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National serological baseline survey of *Toxoplasma gondii* in lambs and sheep

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Abstract

Toxoplasma gondii is one of the most common parasitic infections of human and other warm-blooded animals. One infection route for humans is through ingestion of infected animal tissue. International data on the sero-prevalence of livestock animals indicates that lambs and sheep may pose a significant risk to humans, however no up-to-date data for Australia exists.

A national baseline survey was undertaken to estimate the sero-prevalence of *T. gondii* in lambs and sheep. Blood and tongue samples were collected at abattoirs. The national estimate of sero-prevalence for lambs is 16.4% (approx 95% CI: 9.8% to 22.9%) and 31.9% for sheep (approx 95% CI: 26.5% to 37.3%). Tongue samples are stored at -80ºC so any tissues cysts can be genotyped at a later date.

In the future, this updated national sero-prevalence information can be used to inform risk assessments undertaken for *T. gondii*, while any genotyping data will provide information about the likelihood of lamb and sheep meat being attributed a proportion of overall risk.

Executive summary

Toxoplasma gondii is one of the most common parasitic infections of human and other warm-blooded animals. One infection route for humans is through ingestion of infected animal tissue. International data on the sero-prevalence of livestock animals indicates that lambs and sheep may pose a significant risk to humans, but data for Australia is 20 years old.

The aim of this project was to obtain a national sero-prevalence estimate for *T. gondii* in lambs and sheep. To achieve this aim a national survey of 249 lambs and 388 sheep slaughtered at 12 export abattoirs was undertaken. Blood was collected immediately after sticking and a piece of tongue removed from each sampled animal. The sera were tested for *T. gondii* antibodies using a modified latex agglutination test. Tongue samples are stored at -80ºC so any tissues cysts can be genotyped at a later date.

The national estimate of sero-prevalence was calculated to be 16.4% (approx 95% CI: 9.8% to 22.9%) for lambs and 31.9% (approx 95% CI: 26.5% to 37.3%) for sheep. The higher sero-prevalence for sheep compared to lambs is in line with international experience and reflects an increased likelihood of exposure with age. In addition, considerable variability between the states from which animals originated was observed.

In the future, this updated national sero-prevalence information can be used to inform risk assessments undertaken for *T. gondii*. In addition, any genotype information obtained from the tongues samples will allow for comparisons with genotypes isolated from human cases.

Acknowledgements

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Contents

1 Background

Toxoplasma gondii is one of the most common parasitic infections of humans. In Australia the seroprevalence to *T. gondii* in humans is comparable to other developed countries, with at least 30-40% of the mainland population having antibodies to the parasite (Johnson, 1992; Sfameni, Skurrie and Gilbert, 1986). Since one of its transmission routes to humans is via raw or undercooked meat, it is considered a significant foodborne public health hazard.

Most human infections with the protozoan parasite *T. gondii* are asymptomatic, but severe symptoms can occur in immunocompromised patients, in developing foetuses and in ocular infections in immunocompetent individuals (Edvinsson et al., 2007). Most infections in human and other secondary hosts progress to a chronic stage (formation of tissue cysts) without major complications and remain clinically unapparent (Dubey and Beattie, 1988; Tenter, Heckeroth and Weiss, 2000). However, there is emerging evidence that implicates *T. gondii* in a suite of mental disorders occurring in otherwise healthy people (McAlister, 2005). Blindness and mental retardation can also result in congenitally infected children and severe disease results from infection of foetuses and immuno-compromised patients (Tenter et al., 2000).

For New Zealand, estimates based on existing data suggest about 80 (65-102) infections amongst pregnant woman annually. Exposure of pregnant Australian women reflects that of New Zealand (0.5% per pregnancy), resulting in about 400 (325-510) infections (reviewed in 2007 MLA Risk Profile for *T. gondii)*.

While sheep, goats and beef are all intermediate hosts of *T. gondii*, sheep meat poses the greatest risk to human health (based on 20 year old sero-prevalence data) based on the following two reasons:

- Relatively high sero-prevalence – considerably higher than in beef;
- Relatively high consumption – much higher than goats.

Furthermore, sheep meat, and in particular lamb meat, is not often served 'well done', which allows for viable cysts to persist in the meat (effective cooking kills *T. gondii* cysts). Changes in the diversity of the end-use pathways of these products may also exacerbate the risk posed by sheep and lamb meat.

Sero-prevalence indicates past exposure as cysts remain viable for the life of the animal. Detection of cysts is costly and time consuming (animal experiments), but recent developments in the use of PCR tests to screen for presence of cysts (viable and non-viable) and their genotypes would allow for better estimation of the exposure risk to consumers (2007 MLA Risk Profile for *T. gondii)*.

Consequently, a sero-prevalence survey, with provisions for follow-up PCR screening of tissue, in sheep and lambs would allow the industry to estimate the risk posed by *T. gondii* by this exposure route.

