Australian Government
Department of Agriculture, Water and the Environment

AUSTRALIAN Pork

Pilot prevalence survey of *Toxoplasma gondii* in sow meat

Final Report
APL Project 2014/506

June 2015

South Australian Research and Development Institute
Kate Hodgson, Dave Hamilton and Jessica Tan
Gate 2b, Hartley Grove
Urrbrae S.A. 5064

Acknowledgements

This project was supported by funding from Australian Pork Limited and the Department of Agriculture.

We would like to thank pork processors for their cooperation and collecting and sending samples.

Thanks also go to Geoff Holds, Gayle Smith, Viv Mattei and Linda Friedrich (SARDI) for assisting with microbiological and PCR testing.

Executive Summary

Toxoplasma gondii (*T. gondii*) is a protozoan parasite that infects a wide range of warm-blooded vertebrates, including humans. Infection in humans can result from accidental ingestion via contact with cat faeces, consumption of uncooked or undercooked contaminated meat, drinking contaminated water, infected organ transplant or congenital (in utero) transmission from mother to foetus (Tenter et al. 2000). It is one of the most important foodborne pathogens world-wide with the US Centre for Disease Control and Prevention (CDC) ranking it one of three pathogens (including *Salmonella* and *Listeria*) that account for more than 70% of foodborne related deaths (Scallan et al. 2011). '*Toxoplasma* and pork' is ranked second in the top ten pathogen-food combinations in the US. The World Health Organisation (WHO) ranked *T. gondii* from the meat of small ruminants, pork, beef and game fourth in a global ranking tool of foodborne parasites by 'importance' and their primary food vehicle (FAO/WHO 2014). Infection in humans is very common, estimated at 30% of the world's population, however clinical symptoms are generally only observed in at-risk groups such as the immunocompromised, transplant patients and unborn children.

The survival of viable *T. gondii* in meat products is dependent on the processing steps (particularly freezing and cooking time/temperatures parameters) that can affect the survival of tissue cysts. Infective *T. gondii* cysts have been isolated from some uncooked processed pork products after treatments such as salting, smoking, curing and fermentation. Therefore these products and undercooked fresh pork represent a potential source for human infection.

This project aimed to determine the prevalence of *T. gondii* in sow meat that is commonly used in processed meat products. These products include Uncooked Comminuted Fermented Meats (UCFM), a large proportion of which do not have a process step (e.g. freezing) that would eliminate viable *T. gondii*. In addition, culled sow meat is increasingly being utilised in the production of mince and prime cuts, which with the current focus on avoiding overcooking pork, may result in an increased consumer risk of exposure. As toxoplasmosis is a whole-of-life infection, older animals generally have a higher prevalence.

Previous APL projects utilising comparative serological testing of sows have raised questions about the accuracy (specificity and sensitivity) of serological detection methods used to determine the toxoplasma prevalence in the Australian sow population. Issues associated with serology include the potential for cross-reaction, false positives and false negatives whereas quantitative PCR has been demonstrated to be both sensitive and specific with a reduced likelihood of false positives.

The hearts of sows (n=92) from 62 herds (maximum two/herd) were sampled at three processing facilities in South-East (SE) Australia to estimate the *T. gondii* prevalence in culled sows. Sow hearts are recognised to be a predilection site and are easy for abattoir staff to collect. The hearts were homogenised and digested using acid/pepsin to release cysts from the muscle tissue. The homogenate was centrifuged to concentrate the material. DNA was extracted and examined by qPCR for the presence of both the B1 gene and an alternative more numerous PCR target, a 529-base pair fragment of the *T. gondii* genome.

Targeting both the B1 gene and the 529-bp fragment detected the presence of *T. gondii* DNA. However, the B1 qPCR proved less sensitive and there were issues with the commercial kit controls. It was therefore decided to test all samples for the 529-bp fragment. In total, nine samples were positive, all from different herds. The estimated sow prevalence of *T. gondii*, from these results, is 9.8% (standard deviation of +/- 3.1%) which represents a risk to consumers of potential concern. This pilot prevalence estimate is an indication of the level of infection in culled sows under current management systems. It is worth noting that molecular detection does not reflect the level of viable parasites which can only be assessed using a bioassay. Bioassay in mice would also provide sufficient DNA to enable genotyping of the strain of *T. gondii* detected.

A national baseline survey with a statistically designed sampling plan encompassing a greater geographic range and production systems is necessary to determine the national prevalence.

Table of Contents

List of Tables

List of Figures

1. Background to Research

Toxoplasma gondii (*T. gondii*) is an obligate intracellular protozoan parasite that infects a broad spectrum of mammalian species, including humans. The definitive hosts are members of the family Felidae, for example domestic cats; intermediate hosts are warm-blooded animals such as livestock. It is one of the more common parasitic zoonoses world-wide with an estimated 30% of the world's population infected (FAO/WHO 2014). Despite the high infection rate, clinical disease is generally confined to at-risk groups which include the immunocompromised, transplant patients and the unborn foetus if the mother is infected during pregnancy. Most infections in immunocompetent individuals remain asymptomatic, but severe, potentially life-threatening, complications can arise in immunocompromised patients. Congenital infection can also occur during pregnancy when the parasite is transmitted via the placenta, leading to life-time central nervous system abnormalities, eye disease or foetal death. More recent studies have shown that *Toxoplasma* infection is associated with risk taking behaviours which could indirectly lead to traffic and workplace accidents or suicides; it has also been identified as an important risk factor in schizophrenia (Flegr 2013).

T. gondii has three infectious stages in its life cycle; tachyzoites that multiply rapidly then initiate the formation of the slowly multiplying bradyzoites in tissue cysts and finally sporozoites in sporulated oocysts (Tenter et al. 2000). Oocysts are environmentally resistant cysts excreted by cats in their faeces for the relatively short period of two weeks during primary infection (Dubey et al. 1970). Infection can be acquired by both the definitive and intermediate hosts via various routes. These include horizontally by ingestion of infectious oocysts from the environment or foodborne tissue cysts or vertically via transplacental transmission of tachyzoites or in maternal milk (Tenter et al. 2000). The main human transmission routes include the consumption of raw or undercooked tissue from infected animals, oocyst contaminated water or ingestion of sporulated oocysts excreted by cats (Tenter et al. 2000, Dubey 2004).

Toxoplasmosis has been estimated to be responsible for 24% of deaths attributed to a foodborne pathogen in the United States with an estimated cost of US$3.3 billion based on 2000-2008 data from the US Centers for Disease Control and Prevention (CDC) Economic Research Service (Scallan et al. 2011). A recent review assessed the overall impact of seven foodborne pathogens using disability adjusted life years (DALYs) which is an aggregate of the loss of life and health due to illness compared with 'perfect' health, using time as the common metric (Scallan et al. 2015). Of the total annual 112,000 DALYs due to foodborne illnesses acquired in the US using data from 2002 to 2008, non-typhoidal *Salmonella* (32,900) and *T. gondii* (32,700) caused the most DALYs. *Toxoplasma* and pork is ranked second in the top ten pathogen-food combinations in the US (Guo et al. 2015). In Europe, the Food Safety and Codex unit of the World Health Organisation developed a global ranking tool of foodborne parasites by 'importance' and their primary food vehicle (FAO/WHO 2014). *T. gondii* from the meat of small ruminants, pork, beef and game (red meat and organs) ranked fourth behind *Taenia solium* (pork), *Echinococcus granulosus* and *Echinococcus multilocularis* (both from fresh produce). Pork is recognised as a high risk meat due to the high susceptibility of pigs to *T. gondii* infection (Hill and Dubey 2013).

