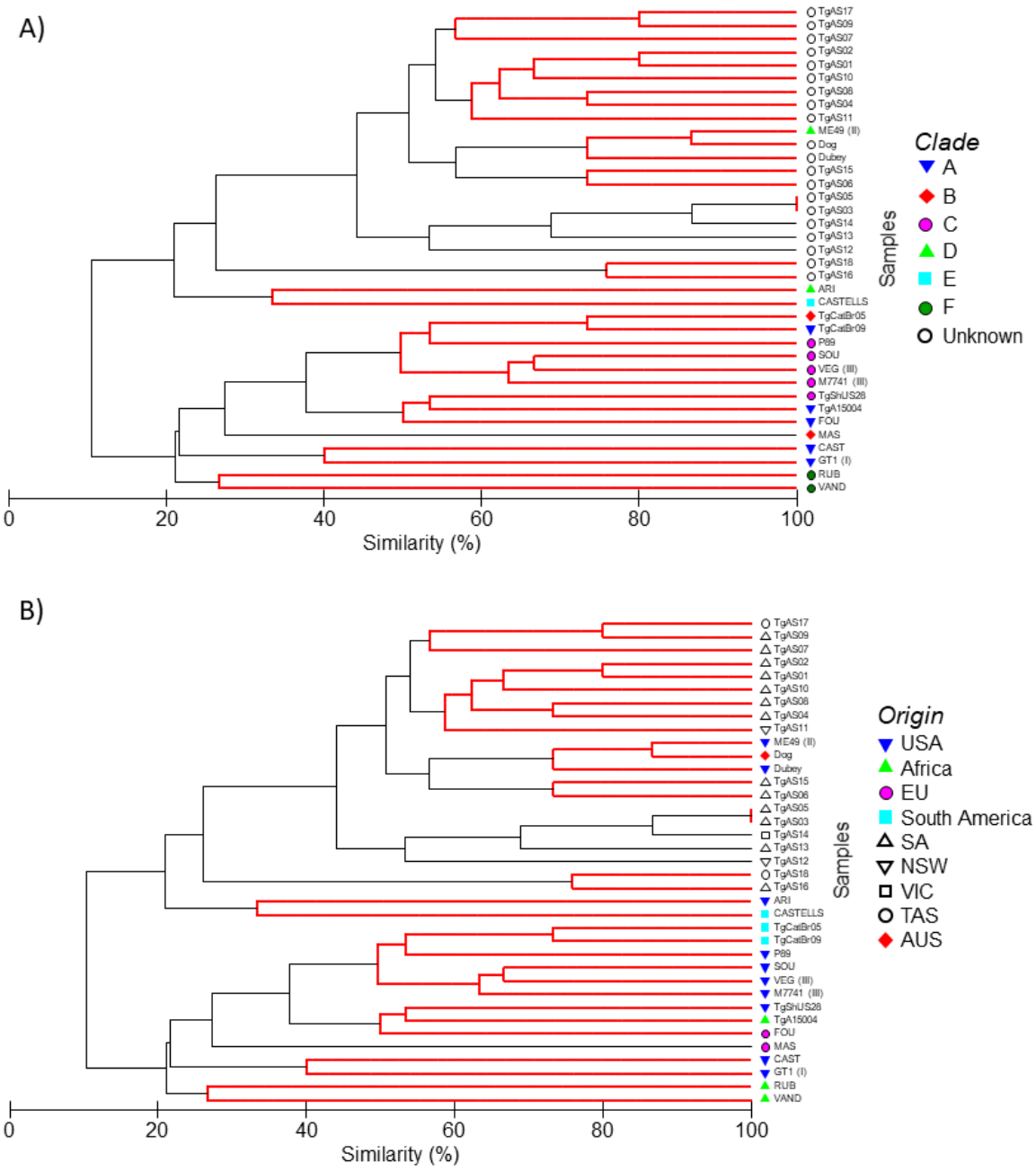

Figure 4: Phylogenetic tree generated from 15 T. gondii microsatellite markers. A) shows T. gondii isolates identified by previously assigned clades or as unknown. B) shows T. gondii isolates identified by geographic origin. Samples connected by red lines are not significantly differentiated, and only the structure shown by black lines in the dendrogram should be interpreted.

3.3.3 Intron analysis

T. gondii intron sequences were more homologous than microsatellite polymorphisms. Again, two major clusters were observed with Australian sheep isolates clustering together along with ME49 (type II) and other clade D (ME49 and ARI) isolates (Figure 5). Australian isolates are highly similar (>95%) in the four intron regions investigated. The Australian *T. gondii* isolates also tended to cluster more broadly with isolates from the USA. The split between Australian *T. gondii* and other isolates is also evident in the network analysis (Figure 6).