2 Project Objectives

1. Estimate the *T. gondii* sero-prevalence in lambs.
2. Estimate the *T. gondii* sero-prevalence in older sheep.
3. Develop a storage bank of tissue (tongue samples) from sero-positive animals for genotyping at a later date.
4. Keep a watch on international and domestic literature regarding methodology for *T. gondii* genotyping, and which organisations are involved in *T. gondii* research.

3 Methodology

3.1 Sampling

3.1.1 Abattoir Selection

For practical reasons, samples were only collected from export abattoirs since these abattoirs source stock from a wide geographic area, which is expected to be similar to that used by domestic abattoirs. This approach does not provide an equal probability for each animal to be sampled, which biases the prevalence estimates. However, due to the large proportion of throughput captured this way, it is unlikely that this bias would be large.

There are a total of 29 export abattoirs around Australia. The number of plants visited was based on an approximate proportional allocation according to the total (sheep+lamb) throughput per state. For practical reasons, the number of abattoirs per state was limited to three. It is expected that the effect of this choice is negligible as abattoirs source stock from a relatively wide area. Consequently, visiting more abattoirs would increase collection costs without necessarily providing better geographical representation, while fewer abattoirs could place too much reliance on the geographic variability of the lots of sheep processed on the days of sampling. The single Tasmanian export abattoir was not visited, since Tasmania only represents 3% of the overall production volume of sheep and lamb meat. This choice was made even though Tasmania has historically had higher sero-prevalence (2007 MLA Risk Profile for *T. gondii*). Proper investigation of sero-prevalence in Tasmania would require a baseline in its own right.

A summary of the number of plants visited per state is given in the following table.

NSW	VIC	QLD	TAS	SA	WA	Total
3	3	1	0	2	3	12

To maximise the number of samples which could be collected on a single day, only the major and geographically diverse plants per state were visited.

The systematic selection of abattoirs does compromise the randomisation aspect of the abattoir and animal selection, but the effects are likely to be negligible. In addition, it does overcome the potential problem of selecting lower throughput abattoirs which may not source stock as widely as the major throughput abattoirs.

3.1.2 Sample Size Determination

Since lamb and sheep meat present different pathways of exposure for humans, a separate baseline was undertaken for each.

Previous research indicates that lambs (<1 year old) have a sero-prevalence of about 20%, lower than the 50% observed in older sheep. Using a margin of error of ±5% and a confidence level of 95%, the required sample sizes are 246 (use 250) and 385 (use 400) for lambs and sheep, respectively, assuming a simple random sample.

Sample numbers for each abattoir were determined as follows. Firstly, a proportional allocation of sample numbers was undertaken for each state, based on the state throughput (separately for lambs and sheep), using a total sample size of 250 for lambs and 400 for sheep. The annual kill figures (1 July 2006 to 30 June 2007) are provided in Table 1 (pers. comm. Paul Smith, AQIS).

Table 1: Number of sheep and lambs slaughtered by state (1 July 2006 to 30 June 2007).

	NSW	VIC	QLD	SA	TAS	WA	Totals
Lamb	2,035,579	4,928,053	20,491	3,002,116	201,916	1,930,484	**12,118,639**
Sheep	3,306,285	2,305,630	731,068	1,152,916	123,140	2,042,590	**9,661,629**
Total	5,341,864	7,233,683	751,559	4,155,032	325,056	3,973,074	**21,780,268**

Then the samples for each state were allocated to selected abattoirs based on the proportional throughput of those abattoirs (ignoring abattoirs from other states). A summary of the sample numbers for lambs and sheep, per abattoir, is given in Table 2.

Ideally, large sheep herds are sampled more intensively than smaller herds. However, at the time of processing it is often not possible to determine the size of the herd from which a particular lot of sheep/lambs originated from. The best surrogate for this is the size of the lot to be slaughtered, which was obtained from the vendor declaration. While some lot sizes were able to be recorded, this was not always the case as some abattoirs sporadically and without notice changed the order of lots to be slaughtered. Nevertheless, four animals were sampled from lots less than 200; six animals were sampled from lots of size 200 to 400 animals; eight animals were sampled from lots greater than 400.

Since lots were not always able to be tracked at the time of sampling, estimation of lot-to-lot variability is not possible.

Table 2: Throughput and sample numbers for lambs and sheep from selected abattoirs.