T. gondii is recognised as a foodborne hazard associated with pigs in Australia as indicated in a baseline study (PRDC 1997) and initial Risk Profile (Pointon et al. 2009) by SARDI. However, the consumer risk from consumption of meats is undefined. To date, prevalence estimates of Australian pigs have been based on inconsistent serological data. Food Standards Australia New Zealand (FSANZ) recently developed a Toxoplasmosis Risk Profile which demonstrates concern regarding potential foodborne disease associated with *T. gondii*:

(http://www.foodstandards.gov.au/publications/Documents/Toxoplasma%20gondii%20-%20jan%202014.pdf).

Producers are required by the Australian Pork Industry Quality Assurance Program (APIQ✓®) to implement a range of procedures to minimise exposure of pigs to cats and rodents. There have been substantial changes in sow housing options, particularly to bedding systems during gestation, which may impact on the risk of infection. The increased potential for exposure of pigs to *T. gondii* in organic,

free-range and outdoor bred production systems has also been reported overseas (Dubey et al. 2012, Hill and Dubey 2013, Bacci et al. 2015). International studies have shown that the prevalence differs between geographic regions, possibly due to differences in altitude, temperature and humidity which impact on oocyst survival (Guo et al. 2015). The prevalence of *T. gondii* is influenced by management systems with confined rearing systems and biosecurity protocols reducing infections. Small herds, free-range and backyard raised pigs also have an increased prevalence (USDA 2011).

There is uncertainty related to the survival of tissue cysts in foods with varying resistance to heat, curing with salt, sucrose and low temperature smoking possibly related to the strain of parasite, uneven treatment through a product and consumer cooking behaviour (Tenter et al. 2000, Guo et al. 2015). An Italian study identified a strong association between consumption of cured pork products or raw meat and risk of infection to susceptible pregnant women (Buffolano et al. 1996). An EU study attributed 30 to 63% of *T. gondii* infections in pregnant women to inadequately cooked or cured meats (Cook et al. 2000). In the UK, viable cysts were detected in one of 67 cured meat samples (Warnekulasuriya et al. 1998). Fresh pork sausage has been identified as important in the transmission of toxoplasmosis in a number of studies (Dias et al. 2005, Vitale et al. 2014). Similarly, a report from Spain has shown persistence of infective *T. gondii* cysts in salted fermented Serrano ham (Gomez-Samblas et al. 2015).

Australian Uncooked Comminuted Fermented Meats (UCFM) products were risk-rated using the qualitative risk assessment 'Toxo Tool' (Hamilton et al. 2008). As a result, 43% of total UCFM production was classed as 'High Risk' and 52% of UCFM products tested were identified as manufactured by processes that do not reduce the risk of viable *T. gondii* cysts. Sow meat is increasingly used in the production of mince and prime cuts which when combined with current trends in cooking to avoid overcooking pork ('pink pork'), can lead to food safety issues associated with the consumption of pork and pork products. In addition, uncooked fermented products are popular in the Australian marketplace. A more recent APL risk assessment study of UCFM production in Australia indicated that 82% of UCFMs do not have an effective *T. gondii* kill step (Hamilton et al. 2011).

This project aimed to characterise contamination of sow meat that is commonly used for meat products, a large proportion of which do not have process steps (such as freezing or cooking to an internal temperature of 66°C (Dubey et al. 1990)) that would eliminate viable *T. gondii*. The impact of the fermentation process on the viability of cysts is also not well elucidated representing an unknown risk to consumers.

2. Objectives of the Research Project

1. Determine the prevalence of sows with *Toxoplasma gondii* cysts in edible meat.
2. Establish a library of DNA that can be used to characterise the genotype of *Toxoplasma gondii* to which consumers may be exposed.

3. Introductory Technical Information

Assessment of *T. gondii* prevalence in meat animals and meat products has been attempted using a variety of methods including bioassay, serological assays and various polymerase chain reaction (PCR) techniques (Su et al. 2010, Guo et al. 2015). Bioassay in mice or cats is the 'gold standard', however it is time consuming and expensive. The serological and molecular techniques are faster and simpler. There are many publications detailing prevalence in meat from various species, including pigs. In a recent review summarising the detection of *T. gondii* in pig tissues, prevalence estimates ranged widely from 7/2,094 (positive/tested) positive pork loin samples to 6/6 positive hams, both determined by bioassay (Guo et al. 2015). Heart samples in studies from the US ranged from 29/300 positive by PCR and 14/38 positive by bioassay (Dubey et al. 2008, Velmurugan et al. 2009).

An Australian baseline study to establish the proportion of seropositive pigs was completed in 1998 and estimated a relatively low prevalence by international standards of 1.3% (n=310) in finishers and 11% (n=511) in culled sows (PRDC 1997). The follow-up study in 2006 reported a *T. gondii* prevalence in finishers of 4.6% (Hamilton et al. 2006). This raised concern as culled sows could be expected to have a higher prevalence due to their age and the meat from these animals is commonly used in uncooked smallgoods. Fermentation of meat products is variable and not a kill step or critical control point (CCP), which leaves the potential for consumption of viable parasites in the final product.

A survey of culled sows (n=412) using a commercial serological kit resulted in a prevalence estimate ranging from 6.3 to 22.6% (Hamilton et al. 2008). Inconsistencies in the serological data were investigated by re-testing the sera with three additional commercial serological kits. There were considerable discrepancies between data from the kits, highlighting the issue of test specificity and sensitivity. The kits had been validated in the countries of origin and none were designed specifically for Australia where there is potential for genetic variability. The risk of the presence of *T. gondii* in UCFM was evaluated based on the prevalence estimates, although uncertain, resulting in a 'high risk' classification. Further investigation of culled sows was completed using three commercial serological tests and a molecular protocol (PCR) (Hamilton et al. 2011). *T. gondii* was not detected by the collaborating molecular laboratory and the serological tests were inconclusive. Mince and UCFM samples were analysed and determined to be positive by PCR but were not indicative of prevalence (Hamilton et al. 2011). Further studies at SARDI were designed to develop PCR capability, resulting in nested PCR and qPCR protocols and a live animal model to assess the viability of *T. gondii* positive samples (Hamilton et al. 2014).

Comparison between studies can be problematic when different detection methods have been used. Furthermore, molecular based detection such as PCR is complicated by the non-homogeneous distribution of the cysts in tissue and the small sample size (20 to 50 mg) compared to the bioassay (up to 500 g of tissue is fed to a cat or the digest of 50 g of tissue is injected into a mouse). These factors greatly reduce the likelihood of detection. This study combined the acid/pepsin digestion method of Dubey with the molecular detection of *T. gondii*. The digestion was designed to mimic the passage of meat through the stomach and release cysts from the muscle tissue, resulting in a more homogeneous suspension. An aliquot of the digest was then analysed for the presence of the *T. gondii* genome by targeting specific genes or genome fragments. It is important to note that PCR assays detect DNA from viable and dead or non- infective organisms and therefore may not accurately reflect the actual risk of infection to consumers.