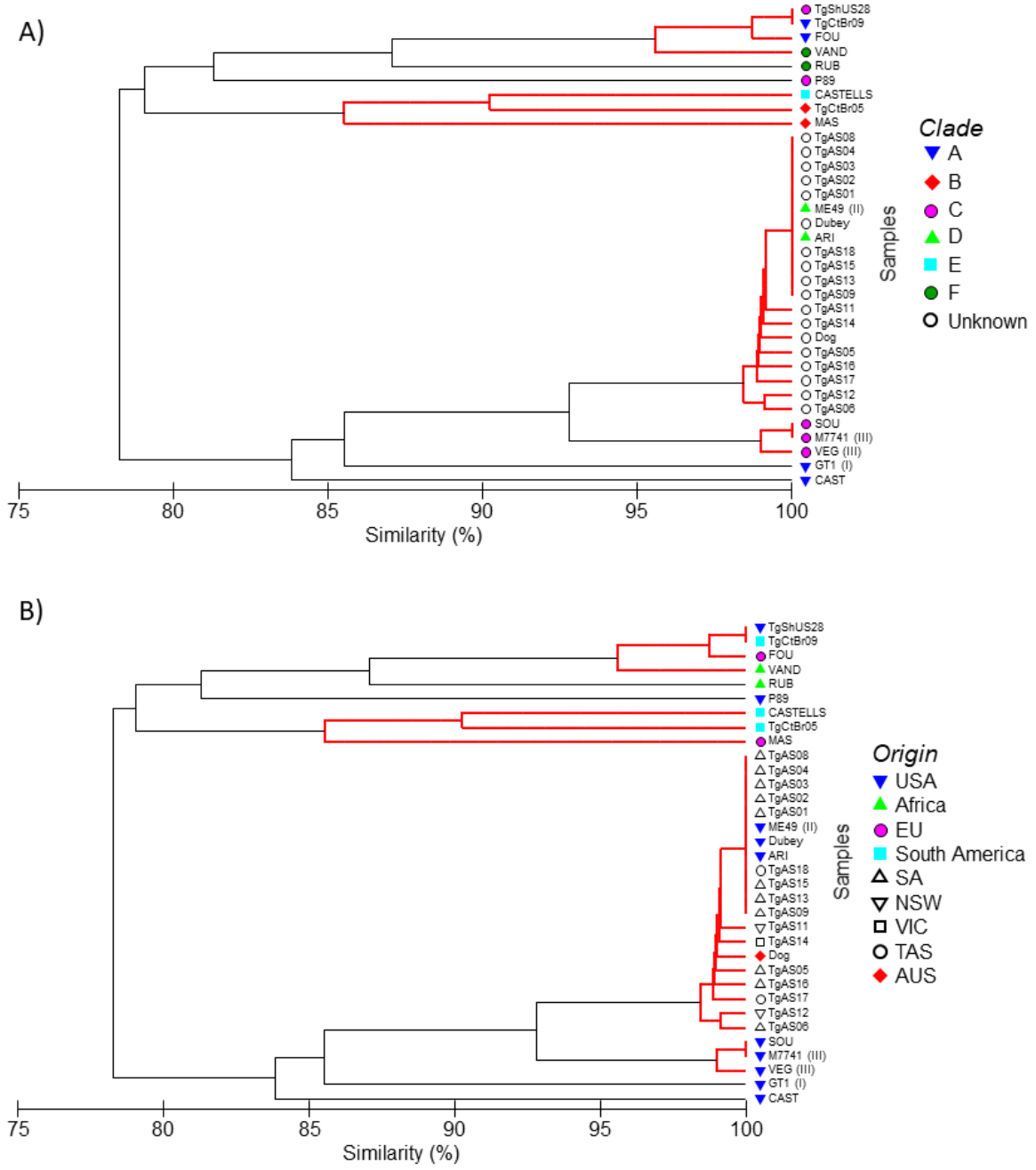

Figure 5: Phylogenetic tree generated from four T. gondii intron sequences. A) shows T. gondii isolates identified by previously assigned clades or as unknown. B) shows T. gondii isolates identified by geographic origin. Samples connected by red lines are not significantly differentiated, and only the structure shown by black lines in the dendrogram should be interpreted.