		Annual Kill		Sample size	
State	**Abattoir**	**Sheep**	**Lambs**	**Sheep**	**Lambs**
NSW	Abattoir 1	1,450,000	365,000	80	9
	Abattoir 2	1,022,000	262,000	57	7
	Abattoir 3	0	1,000,000	0	26
	Total			*137*	*42*
QLD	Abattoir 4	525,000	180,000	30	0
	Total			*30*	*0*
SA	Abattoir 5	946,267	876,177	48	25
	Abattoir 6	0	1,300,000	0	37
	Total			*48*	*62*
VIC	Abattoir 7	1,000,000	50,000	53	3
	Abattoir 8	806,868	373,260	43	20
	Abattoir 9	0	1,500,000	0	79
	Total			*95*	*102*
WA	Abattoir 10	1,520,000	204,000	75	5
	Abattoir 11	163,000	768,000	8	20
	Abattoir 12	20,000	530,000	1	14
	Total			*85*	*40*
Total*				***395***	***246***

* The total sample numbers do not equal 250 and 400 due to rounding error.

3.1.3 Sample Collection

Serum and tongue samples were collected, from the same animal, at the abattoirs immediately after sticking. The serum sample was collected to determine the sero-prevalence in lambs and sheep. Tongue samples were collected to allow for potential follow up testing for tissue cysts via PCR. In particular, tongue, along with diaphragm and brain tissue, has been identified as a preferential site for tissue cysts. Tongue has been chosen since it is much easier to sample from the same animal from which serum samples are collected (can be collected at the same time) than heart or diaphragm which would have to be sampled later in the slaughter chain, potentially resulting in sampling a different animal.

Serum and tongue samples are stored at -80ºC at the SARDI laboratory at Glenside. Freezing will affect the viability of tissue cysts. However, this is considered to be of little importance as detection of tissue cysts in tongues is of primary interest and these cysts can be considered viable in the live animal.

As far as possible, NLIS tag information was recorded at the time of sampling. However, not all animals presented with NLIS tags at the time of slaughter. Some animals had alternate identification tags (information was recorded), some tags were illegible and some animals were presented without tags.

3.1.4 Serological Testing

Serum samples were tested using the Eiken Indirect Latex Agglutination Test (ILAT), which was recommended as a very good test (pers. comm.. R Dempster, Intervet NZ Ltd). Samples were diluted two-fold, with dilutions ranging from 1:16 to 1:1048. Samples were determined to be positive when a reaction occurred at dilutions greater than or equal to 1:32.

4 Results and Discussion

4.1 National Sero-prevalence estimates

In total, 279 lamb serum samples and 424 sheep serum samples were collected, of which 30 lamb sera and 36 sheep sera were unsuitable for analysis due to sample degradation. Consequently, the resulting sample sizes are 249 for lambs and 388 for sheep.

Of these, 37 (14.9%; 95% CI[1]: 10.4-19.3%) lambs and 126 (32.5%; 95% CI: 27.8-37.1%) sheep were sero-positive for *T. gondii* antibodies at dilutions of 1:32 or greater. These observations are consistent with those reported internationally by Dubey and Beattie (1988).

A summary of titres is given in Table 3. It should be noted that the age of the lambs is unknown but they can be expected to be at least 16 weeks of age. Thus it is unlikely that very low titres of positive samples are due to maternal antibodies.

Table 3: 'Raw' prevalence estimates and titre distribution of *T. gondii* in lambs and sheep.

	% positive	≤1:16	1:32	1:64	1:128	1:256	1:512	1:1024	Total
Lambs	14.9	212	25	11	0	1	0	0	249
Sheep	32.5	262	49	34	23	7	8	5	388

The largest discrepancy between expected and actual sample numbers was observed in the lamb samples from NSW and sheep samples from WA – 20 of 46 lambs samples collected in NSW and 27 of 98 sheep samples collected in WA were unusable. The most likely explanation is that some ingesta content was collected with the blood, making the resulting serum unusable. This is not detectable until after clot retraction.

4.2 Sero-prevalence estimates by geography

The state of origin could not be determined for 11 of the 249 lambs sampled and for 110 of the 388 sheep sampled. This is likely due to the still recent introduction of the NLIS tags, which would be expected to be less of an issue for younger animals than older animals.

It should be noted that while samples were collected from a Queensland plant, no animals originated from that state. A summary of the number of serum samples by state of origin and state of slaughter is provided in Table 4.

[1] These confidence intervals are approximate intervals only.

Table 4: Sample numbers for each combination of 'state of origin' (rows) and 'state of slaughter' (columns) for lambs and sheep.

State of Origin	State of Slaughter									
	Lambs					Sheep				
	NSW	QLD	SA	VIC	WA	NSW	QLD	SA	VIC	WA
NSW	21			3		88	40	1		
SA			28	60				26		
TAS								2	9	
VIC			35	51		16			25	
WA					40				9	62

A summary of the prevalence estimate for lambs and sheep by state of origin is provided in Table 5.