4. Research Methodology

4.1 Sample collection

The survey was originally designed to sample 110 sows from APIQ✓® accredited herds, with the intention of sampling no more than two sows per herd (55 herds), representing a cross-section of the population from South-East (SE) Australia. Sow hearts are recognised to be a predilection site and are easy for abattoir staff to collect. The original sample size of 110 was calculated based on the assumption of the detection of one positive at >3% prevalence (with 95% confidence). Logistic limitations resulted in the sampling of 100 hearts from three processing facilities in SE Australia. The hearts were collected by abattoir employees, frozen on the day of collection and transported in that state to the SARDI Food Safety laboratory at Waite where they were stored at −20°C prior to processing. Eight samples from one plant (Abattoir 1) had insufficient tissue for analysis and these were excluded from the survey. In total, 92 sow hearts from 62 herds were analysed.

4.2 Acid pepsin digestion

The hearts were thawed at 4°C. The connective tissue, fat and epithelium from muscular tissues were removed and the remaining muscle cut with sterile scalpels into 1–2 cm pieces. A 50 g portion of each was homogenised then blended with saline for the acid/pepsin digestion. The method is based on the Dubey protocol (Dubey 1998):

1. Remove connective tissue, fat, epithelium from muscular tissues using nonporous, hard plastic cutting boards and scalpels for solid portions of tissue. Cut muscle into small (1–2 cm) pieces.
2. Grind muscle (50 g) in a blender for 15 seconds at low speed without saline. Add 125 mL of saline and blend at high speed for 30 seconds.
3. Pour the homogenate into a filter stomacher bag. Rinse the blender with a further 125 mL of saline and add this rinse to the bag.
4. Add 250 mL of freshly prepared pre-warmed (37°C) acid pepsin solution and incubate at 37°C with shaking for 1 hour.
5. Mix well and filter the digest utilising the filter stomacher bag (or gauze if necessary) and pour off 250 mL the liquid phase into a 500 mL wide mouth centrifuge bottle and centrifuge at 1200 × g for 10 min.
6. Pour off the supernatant. Resuspend the sediment with 20 mL of phosphate buffered saline using disposable plastic pipettes and transfer the homogenate to a 50 mL centrifuge tube.
7. Add a few drops of phenol red indicator or use pH indicator strips and neutralise the homogenate with of freshly prepared 1.2% sodium bicarbonate (pH 8.3). Colour change to orange (pH = 7.0±0.2). Gently mix the sample.
8. Centrifuge the sample at 1200 × g for 10 min.
9. Pour off the supernatant and add 5–10 mL of saline.
10. The digests were analysed immediately or stored at −20°C for DNA extraction in batches.

4.3 DNA extraction and purification

The DNA of each heart digest was undertaken using the Wizard Genomic DNA Extraction Kit (Promega, Wisconsin USA) following the manufacturer's instructions with modifications:

1. Dispense 17 × 600 µL Nuclei lysis solution into eppendorfs.
2. Add 17.5 µL of 20 mg/mL Proteinase K.
3. Add 25 µL of heart digest.
4. The extraction blank (EB) was 25 µL mQ water (filter purified water)
5. Incubate overnight (O/N) @ 55°C with gentle shaking @ 350 rpm.
6. Add 200 µL Protein precipitation solution & vortex for 20 sec.
7. Chill on ice for 5 min.
8. Centrifuge for 4 min @ 16000 × g.
9. Transfer the supernatant carefully to fresh eppendorfs with 600 µL isopropanol @ room temperature (RT).
10. Mix by inversion till white threads are visible (if possible).
11. Centrifuge for 60 sec @ 16000 × g → small white pellet.
12. Carefully decant the supernatant.
13. Add 600 µL of RT 70% ethanol & gently invert several times to wash the pellet.
14. Centrifuge for 60 sec @ 16000 × g, RT.
15. Carefully aspirate off the ethanol.
16. Invert on clean absorbent paper and air-dry for 15 min.
17. Add 100 µL DNA Rehydration solution.
18. Incubate @ 65°C for 60 min or O/N @ 4°C.
19. Store @ −20°C for PCR.

4.4 Real time polymerase chain reaction (qPCR)

4.4.1 LSI VetMAX™ Toxoplasma gondii kit

The LSI VetMAX™ *Toxoplasma gondii* kit was used according to the manufacturer's instructions for the detection of the B1 gene of *T. gondii* by qPCR:

1. Agitate the <Mix Toxo> briefly and centrifuge.
2. Aliquot 20 µL per sample into PCR tubes including a no template control (<Mix Toxo> only) and the external positive control (EPC).
3. Add 5 µL of EPC or sample to each tube.
4. Run as per the TaqVet Toxo cycle on the Qiagen Rotorgene:

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

4.4.2 Toxoplasma gondii 529-bp repeat element PCR

A novel 529-bp fragment of the *T. gondii* genome was identified by Homan (Homan et al. 2000). A selection of samples were analysed using the primers TOX4 and TOX5 developed by Homan.

The primers used are detailed in Table 1.

Table 1. T. gondii 529bp fragment primers

Target	Primer name	Primer sequence
529bp	TOX4 Fw	CGCTGCAGGGAGGAAGACGAAAGTTG
fragment	TOX5 Rev	CGCTGCAGACACAGTGCATCTGGATT

The PCR mastermix is detailed in Table 2.

Table 2. Mastermix for T. gondii 529bp PCR

Mastermix TOX 529bp	Required conc	Vol required µL
PCR buffer II (10 x)	1 x	2.5
TOX4 (10 µM)	0.5 µM	1.25
TOX5 (10 µM)	0.5 µM	1.25
dNTPs (10 mM, 2.5 ea)	0.8 mM (0.2 ea)	2
$MgCl_2$ (25 mM)	2 mM	2
AmpliTaq		0.25
Ultrapure H_2O		10.75
Total MM		20
DNA template		5

The cycling conditions were:

Step 1: 94°C for 7 min

Step 2: 94°C for 1 min
55°C for 1 min
72°C for 1 min
] 33 cycles

Step 3: 72°C for 10 min

Step 4: Hold @ 4°C

The PCR products were visualised by gel electrophoresis, see *4.4.4*.

4.4.3 Mammalian house-keeping gene PCR

DNA extracts were analysed for a mammalian house-keeping gene indicating the presence of DNA from the host pig heart (Frericks and Esser 2008).

The primers used are detailed in Table 3 and the mastermix in Table 4.

Table 3. 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

Table 4. Mastermix for the house-keeping gene PCR

Mastermix Cxxc1	Required	Vol required µL
2 x MyTaq red		12.5
Cxxc1-F (10 µM)	10 pmol	1
Cxxc1-R (10 µM)	10 pmol	1
Extra mQ water		5.5
DNA template		5
Final volume µL		25

The cycling conditions were:

Step 1: 94°C for 15 min

Step 2: 94°C for 20 sec
55°C for 15 sec
72°C for 20 sec
] 45 cycles

Step 3: 72°C for 3 min

Step 4: Hold @ 4°C

The PCR products were visualised by gel electrophoresis, see *4.4.4*.

4.4.4 Gel electrophoresis of PCR products

The products of PCRs were run on a 1.5% agarose gel containing GelRed for 45–60 min at 150 V.