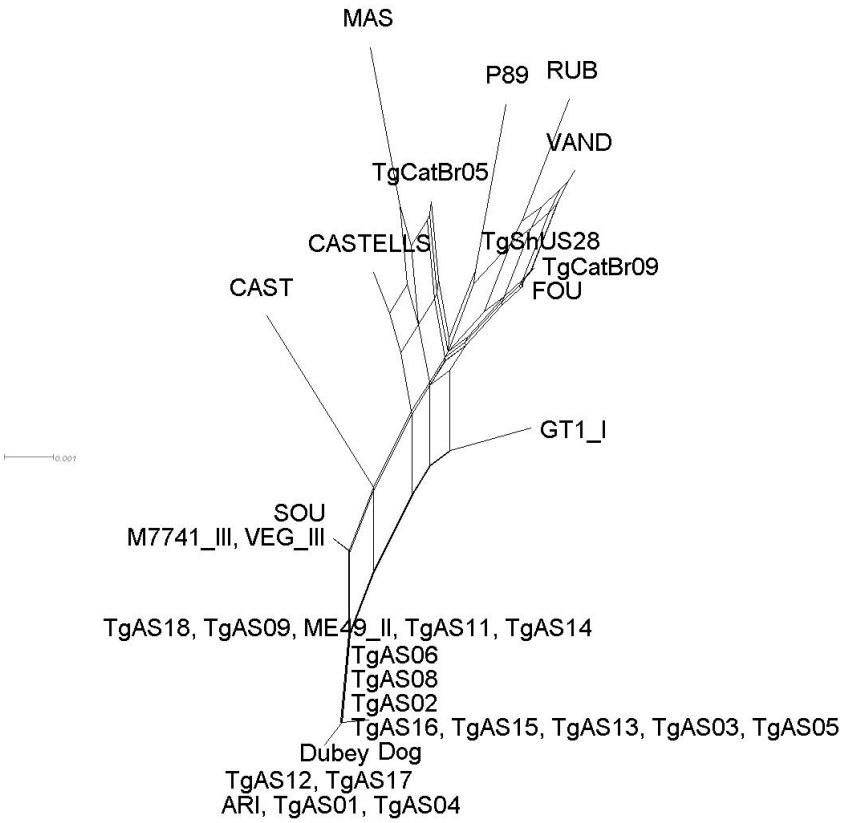

Figure 6: Neighbor-net analysis of polymorphisms in T. gondii intron sequences. The network has 41 splits (bipartitions, that represent groupings in the data obtained from 34 T. gondii isolates). Each split is represented by a different set of parallel edges (boxes). The scale bar represents the edge lengths that are proportional to split weights.

3.3.4 Combined microsatellite and intron phylogenetic analysis

The combined phylogenetic analysis of the microsatellite and intron polymorphisms generated similar results to the individual microsatellite and intron analysis. Two major clades were observed with Australian isolates (sheep and dog) clustering closely and most similar to ME49 (type II), other clade D isolates and isolates from the USA (Figure 7). The same pattern is observed in the network analysis (Figure 8).