1. Prepare an agarose gel cast and comb of appropriate size.
2. Prepare a 1.5% agarose gel (e.g. 1.5 g, 100 mL TAE buffer) by melting in the microwave.
3. Allow to cool but not set and add 10 µL GelRed (1 µL per 10 mL agarose) mixing gently.
4. Pour into the agarose gel cast and set.
5. Remove the comb.
6. Aliquot 2 µL of the DNA ladder and 2-10 µL DNA products into the wells.
7. Run the gel @ 100 V for 90 min or 150 V for 50-60 min.
8. Visualise the bands in the Gel-Doc.

4.4.5 Toxoplasma gondii 529-bp repeat element real time PCR (qPCR)

The DNA extracts were analysed by qPCR for the 529 bp fragment using the primers and probes developed by Opsteeg and colleagues (Opsteegh et al. 2010).

The primers and probe used are detailed in Table 5 and the qPCR mastermix in Table 6.

Table 5. Primers and probe for Toxo529 qPCR

Target	**Primer name**		**Sequence**
529bp fragment	Tox-9F	Fw	AGGAGAGATATCAGGACTGTAG
	Tox-11R	Rev	GCGTCGTCTCGTCTAGATCG
	Tox-TP1	Probe	CCGGCTTGGCTGCTTTTCCT
Probe labelled at 5' end with 6-FAM & 3' end with BHQ1			

Table 6. Mastermix for Toxo529 qPCR

Mastermix Tox529bp	**Required conc**	**Vol required µL**
Platinum qPCR SuperMix-UDG (2×)	1 x	12.5
Tox-9F (10 µM)	0.7 µM	1.75
Tox-11R (10 µM)	0.7 µM	1.75
Tox-TP1 probe (10 µM)	0.1 µM	0.25
Ultrapure H_2O		3.75
Total MM		20
DNA template		5

The cycling conditions were:

Step 1: 50°C for 2 min

Step 2. 95°C for 2 min

Step 3. 95°C for 10 sec
58°C for 20 sec
72°C for 20 sec
] 45 cycles

The qPCR was run on the Qiagen Rotorgene.

5. Results

5.1 LSI VetMAX™ Toxoplasma gondii kit

The LSI VetMAX™ *Toxoplasma gondii* kit had been assessed using the acid/pepsin digest of pork mince spiked with the homogenised brain of an infected mouse as described in APL Project 2011/1017.401. The technique was repeated with the muscle of infected mice and sow hearts spiked with infected murine brain. *T. gondii* DNA was detected in the infected muscle and each spiked heart sample, see Table 7. The kit includes a ready-to-use mastermix with one set of primers for the B1 gene and another primer set for detection of an internal positive control (IPC). The IPC is an endogenous gene present in organs and enables verification of the DNA extraction efficiency and the presence or absence of inhibitors in the same sample tube or well. Many foods contain substances inhibitory to PCR which can lead to false negatives or reduced quantification values. The IPC should be detected in DNA extracts from mammals including mice and pigs. The mouse brain and muscle samples analysed were positive for the IPC, including the spiked heart tissue. However, the IPC gene was not detected in the sow heart control, see Table 7.

Table 7. TaqVET qPCR T. gondii B1 gene and IPC Cts

No.	Sample	Type	Ct* *T. gondii* B1 gene	Ct IPC
1	<Mix Toxo>	NTC		
2	mQ	NTC		
3	EPC Toxo	Positive Control	26.86	
4	Murine brain	Unknown	32.77	22.39
5	"	Unknown	31.90	22.32
8	Murine muscle	Unknown	33.70	11.01
9	"	Unknown	38.15	27.69
10	Sow heart control	Unknown		
11	"	Unknown		
12	Sow heart + brain 1	Unknown	41.80	29.25
13	"	Unknown	41.14	25.69
14	Sow heart + brain 2	Unknown	39.40	25.99
15	"	Unknown	42.29	27.31

* The threshold cycle (Ct) is the number of PCR cycles at which the fluorescent signal of the reaction crosses the threshold, that is, the signal is significant and above the background signal. This indicates detection of the target DNA.

Table 8 details examples of the data from selected sow heart samples. Note that the IPC has not been detected in any samples, including the two with positive detection of the *T. gondii* B1 gene, FS15-0023 and FS15-0027.

Table 8. TaqVET qPCR T. gondii B1 gene and IPC Cts of heart samples

No.	Name	Type	Ct *T. gondii* B1 gene	Ct IPC
1	Mix Toxo	NTC		
2	EPC Toxo	Positive Control	27.18	
3	"	Positive Control	27.50	
4	EB 3.02.15	Blank		
5	FS15-0005	Unknown		
6	"	Unknown		
7	FS15-0021	Unknown		
8	"	Unknown		
9	FS15-0023	Unknown	41.14	
10	"	Unknown	37.42	
11	FS15-0024	Unknown		
12	"	Unknown		
13	FS15-0027	Unknown	41.52	
14	"	Unknown		

T. gondii DNA present in the heart DNA extracts could be detected, however, the efficiency of the DNA extraction and presence of inhibitors could not be evaluated. The manufacturer's (LSI) technical help eventually informed us that the kit is designed for ruminants which explained the negative IPC results for the sow heart control. This could potentially compromise the data from the survey samples, so an alternative PCR was investigated.

5.2 Toxoplasma gondii 529-bp repeat element PCR

As background, the novel 529-bp fragment of the *T. gondii* genome is repeated 200 to 300-fold in each genome (Homan et al. 2000). The sensitivity of PCR depends on the copy number of the target gene amplified so this sequence was determined to be more sensitive and specific than the widely used B1 gene with 35 copies per genome. A selection of samples were analysed using the primers TOX4 and TOX5 developed by Homan et al. (2000) and the results verified previous data, see Figure 1. The Vero DNA is the negative control and ME49 DNA the positive control.

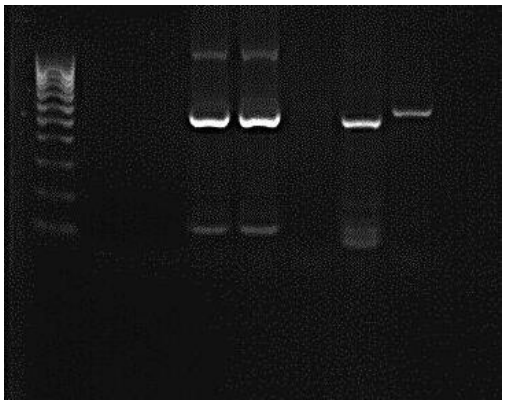

Figure 1. Toxo529 PCR products

Lane 1 DNA ladder, lane 2 NTC, lane 3 Vero, lanes 4 & 5 ME49 10^{-2}, lane 6 ME49 10^{-3}, lane 7 murine brain, lane 8 murine muscle.

The DNA extracts were then analysed using the qPCR protocol which targets the 529-bp fragment (Opsteegh et al. 2010). The data confirmed an increase in sensitivity in comparison to the TaqVET qPCR, as indicated by the lower Ct values for the purified DNA from the infected mouse brain and muscle (the lower Ct means more copies of the target are present). The data are detailed in Figure 2 and Table 9. DNA in the ME49 10^{-3} dilution was also detected whereas no product was observed when analysed using the Homan primers and end-point PCR (Figure 2). Note that *T. gondii* DNA was also detected in one replicate of the sow heart control. The control heart tissue was a homogenate of two hearts collected from one processing facility. It appears there were cysts present. Ideally, hearts from sows known to be parasite free would be used as controls, however these were not available.

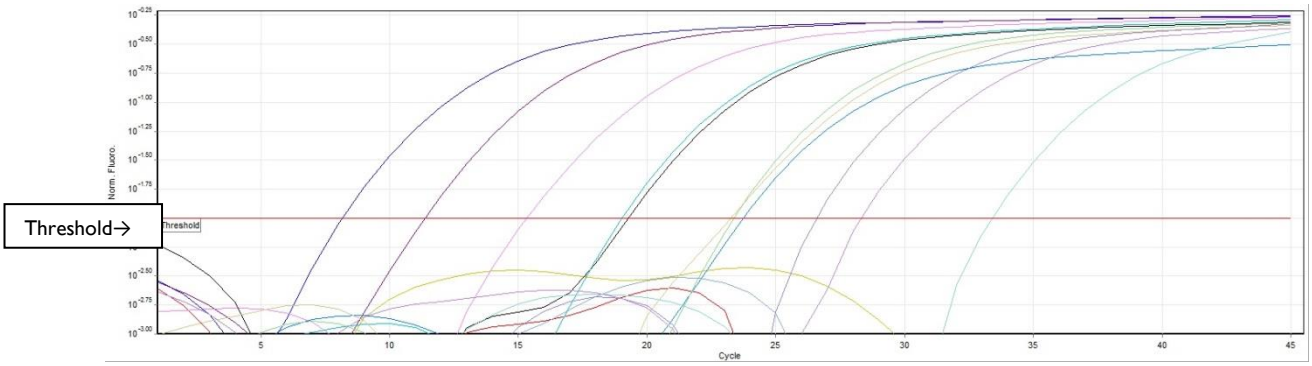

Figure 2. Toxo529 qPCR amplification curves

The threshold fluorescence in this reaction has been set at 0.01(red horizontal line). The PCR cycle number where the fluorescence amplification for a sample or control crosses the threshold is called the Ct. As the quantity of target DNA decreases, the cycle number at which significant amplification is seen increases. The number of cycles required to cross the threshold increases when there is less target DNA present.

Table 9. Toxo529 qPCR Cts

No.	Sample	Type	Ct
1	NTC	Unknown	
2	Vero	Negative Control	
3	ME49	Positive Control	8.16
4	ME49 10^{-1}	Positive Control	11.37
5	ME49 10^{-2}	Positive Control	15.33
6	ME49 10^{-3}	Positive Control	23.73
11	Murine brain	Unknown	19.27
12	"	Unknown	19.02
13	Murine muscle	Unknown	23.25
14	"	Unknown	23.35
15	Sow heart control	Unknown	33.41
16	"	Unknown	
17	Sow heart + brain	Unknown	26.56
18	"	Unknown	28.29

5.2.1 qPCR controls

The *T. gondii* 529 bp end point PCR and qPCR did not include an internal positive control for verification of the DNA extraction and purification efficiency or a mechanism to evaluate the presence or absence of PCR inhibitors. Consequently, each DNA extract was analysed for a mammalian house-keeping gene indicating the presence of DN

A from the host pig heart, see Figure 3.

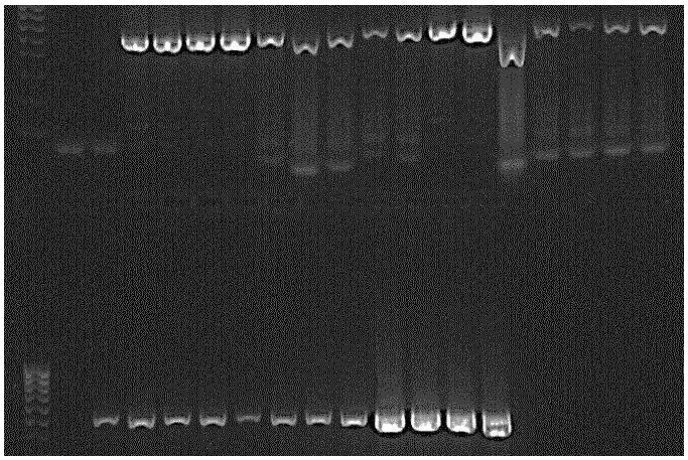

Figure 3. House-keeping gene products
Row 1; Lane 1 DNA ladder, lane 2 NTC, lane 3 EB, lanes 4-19 samples:
Row 2; Lane 1 DNA ladder, lanes 2 EB, lanes 3-13 samples.

Samples with unacceptable DNA extractions, as indicated by the intensity of the bands, were re-extracted and purified prior to qPCR analysis.

Each DNA extract was analysed using the Toxo529 qPCR in triplicate. The level of inhibition was determined for each sample by adding a 1 µL aliquot of purified DNA from *T. gondii* ME49 to one of the triplicate test aliquots. The Ct value derived from this was compared with the Ct of the ME49 DNA only. Inhibition was indicated by a higher Ct value, for the sample with ME49 DNA than for the ME49 DNA only; for example by four or more units.

5.3 Analysis of survey samples

In total, 100 samples were received. There were eight with insufficient quantity for analysis, that is, less than 50 g after the fat and connective tissue were removed. In total, 92 sow hearts from 62 herds were analysed.

An example of the Toxo529 qPCR data is shown in Table 10.

Table 10. Example of Toxo529 qPCR results for a sub-set of samples analysed

No.	Name	Type	Ct	Ct Comment
1	NTC	NTC		
2	Vero	Negative Control		
3	ME49 5uL	Positive Control	18.46	
4	ME49 1uL	Positive Control	20.89	
5	FS15-0021	Unknown		
6	"	Unknown		
7	FS15-0023	Unknown	30.24	Positive
8	"	Unknown	30.83	Positive
9	FS15-0027	Unknown	29.55	Positive
10	"	Unknown	29.15	Positive
11	FS15-0028	Unknown		
12	"	Unknown		
13	FS15-0093	Unknown		
14	"	Unknown		
15	FS15-0094	Unknown		
16	"	Unknown		
17	FS15-0095	Unknown		
18	"	Unknown		
25	FS15-0111	Unknown		
26	"	Unknown		
27	FS15-0112	Unknown		
28	"	Unknown		
29	FS15-0021 + ME49 1uL	Unknown	21.55	
30	FS15-0023 + ME49 1uL	Unknown	20.89	
31	FS15-0027 + ME49 1uL	Unknown	22.14	
32	FS15-0028 + ME49 1uL	Unknown	22.36	
36	FS15-0093 + ME49 1uL	Unknown	21.45	
37	FS15-0094 + ME49 1uL	Unknown	21.21	
38	FS15-0095 + ME49 1uL	Unknown	21.25	
39	FS15-0111 + ME49 1uL	Unknown	20.88	
40	FS15-0112 + ME49 1uL	Unknown	21.03	
41	EB 3.02.15	Unknown		

*Note the Cts of the DNA aliquots with the additional 1 µL of ME49 DNA indicate no or limited inhibition of the qPCR (the maximum difference in Ct was 1.47 units). A sample would be expected to produce a Ct value 3.2 units higher than another sample with ten times the target DNA in a qPCR reaction operating at 100% efficiency. The Ct value is inversely proportional to the amount of DNA target present – the lower the Ct the more target DNA in the sample. More PCR cycles are required for the sample with less target DNA to generate enough DNA copies and a high enough fluorescence signal to cross the threshold.