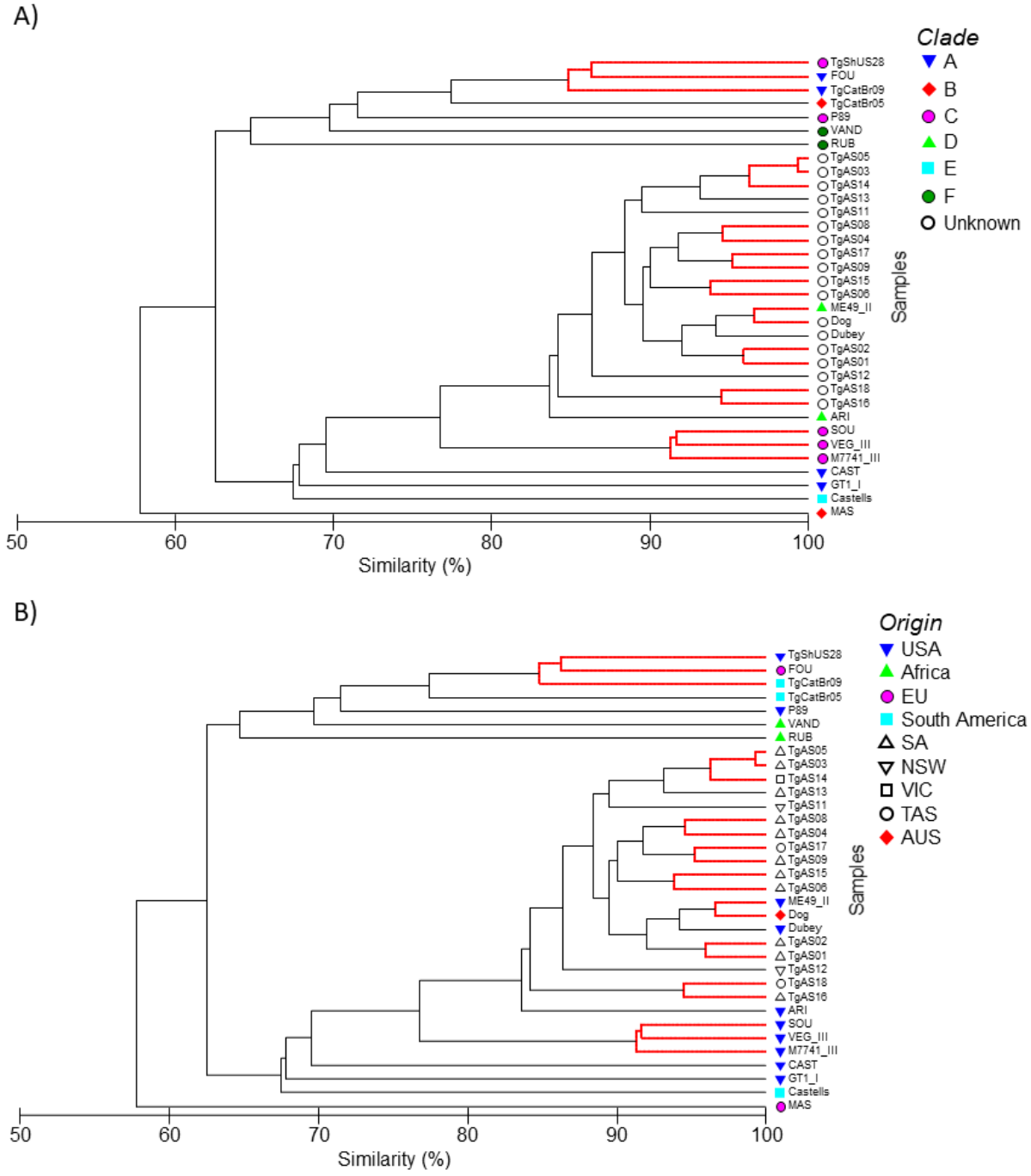

Figure 7: Phylogenetic tree generated from 15 T. gondii microsatellite markers and 4 intron sequences. A) shows T. gondii isolates identified by previously assigned clades or as unknown. B) shows T. gondii isolates identified by geographic origin. Samples connected by red lines are not significantly differentiated, and only the structure shown by black lines in the dendrogram should be interpreted.