The improved sensitivity of the assay can be seen by comparison of the Cts of samples FS15-0023 and FS15-0027 from the LSI VetMAX™ kit and the Toxo529 qPCR, see Table 11.

Table 11. Comparison of Cts from the LSI VetMAX kit and Toxo529 qPCR

Name	Type	Ct VetMAX™ kit	Ct Toxo529 qPCR
FS15-0023	Unknown	41.14	30.24
"	Unknown	37.42	30.83
FS15-0027	Unknown	41.52	29.55
"	Unknown		29.15

Presumptive positive samples were re-tested by qPCR for confirmation. One sample, FS15-0025, was not confirmed to be positive. The Ct value of the original positive aliquot was very high at 40.03. The amplification curve was consistent with actual detection but at a very low concentration. Consequently, this sample was determined to be positive at a trace level.

Only one sample, FS15-0764, was shown to have poor DNA extraction efficiency and/or PCR inhibition with no housekeeping Cxxc1 PCR product detected. This sample was positive for the *T. gondii* 529-bp target. The qPCR ME49 inhibition control also indicated PCR inhibition, however there must have been sufficient target and amplification signal for detection. This was the only sample that displayed significant PCR inhibition.

The *T. gondii* 529-bp fragment was detected in nine samples. All nine were from different herds, see Table 12. From these results, the estimated prevalence of *T. gondii* in sow hearts from SE Australian processing facilities is 9.8% (standard deviation of +/- 3.1%).

Table 12. Summary of samples and results of T. gondii 529-bp qPCR

Processing facility	Samples received	No. with insufficient sample	Total no. of herds	No. of herds analysed	No. of positive samples
1	25	8	13	12	0
2	42	0	21	21	3
3	33	0	29	29	6
Total	100	8	63	62	9
Total analysed	92			62	9

6. Discussion

Internationally, the prevalence of finisher and older pigs, such as sows, testing positive for *T. gondii* infection by serology has decreased significantly with changes in production and management of hygiene, confinement and prevention by vaccination (Tenter et al. 2000, Guo et al. 2015). Grower pigs raised under intensive management have been found to have seroprevalences of <10% in many countries (Tenter et al. 2000). Previous studies of the Australian pig herd have raised uncertainty about the sensitivity and specificity of serological testing (Hamilton et al. 2006, Hamilton et al. 2008, Hamilton et al. 2011). Prevalences have varied from 0.74 to 22.9% depending on the method and commercial kit used for the analysis. Furthermore, comparison of the serology results and molecular nested PCR to detect the *T. gondii* B1 gene in APL project 2009/2306 was inconclusive as none of the serologically positive samples were definitively positive by nested PCR for the *T. gondii* B1 gene.

Molecular techniques involve the detection of specific genes of the *T. gondii* genome – the most widely used being the 35-copy B1 gene. The target of the LSI TaqVET qPCR originally proposed as the test of choice for this survey was the B1 gene. This qPCR kit detected the gene in spiked sow heart samples. However, in this study, it became apparent that the internal positive control designed to indicate the efficiency of the DNA extraction and presence or absence of inhibitors was detecting mouse DNA and could not detect pig DNA. Consequently, in the absence of an alternative kit for B1 gene detection by qPCR, another prospective target was investigated, the 200 to 300-repeat 529-bp fragment of the genome (Homan et al. 2000, Reischl et al. 2003, Opsteegh et al. 2010). The probability of detecting this target is higher compared to the B1 gene due to the greater number of copies in every genome, that is, 200 – 300 copies of the 529 fragment compared to 35 copies of the B1 gene.

The distribution of cysts in various tissues of the host animal is uneven and varies in concentration within a tissue. The highest concentration is found in the brain, however, the brain does not constitute a major food source. The second highest parasite load for pigs has been reported in the lungs and heart (Juránková et al. 2014). In order to increase the probability of detecting *T. gondii* in a tissue with scattered and limited numbers of cysts, we employed an acid/pepsin digestion technique that resulted in a more homogeneous suspension of material. The DNA of a subsample of this digest was then analysed for a *T. gondii* gene or fragment by qPCR. Despite this digestion, however, the proportion of *T. gondii* DNA in the extract of an infected animal would be a fraction of the background sow host DNA.

PCR can be inhibited by substances within a food matrix. The level of inhibition was determined for each sample by adding a 1 µL aliquot of purified DNA from *T. gondii* ME49 to one of three test aliquots. The Ct derived from this was compared with the Ct of the ME49 DNA only. Inhibition would be indicated by a significantly higher Ct value (>3 units for example) for the sample with ME49 DNA than for the ME49 DNA only. An alternative to using ME49 DNA is to develop a plasmid that contains a copy of the target gene or DNA fragment. This could be used as a positive control and inhibition control without the need to propagate *T. gondii* in tissue culture. A preliminary attempt to produce a plasmid by ligation of the 529-bp fragment and the smaller 165 bp fragment into JM109 high efficiency competent cells using the Promega pGEM®-T Easy Vector System was unsuccessful but worth pursuing for future studies.

The prevalence determined in this study is likely an underestimate due to the limited sensitivity of the method, although it must be noted that PCR detection is not an indicator of the presence of infective or viable parasites. A number of modified extraction techniques have been developed in recent years to improve the low sensitivity due to the non-homogeneous distribution of cysts and low relative DNA concentrations. These include a sequence-specific magnetic capture method for the isolation and concentration of the *T. gondii* DNA using 100 g samples (Opsteegh et al. 2010) and loop-mediated isothermal amplification (LAMP) (Lin et al. 2012). The sensitivity of the technique could be increased by implementing a concentration step. The magnetic capture method is likely to be more sensitive due to the extraction from a 100 g sample followed by concentration of *T. gondii* specific DNA from 12 mL of the digest. The method employed in this survey started with a 50 g sample and extraction of DNA (host and parasite) from 25 µL of digest. Magnetic capture would be particularly useful when analysing

tissues with very low cyst concentrations such as skeletal muscle from the fore and hind limbs (Juránková et al. 2014).

A limited number of studies have investigated the level of *T. gondii* in heart tissue of pigs by molecular methods. A survey of livestock exposure to *T. gondii* in St. Kitts and Nevis, West Indies detected antibodies in sera from 48% (49/103) of pigs from multiple farms at slaughter and 55% (60/109) of meat juice from hearts (Hamilton et al. 2015). The molecular analysis of heart tissue was completed using the same techniques as in our study except the DNA was extracted from 2 mL of acid/pepsin digest compared to 25 µL. The researchers scaled up the manufacturer's protocol to account for the increased volume of starting material. This may have increased the likelihood of detection of the target DNA. This would require more Promega DNA extraction kits at extra expense. In addition, our instrumentation was limited and not suitable for handling the larger volumes required. The 529-bp fragment was detected in 21% (23/109) of hearts. Samico Fernandez carried out a seroprevalence study of pigs at slaughter in Brazil with 12.5% (38/305) positive (Samico Fernandes et al. 2012). The *T. gondii* B1 gene was detected by nested PCR in DNA extracted from 25 mg of heart tissue of sero-positive samples resulting in 55.2% (21/38) positive. This equates to 6.9% (21/305) of the total number tested.