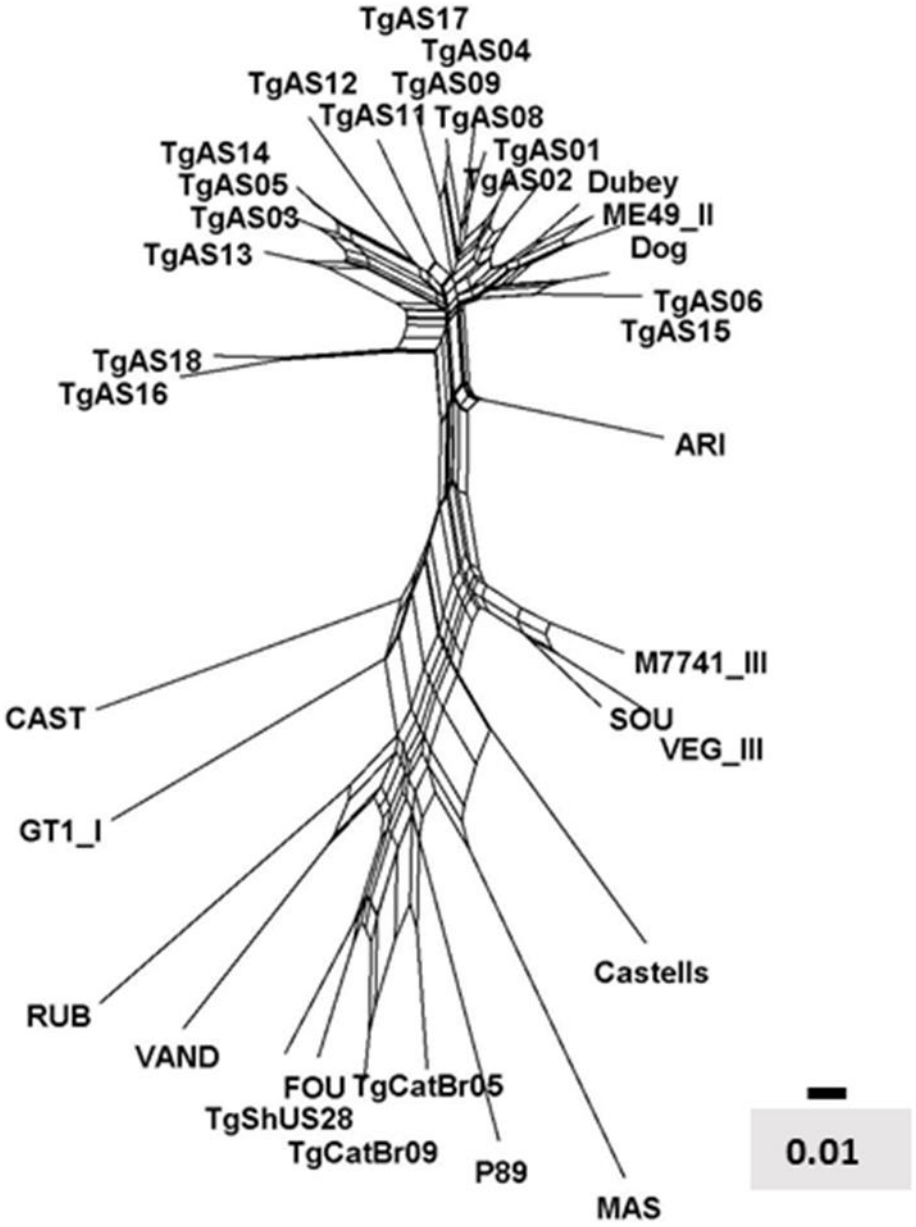

Figure 8: Neighbor-net analysis of polymorphisms in T. gondii 15 microsatellites and four intron sequences. The network has 108 splits (bipartitions, that represent groupings in the data obtained from 34 T. gondii isolates). Each split is represented by a different set of parallel edges (boxes). The scale bar represents the edge lengths that are proportional to split weights.

3.4 Key results

- The first Australian *T. gondii* (Tg-AS02) isolated from meat producing sheep has been fully characterised by whole genome sequencing. This to our knowledge is the first Australian *T. gondii* isolate, regardless of host, to be fully characterised. The genome size of Tg-AS02 is 69Mb, consistent with other *T. gondii* reported. Phylogenetic analysis using SNPs from the

full genome has shown that Tg-AS02 is most similar to ME49, a type II isolate, originally isolated from sheep in the USA, and other clade D isolates. Based on the limited analysis of 21 whole genome sequence comparisons, there was no relationship observed in host range with the other clade D isolates; ARI was isolated from a human transplant patient in USA, TgCtPRC2 from a Chinese cat and COUG from a Canadian cougar. Interestingly, three out of four of these clade D isolates come from Northern America where isolates are highly clonal.

- Lower resolution, but faster and cheaper typing methods, namely microsatellite and intron analysis, have been established and implemented to investigate the broader Australian *T. gondii* strains isolated in this study. Where information for the investigated microsatellite and intron sequences was available in the public domain for the selected global isolates investigated in the above whole genome sequence analysis, these were also included in our lower resolution phylogenetic analysis. This analysis generated results similar to whole genome sequencing, in that Tg-AS02 is most like ME49 (type II) and another clade D isolate, namely ARI. These results provide confidence that this lower resolution phylogenetic analysis is valuable in evaluating relationship amongst newly isolated *T. gondii* and those previously reported.
- Combined microsatellite and intron phylogenetic analysis of our Australian and a limited number of global *T. gondii* isolates have shown that the 18 Australian sheep isolates are highly similar (clonal), regardless of geographic location. Further interpretation regarding geographic relationships amongst these isolates should be done with caution. Although SA had 11 isolates in the analysis, NSW, VIC and TAS only contained one or two isolates each. As *T. gondii* collections and sequence information deposited in public genome databases grow, the value of this information will increase.
- The Australian sheep *T. gondii* isolates are also closely related to Tg-Dog, another Australian type II isolate. Type II isolates have also been reported in Australian cats. Virulence of the Australian *T. gondii* isolates in mice-bio-assays was not investigated in this study
- The 18 Australian sheep isolates are genetically distinct from other global strains apart from ME49 (sheep isolate, type II, clade D), ARI (human isolate, haplogroup 12, clade D) and Dubey (pig isolate, type unknown). Haplogroup 12 isolates have also been reported from pigs and sheep in the USA, although type II dominates in these animals.
- South American strains appear to be less clonal and more virulent in both mice and immunocompetent humans (Carme et al. 2009; Grigg et al. 2015). In this study, the 18 Australian isolates did not group with the three South American isolates examined (2 from Brazilian cats and 1 from a Uruguayan sheep – Table 14), however the genomic regions we examined are not virulence determinant so caution should be exercised in extrapolating grouping to potential virulence.
- In comparison to other countries, Australian investigation of *T. gondii* particularly in relation to culture isolation and typing are limited. The only other Australian isolate we are aware of which has been cultured was from a dog in 2009 (Al-Qassab et al., 2009). This isolate was characterised by another low-resolution typing tool (PCR-RFLP), targeting different polymorphic markers than those used in our study, and was typed as II. We included this same isolate (Tg-Dog, also known as TgDgAu1) in our current study and it grouped closely with our Australian sheep *T. gondii* cluster and ME49 (type II), supporting the previously reported typing.

4. Conclusion

4.1 Key findings

- *Toxoplasma gondii* was able to be successfully cultured from naturally infected slaughter sheep, using fresh abattoir material (sheep heart).
- This project represents the first reported successful *T. gondii* isolation from Australian sheep.
- By selecting heart material from serologically positive animals sourced from Kangaroo Island and using a process of acid pepsin digestion followed by single mouse bio-assay (no replicates), viable *T. gondii* was successfully isolated from 9/18 sheep – an initial 50% success rate.
- Although mouse bio-assay is the standard method used for *T. gondii* isolation, in a Stage 1 trial we were also able to successfully culture the organism by direct seeding of tissue culture with heart acid digest, thus potentially reducing the reliance on mouse bio-assay.
- Gold standard *T. gondii* positive and negative sera was successfully produced in Stage 2, allowing accurate calibration of the serology test kit, and adjustment of the cut-off to 1:60, maximising its sensitivity and specificity for use in the Stage 3 national survey, thereby minimising false negatives and false positives.
- In Stage 2, an Australian isolate from the brain of a dog (Tg-Dog) stimulated a strong serological response in sheep and goats without an associated detectable viable infection, unlike the Australian sheep isolates utilised. This suggests it may be attenuated and would make a promising candidate the future development of a vaccine.
- In the Stage 3 national sheep survey, acid digestion followed by either mouse bio-assay or direct cell culture proved to have a similar success rate in isolating *T. gondii* (Table 12). This represents a significant advance in *T. gondii* research methodology, with the potential to reduce costs and animal ethics issues.
- The Stage 3 national survey of all 6 states indicated a national serological prevalence of 11.5% (46/401), with a range by state of 0 to 44% (Table 11). By comparison, a 2009 survey of Australian sheep and lambs (MLA project A.MFS.0129) determined a national prevalence of 31.9% and 16.4%, respectively.
- In Stage 3, a further nine *T. gondii* isolates were cultured from NSW, SA, Tasmania and Victoria.
- The first Australian culture collection of 18 *T. gondii* isolates (Tg-AS01 – Tg-AS18) specific to livestock/sheep has been established and cryo-preserved for future research purposes.
- An Australian sheep isolate of *T. gondii* (Tg-AS02) has been fully characterised by whole genome sequencing. Genetically, this isolate is most like ME49, a type II isolate collected from sheep in the USA in 1965. Type II isolates have been reported to prevail in pigs and sheep in northern America and are less virulent that type I isolates in mice.
- The 18 Australian sheep isolates do not group genetically with the three South American isolates they were compared with (South American isolates are believed to be more virulent).
- Lower resolution, but faster and cheaper typing methods (microsatellite and intron analysis) have been established and implemented to investigate all the *T. gondii* strains (Tg-AS01 -18) isolated in this study. The results indicate that all 18 isolates are genetically highly similar, regardless of geographic location.

4.2 Benefits to industry

1/. The project highlights the need for the industry to develop a risk management strategy to deal with the *T. gondii* issue, including communication within the industry and consideration of possible mitigation strategies.

The acid pepsin digestion process utilised in this project mimics a normal *T. gondii* infection pathway – ingestion of chronic bradyzoite cysts from an intermediate host (in this case sheep meat) by another monogastric omnivorous intermediate host such as a human. Gastric juices in the new host release and activate encysted bradyzoites to allow infection via the alimentary canal of the new host.

The high success rate in isolating *T. gondii* by this relatively crude method resolves the uncertainty as to the likelihood of the organism remaining viable in naturally infected animals and therefore the potential risk posed by the consumption of infected sheep meat.

Fortunately, there are simple remedial steps available to reduce or remove that risk to consumers:

- Freezing the meat <-20°C for several days prior to consumption.
- Cooking the meat so the centre reaches 70°C.

These steps may not be popular with all sectors of the industry, for example some producers of dry fermented sausage are opposed to using frozen product and some consumers prefer unfrozen meat. However, the industry now has firm data to bring to any discussion.

2/. The industry now has a serological test for *T. gondii* calibrated against Australian strains for future prevalence studies in domestic meat animals, for example goats or lamb.

3/. The industry has available a library of cryopreserved Australian isolates that can be utilised for future research such as the development of vaccines if required. There has already been a preliminary approach from a company wishing to use the Australian isolate to test the efficacy of their *T. gondii* vaccine.

4/. The success of the direct inoculation of acid pepsin digest onto cell culture to isolate *T. gondii* and demonstrate viability in meat products, without the need for an intermediate mouse bio-assay, means that future viability studies can be done more easily and at reduced cost.

5. Future research and recommendations

- Issue
  T. gondii was readily isolated from fresh sheep tissue, demonstrating the viability of the muscle cysts present and underlining the potential risk they represent to consumers. The hearts, however, were processed within 1 or 2 days of slaughter. It is uncertain to what extent normal sheep meat storage parameters reduces that viability.