A recent review of international studies summarised seroprevalences in sows from 2004 to 2013, which ranged between 0% (Malaysia), 4.26% (Slovakia), 9.3% and 16.5% (Germany), 12.4% (Romania), 12.8% and 20.7% (Brazil), 14.4% (China), 15.5% and 24.2% (Spain), 32.3% (Vietnam), 36% (Switzerland) and 37.8% (Argentina) (Guo et al. 2015). In the US the National Animal Health Monitoring System (NAHMS) has reported sow seroprevalences of 20% in 1990, 15% in 1995 and 6% in 2000; for grower/finishers, figures of 3.2% in 1995 and 0.9% in 2000 have been reported (http://www.aphis.usda.gov/wps/portal/aphis/ourfocus/animalhealth/sa_monitoring_and_surveillance/sa_nahms/ct_nahms_swine_studies/).

These studies used a variety of methods and cut-offs or titres to determine when a sample is positive. This means comparison of the data is problematic.

Serology indirectly indicates the presence of the organism through detection of antibodies with the possibility for cross-reaction and false positives. There is also an issue with false negatives due to reduction over time in the immunological response (that is, no longer producing the antibodies to the antigen). In contrast, PCR directly detects the DNA of the organism and while false negatives are possible, false positives are less likely.

The 10% estimate is therefore a minimum prevalence and to more accurately assess the Australian risk, a national baseline survey with a randomised sampling framework based on proportional pig numbers is necessary (approximately 300 samples would give 95% confidence in the national prevalence estimate).

7. Implications & Recommendations

Issue

There were problems associated with the LSI VetMAX™*Toxoplasma gondii* kit for the detection of the B1 gene of *T. gondii* by qPCR. This was found to be unsuitable for pig tissues as it was designed for ruminants. As there are only 35 copies of the B1 gene target of this kit present in the *T. gondii* genome, the alternative target, the 529-bp fragment was more sensitive as there are 200 to 300 copies per genome.

Recommendation

Future studies using PCR should focus on the 529-bp fragment which is repeated 200 to 300 times on the *T. gondii* genome.

Issue

This project has established that acid/pepsin digestion of tissue increases the recovery of *T. gondii* for qPCR. The DNA extracted from the digested heart tissue still contains considerable amounts of sow DNA and the *T. gondii* DNA, if present, represents a very small proportion of the total. The sensitivity could be further improved using the sequence-specific magnetic capture method for the isolation and concentration of the *T. gondii* DNA which would increase the concentration of *T. gondii* specific DNA relative to the sow DNA (Opsteegh et al. 2010).

Recommendation

Develop the sequence-specific magnetic capture method to increase the concentration of *T. gondii* DNA and incorporate with the acid/pepsin digestion.

Issue

The current qPCR protocol for the 529-bp fragment does not include standardised controls for either DNA extraction efficiency or PCR inhibition. In this study we used DNA from a laboratory strain of *T. gondii* ME49 which required culturing of the live parasite followed by DNA extraction and purification. There are potential OHS&W and cross-contamination issues associated with this.

Recommendation

Develop a plasmid as a positive and inhibition control which would bypass the need to culture *T. gondii* in a cell line for assays and provide a simple, economic and consistent standard for further assays.

Issue

The current study was limited to the opportunistic collection of 100 samples from SE Australia for budgetary and logistic reasons, with the result that uncertainty remains as to the national status of *T. gondii* infection in culled sows.

Recommendation

A national baseline survey be conducted with an expanded sample size encompassing a greater geographic range and production systems to better determine the national prevalence.

Issue

The DNA extracted using the methods in this study would not be suitable for genotyping. Molecular detection does not reflect the level of viable or infective *T. gondii* parasites in sow tissues, however, it does indicate the presence of DNA from the parasite. Genotype is of increasing interest internationally and in other meat species.

Recommendation

That future studies combine the qPCR detection of *T. gondii* DNA with the mouse bioassay to determine infectivity and provide DNA from the isolated parasite suitable for genotyping.

Issue

It appears that 10% of Australian sows are infected with *T. gondii*. Processors are unlikely to be aware of the potential risks to consumers or steps that could be taken to mitigate these risks.

Recommendation

That this information be communicated to processors in a non-alarming manner. The availability of the Toxo Tool, with training if needed, should be promoted and information regarding potential kill steps in manufacturing processes provided to assist them to deal with the risk.

8. Technical Summary

The acid/pepsin digestion procedure followed by DNA extraction of tissues infected with *T. gondii* has been validated.

The LSI VetMAX™ *Toxoplasma Gondii* kit is unsuitable for analysis of DNA extracted from porcine samples.

The molecular detection of *T. gondii* using PCR for the 529-bp fragment of the genome is more sensitive than for the B1 gene. This is likely due to the number of copies of the fragment, 200 to 300, compared to the B1 gene with 35 copies.

The qPCR detection of the 529-bp fragment is more sensitive than conventional end point PCR.

9. Literature Cited

Bacci, C., A. Vismarra, C. Mangia, S. Bonardi, I. Bruini, M. Genchi, L. Kramer and F. Brindani (2015). "Detection of Toxoplasma gondii in free-range, organic pigs in Italy using serological and molecular methods." International Journal of Food Microbiology **202**: 54-56.

Buffolano, W., R. E. Gilbert, F. J. Holland, D. Fratta, F. Palumbo and A. E. Ades (1996). "Risk factors for recent toxoplasma infection in pregnant women in Naples." Epidemiology and Infection **116**(3): 347-351.

Cook, A. J. C., R. E. Gilbert, W. Buffolano, J. Zufferey, E. Petersen, P. A. Jenum, W. Foulon, A. E. Semprini and D. T. Dunn (2000). "Sources of toxoplasma infection in pregnant women: European multicentre case-control study." British Medical Journal **321**(7254): 142-147.

Dias, R. A., I. T. Navarro, B. B. Ruffolo, F. M. Bugni, M. V. Castro and R. L. Freire (2005). "Toxoplasma gondii in fresh pork sausage and seroprevalence in butchers from factories in Londrina, Parana State, Brazil." Rev Inst Med Trop Sao Paulo **47**(4): 185-189.

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

Dubey, J. P. (2004). "Toxoplasmosis – a waterborne zoonosis." Veterinary Parasitology **126**(1–2): 57-72.

Dubey, J. P., D. E. Hill, D. W. Rozeboom, C. Rajendran, S. Choudhary, L. R. Ferreira, O. C. H. Kwok and C. Su (2012). "High prevalence and genotypes of Toxoplasma gondii isolated from organic pigs in northern USA." Veterinary Parasitology **188**(1-2): 14-18.

Dubey, J. P., D. E. Hill, N. Sundar, G. V. Velmurugan, L. A. Bandini, O. C. H. Kwok, V. Pierce, K. Kelly, M. Dulin, P. Thulliez, C. Iwueke and C. Su (2008). "Endemic toxoplasmosis in pigs on a farm in Maryland: Isolation and genetic characterization of Toxoplasma gondii." Journal of Parasitology **94**(1): 36-41.

Dubey, J. P., A. W. Kotula, A. Sharar, C. D. Andrews and D. S. Lindsay (1990). "Effect of high temperature on infectivity of Toxoplasma gondii tissue cysts in pork." Journal of Parasitology **76**(2): 201-204.