  Recommendation
  A study be funded to investigate the loss of bradyzoite viability over time at normal refrigeration temperatures.

- Issue
  In the Stage 3 national survey, Tasmania had a very high prevalence of *T. gondii* in sheep (44%). While based on a small sample size (nine sheep for the whole state), Tasmania has been earlier found to have one of the highest stray and feral cat seroprevalences in the world (84%).

Recommendation
A more representative serological survey should be carried out on Tasmanian sheep to more accurately estimate the true prevalence of *T. gondii*.

- Issue
  Although mature sheep are likely to have higher prevalence of infection with *T. gondii*, due to the life-time persistence of viable infection, it is lamb that is most likely to be eaten rare and therefore provide the greatest foodborne risk to consumers.

  Recommendation
  A serological survey should be undertaken to estimate the current prevalence of *T. gondii* in Australian lamb.

- Issue
  In this study we were able to draw on publicly available *T. gondii* genotype data. This access greatly assisted the project completion.

  Recommendation
  That MLA consider contributing the *T. gondii* Tg-AS02 DNA sequence to a public database.

- Issue
  There has been interest shown in accessing the Australian sheep isolate collection for experimental use (e.g. for use in testing the efficacy of a *T. gondii* vaccine).

  Recommendation
  That MLA agree in principal to provide access to the isolate library for research purposes.

Potential publications:
- Isolation article
- Gold standard sheep and goat trial article
- Genotyping article

6. References

Ajzenberg, D., Banuls, A.L., Tibayrenc, M. and Dardé, M.L., 2002. Microsatellite analysis of Toxoplasma gondii shows considerable polymorphism structured into two main clonal groups. *International journal for parasitology*, *32*(1), pp.27-38.

Ajzenberg, D., Banuls, A.L., Su, C., Dumetre, A., Demar, M., Carme, B. and Dardé, M.L., 2004. Genetic diversity, clonality and sexuality in Toxoplasma gondii. *International journal for parasitology*, *34*(10), pp.1185-1196.

Ajzenberg, D., Collinet, F., Mercier, A., Vignoles, P. and Dardé, M.L., 2010. Genotyping of Toxoplasma gondii isolates with 15 microsatellite markers in a single multiplex PCR assay. *Journal of clinical microbiology*, *48*(12), pp.4641-4645.

Al-Qassab, S., Reichel, M., Su, C., Jenkins, D., Hall, C., Windsor, P., Dubey, J., and Ellis, J., 2009. Isolation of Toxoplasma gondii from the brain of a dog in Australia and its biological and molecular characterization. *Veterinary Parasitology*, *164*(1-2), pp.335-339.

Carme, B., Demar, M., Ajzenberg, D., and Darde, M.L., 2009. Severe acquired toxoplasmosis caused by wild cycle of *Toxoplasma gondii*, French Guiana. *Emerging Infectious Disease*, *15*(4), pp.656–658.

Dubey, J.P., 1998. Refinement of pepsin digestion method for isolation of Toxoplasma gondii from infected tissues. *VeterinaryPparasitology*, *74*(1), pp.75-77.

Fancourt, B.A. and Jackson, R.B., 2014. Regional seroprevalence of Toxoplasma gondii antibodies in feral and stray cats (*Felis catus*) from Tasmania. *Australian Journal of Zoology*, *62*(4), pp.272-283.

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

Grigg, M. and Sundar, N., 2009. Sexual recombination punctuated by outbreaks and clonal expansions predicts Toxoplasma gondii population genetics. *International journal for parasitology*, *39*(8), pp.925-933.

Grigg. M.E., Dubey, J.P., and Nussenblatt, R.B., 2015. Ocular toxoplasmosis: lessons from Brazil. *American Journal of Ophthalmology, 159*(6), pp.999–1001.

Hamilton, D., Hodgson, K., May, D., Ellis, J., McAllister, M., Kiermeier, A., and Pointon, A., 2014. Establishing the risk of Toxoplasmosis associated with the consumption of pork and pork products. Australian Pork Limited. Project code 2011/1017.401 final report.

Kiermeier, A., Hamilton, D., and Smith, G., 2008. National serological baseline survey of Toxoplasma gondii in lambs and sheep. Meat and Livestock Australia. Project code A.MFS.0129 final report.

Khan, A., Jordan, C., Muccioli, C., Vallochi, A.L., Rizzo, L.V., Belfort Jr, R., Vitor, R.W., Silveira, C. and Sibley, L.D., 2006. Genetic divergence of Toxoplasma gondii strains associated with ocular toxoplasmosis, Brazil. *Emerging infectious diseases*, *12*(6), p.942.