Dubey, J. P., N. L. Miller and J. K. Frenkel (1970). "The Toxoplasma gondii oocyst from cat feces." Journal of Experimental Medicine **132**(4): 636-662.

FAO/WHO (2014). Multicriteria-based ranking for risk management of food-borne parasites. Microbiological Risk Assessment Series Rome. Food and Agriculture Organization of the United Nations/World Health Organization.

Flegr, J. (2013). "How and why Toxoplasma makes us crazy." Trends in Parasitology **29**(4): 156-163.

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

Gomez-Samblas, M., S. Vílchez, J. C. Racero, M. V. Fuentes and A. Osuna (2015). "Quantification and viability assays of Toxoplasma gondii in commercial "Serrano" ham samples using magnetic capture real-time qPCR and bioassay techniques." Food Microbiology **46**(0): 107-113.

Guo, M., J. P. Dubey, D. Hill, R. L. Buchanan, H. Ray Gamble, J. L. Jones and A. K. Pradhan (2015). "Prevalence and risk factors for toxoplasma gondii infection in meat animals and meat products destined for human consumption." Journal of Food Protection **78**(2): 457-476.

Hamilton, C. M., P. J. Kelly, P. M. Bartley, A. Burrells, A. Porco, D. Metzler, K. Crouch, J. K. Ketzis, E. A. Innes and F. Katzer (2015). "Toxoplasma gondii in livestock in St. Kitts and Nevis, West Indies." Parasites and Vectors **8**(1).

Hamilton, D., K. Hodgson, D. May, J. Ellis, M. McAllister, A. Kiermeier and A. Pointon (2014). Establishing the risk of Toxoplasmosis associated with the consumption of pork and pork products, APL Project 2011/1017.401.

Hamilton, D., G. Holds, A. Kiermeier, M. O'Callaghan, D. Brake, M. Lorimer, J. Slade, D. Davos, M. Heuzenroeder, I. Ross, S. Lester, R. Nichols, B. Mullan, R. Buddle and A. Pointon (2006). Food Safety Risk Management to Maximise Market Safety, APL Project No. 2019. South Australian Research & Development Institute.

Hamilton, D., G. Holds, D. May, R. Flint, J. Slade, L. Pallant, A. Thompson and A. Kiermeier (2011). Food Safety Priorities - Baseline Survey of Carcases and Mince and *Toxoplasma gondii* Genotyping. Final Report, APL Project 2009/2306. South Australian Research & Development Institute.

Hamilton, D., G. Holds, D. May, R. Flint, J. Slade, L. Pallant, A. Thompson and A. Kiermeier (2011). Underpinning Knowledge for Prerequisite Programs for Food Safety Regarding Pork, APL Project 2010/1016.346. South Australian Research and Development Institute.

Hamilton, D., G. Holds, G. Smith, M. Lorimer, J. Slade, D. Davos and A. Kiermeier (2008). Safety of domestically retailed pork and pork products, APL report No. 2198. South Australian Research & Development Institute.

Hill, D. E. and J. P. Dubey (2013). "Toxoplasma gondii prevalence in farm animals in the United States." International Journal for Parasitology **43**(2): 107-113.

Homan, W. L., M. Vercammen, J. De Braekeleer and H. Verschueren (2000). "Identification of a 200- to 300-fold repetitive 529 bp DNA fragment in Toxoplasma gondii, and its use for diagnostic and quantitative PCR." International Journal for Parasitology **30**(1): 69-75.

Juránková, J., W. Basso, H. Neumayerová, V. Baláž, E. Jánová, X. Sidler, P. Deplazes and B. Koudela (2014). "Brain is the predilection site of Toxoplasma gondii in experimentally inoculated pigs as revealed by magnetic capture and real-time PCR." Food Microbiology **38**: 167-170.

Lin, Z., Y. Zhang, H. Zhang, Y. Zhou, J. Cao and J. Zhou (2012). "Comparison of loop-mediated isothermal amplification (LAMP) and real-time PCR method targeting a 529-bp repeat element for diagnosis of toxoplasmosis." Veterinary Parasitology **185**(2-4): 296-300.

Opsteegh, M., M. Langelaar, H. Sprong, L. den Hartog, S. De Craeye, G. Bokken, D. Ajzenberg, A. Kijlstra and J. V. der Giessen (2010). "Direct detection and genotyping of Toxoplasma gondii in meat samples using magnetic capture and PCR." International Journal of Food Microbiology **139**(3): 193-201.

Pointon, A., P. Horchner, D. Hamilton, K. Nairn, P. Pattison, P. Mitchell and J. Slade (2009). Pork on-Farm HACCP Plan, 2009/2260. South Australian Research and Development Institute.

PRDC (1997). An empirical risk-based assessment of post-mortem pig meat inspection in Australia. A. Pointon, Pig Research and Development Corporation.

Reischl, U., S. Bretagne, D. Krüger, P. Ernault and J. M. Costa (2003). "Comparison of two DNA targets for the diagnosis of Toxoplasmosis by real-time PCR using fluorescence resonance energy transfer hybridization probes." BMC Infectious Diseases **3**.

Samico Fernandes, E. F. T., M. F. T. Samico Fernandes, P. C. P. Kim, P. P. F. De Albuquerque, O. L. De Souza Neto, A. De S. Santos, É. P. B. X. De Moraes, E. G. F. De Morais and R. A. Mota (2012). "Prevalence of toxoplasma gondii in slaughtered pigs in the state of Pernambuco, Brazil." Journal of Parasitology **98**(3): 690-691.

Scallan, E., R. M. Hoekstra, F. J. Angulo, R. V. Tauxe, M.-A. Widdowson, S. L. Roy, J. L. Jones and P. M. Griffin (2011) "Foodborne Illness Acquired in the United States—Major Pathogens." Emerging Infectious Diseases **17** DOI: 10.3201/eid1701.P11101.

Scallan, E., R. M. Hoekstra, B. E. Mahon, T. F. Jones and P. M. Griffin (2015). "An assessment of the human health impact of seven leading foodborne pathogens in the United States using disability adjusted life years." Epidemiology and Infection.

Su, C., E. K. Shwab, P. Zhou, X. Q. Zhu and J. P. Dubey (2010). "Moving towards an integrated approach to molecular detection and identification of Toxoplasma gondii." Parasitology **137**(1): 1-11.

Tenter, A. M., A. R. Heckeroth and L. M. Weiss (2000). "Toxoplasma gondii: From animals to humans." International Journal for Parasitology **30**(12-13): 1217-1258.

USDA (2011). Seroprevalence of *Trichinella* and *Toxoplasma* in U.S. grower/finisher pigs., U.S. Department of Agriculture.

Velmurugan, G. V., C. Su and J. P. Dubey (2009). "Isolate designation and characterization of toxoplasma gondii isolates from pigs in the United States." Journal of Parasitology **95**(1): 95-99.

Vitale, M., G. Tumino, S. Partanna, S. La Chiusa, G. Mancuso, M. La Giglia and V. D. Lo Presti (2014). "Impact of Traditional Practices on Food Safety: A Case of Acute Toxoplasmosis Related to the Consumption of Contaminated Raw Pork Sausage in Italy." Journal of Food Protection **77**(4): 643-646.

Warnekulasuriya, M. R., J. D. Johnson and R. E. Holliman (1998). "Detection of Toxoplasma gondii in cured meats." International Journal of Food Microbiology **45**(3): 211-215.