Khan, A., Fux, B., Su, C., Dubey, J.P., Dardé, M.L., Ajioka, J.W., Rosenthal, B.M. and Sibley, L.D., 2007. Recent transcontinental sweep of Toxoplasma gondii driven by a single monomorphic chromosome. *Proceedings of the National Academy of Sciences*, *104*(37), pp.14872-14877.

Lau, Y.L., Lee, W.C., Gudimella, R., Zhang, G., Ching, X.T., Razali, R., Aziz, F., Anwar, A. and Fong, M.Y., 2016. Deciphering the draft genome of Toxoplasma gondii RH strain. *PLoS One*, *11*(6), p.e0157901.

Lorenzi, H., Khan, A., Behnke, M.S., Namasivayam, S., Swapna, L.S., Hadjithomas, M., Karamycheva, S., Pinney, D., Brunk, B.P., Ajioka, J.W. and Ajzenberg, D., 2016. Local admixture of amplified and diversified secreted pathogenesis determinants shapes mosaic Toxoplasma gondii genomes. *Nature communications*, *7*(1), pp.1-13.

Opsteegh, M., Langelaar, M., Sprong, H., Den Hartog, L., De Craeye, S., Bokken, G., Ajzenberg, D., Kijlstra, A. and Van Der Giessen, J., 2010. Direct detection and genotyping of Toxoplasma gondii in meat samples using magnetic capture and PCR. *International journal of food microbiology*, *139*(3), pp.193-201.

Su, C., Khan, A., Zhou, P., Majumdar, D., Ajzenberg, D., Dardé, M.L., Zhu, X.Q., Ajioka, J.W., Rosenthal, B.M., Dubey, J.P. and Sibley, L.D., 2012. Globally diverse Toxoplasma gondii isolates comprise six major

clades originating from a small number of distinct ancestral lineages. *Proceedings of the National Academy of Sciences*, *109*(15), pp.5844-5849.

Weiss, L.M. and Kim, K. eds., 2011. *Toxoplasma gondii: the model apicomplexan. Perspectives and methods*. Elsevier.

7. Appendix

Appendix 1: Complete serological results from gold-standard sheep and goat trial

Sheep/goat ID	***T. gondii* strain**	**Dose**	**Sheep/goat Toxo-Screen DA result (IU/mL)**							
			0 days	**7 days**	**14 days**	**21 days**	**28 days**	**49 days**	**70 days**	**82-93 days**
G1	Tg-Dog	60,000	-	-	100	800	800	13122	39366	13122
G3	Tg-AS01	100,000	-	-	4	1458	4374	4374	4374	4374
G26	Tg-AS02	100,000	-	-	162	1458	1458	4374	13122	1458
G27	Tg-AS04	100,000	-	-	400	1458	13122	4374	4374	4374
G17	Tg-AS09	100,000	-	16	1600	1458	1458	13122	4374	4374
G7	control	n/a	-	-	-	-	-	-	-	-
G16	control	n/a	-	-	-	-	-	-	-	-
G20	control	n/a	-	-	-	-	-	-	-	-
G21	control	n/a	-	-	-	-	-	-	-	-
G23	control	n/a	-	-	-	-	-	-	-	-
G24	control	n/a	-	-	-	-	-	-	-	-
S37	Tg-Dog	60,000	-	16	100	162	486	4374	4374	4374
S32	Tg-Dog	1,000	-	-	100	162	1458	1458	1458	1458
S33	Tg-AS01	100,000	-	-	100	486	1458	4374	1458	4374
S41	Tg-AS01	1,000	-	-	16	162	1458	13122	4374	4374
S31	Tg-AS02	100,000	-	16	400	4374	1458	13122	1458	1458
S34	Tg-AS02	1,000	-	-	150	162	1458	1458	1458	1458
S47	Tg-AS04	100,000	-	-	486	1458	1458	1458	1458	1458
S36	Tg-AS04	1,000	-	-	20	1458	1458	1458	1458	1458
S39	Tg-AS09	100,000	-	16	200	1458	1458	4374	1458	4374
S42	Tg-AS09	1,000	-	-	100	1458	1458	13122	4374	1458
S35	control	n/a	-	-	-	-	-	-	-	-
S38	control	n/a	-	-	-	-	-	-	-	-
S40	control	n/a	-	-	-	-	-	-	-	-
S43	control	n/a	-	-	-	-	-	-	-	-
S44	control	n/a	-	-	-	-	-	-	-	-
S45	control	n/a	-	-	-	-	-	-	-	-
S46	control	n/a	-	-	-	-	-	-	-	-
S48	control	n/a	-	-	-	-	-	-	-	-
S49	control	n/a	-	-	-	-	-	-	-	-
S50	control	n/a	-	-	-	-	-	-	-	-