Table 5: Prevalence estimates and titre distribution for lambs and sheep by state of animal origin

	% positive	negative (≤1:16)	1:32	1:64	1:128	1:256	1:512	1:1024	Total
Lambs									
NSW	25.0	18	4	2					24
SA	11.4	78	9	1					88
TAS	NA	0							0
VIC	12.8	75	7	3		1			86
WA	15.0	34	1	5					40
Sheep									
NSW	27.9	93	20	8	7	1			129
SA	50.0	13	3	7	2	1			26
TAS	18.2	9	0	0	1		1		11
VIC	36.6	26	4	6	1		1	3	41
WA	33.8	47	9	6	6	1	2		71

From Table 5 it can be seen that, for lambs, the highest prevalence estimate was obtained for NSW, which was twice as high as the estimates for SA and Victoria. In contrast, for older sheep, the highest prevalence estimate was obtained from SA, while the lowest estimate came from Tasmania (only 11 animals tested). Nevertheless, no statistically significant differences were detected between the prevalence estimates using Fisher's exact test (lambs: P-value = 0.38; sheep: P-value = 0.19).

An appropriate national prevalence estimate can be obtained by weighting the state prevalence estimates by the corresponding state livestock figures according to the method described in Appendix 1 – Weighted prevalence estimation. The state livestock numbers were obtained from ABS estimates (Catalogue number 7111.0 Livestock numbers-Year ended 30 June) and are shown in Table 6..

Table 6: Livestock number estimates for sheep and lambs by state (ABS, Catalogue number 7111.0 Livestock numbers-Year ended 30 June)

	NSW	VIC	QLD	SA	TAS	WA	Totals
Lamb	20,787,000	12,827,000	3,732,000	7,627,000	2,056,000	15,509,000	62,538,000
Sheep	7,827,000	4,895,000	898,000	3,730,000	704,000	5,605,000	23,659,000
Total	28,614,000	17,722,000	4,630,000	11,357,000	2,760,000	21,114,000	86,197,000

Consequently, the weighted prevalence estimates for lambs and sheep are 16.4% (approx 95% CI: 9.8% to 22.9%) and 31.9% (approx 95% CI: 26.5% to 37.3%), respectively. It should be noted that the confidence intervals are larger than the planned ±5% which is due to the smaller than expected number of animals which could be identified by state of origin.

4.2.1 Geographical distribution

As indicated above, not all animals sampled had an NLIS tag. However, for those that were tagged a summary by region could be obtained form the Property Identification Code (PIC) of the tag. These summaries are presented below. In general this shows that while some regions were sampled more heavily, there generally is good spread over many geographical regions. Consequently it can be concluded that a reasonable geographical distribution of animals was achieved. However, it is not possible to assess whether the geographical regions/municipals/districts are represented on a basis proportional to livestock numbers.

4.2.1.1 NSW

The municipal code is given by 3rd and 4th position of PIC.

```
Municipal: 01 02 03 04 05 11 14 16 17 22 25 27 29 34 42 43 44 50 51 54 55 62 63
Samples:    2 44  2  1  8  1  3  5  8  1 17  8  7  4  1  7  4  3 11  1  6  1  7
```

Note that the animals for municipal 02 were all sheep and originated from 12 different properties, with a maximum of eight animals from any one property. Similarly, animals from municipal 25 were all sheep, originating from four properties, with a maximum of six animals from any one property.

4.2.1.2 SA

The region code is given by 3rd and 4th position of PIC.

```
Region: 10 11 16 23 24 25 26 27 28 30 32 33 34 36 40 50 51 52 53 54 55 56 58 60 62 70 72 80
Samples: 6  3 10  6  3  9  2 16  3  8  2  3  4  2  1 12  1  2  1  1  5  1  1  1  3  1  3  4
```

Note that the animals for region 27 were mainly lambs (two sheep) and originated from five different properties, with a maximum of four animals from any one property. Similarly, animals from region 50 (Kangaroo Island) originated from three different properties, with a maximum of eight animals from one property. For the KI animals three of four sheep were positive (from two properties) and only one of eight lambs were positive for *T. gondii*.

4.2.1.3 TAS:

The municipal code is given by the 3rd and 4th position in the PIC.

```
Municipal: NN WE
Samples:    6  2
```

4.2.1.4 VIC:

The municipal code is given by the 2nd and 3rd position in the PIC.

Municipal:	AR	BT	CE	CL	CP	GA	GM	GP	HP	LN	MB	MH	ML	MY	NG	NI	PY	SB	SF	SH	WC
Samples:	19	1	1	4	1	2	1	3	2	24	1	3	1	12	6	2	5	4	10	7	6

Note that the animals for shire AR consisted of 5 lambs and 14 sheep which originated from 4 different properties, with a maximum of 8 animals from one property. Similarly, animals from shire LN were all sheep, originating from 5 properties, with a maximum of 6 animals from any one property. All animals from shire LN were lambs.

4.2.1.5 WA:

The shire code is given by the 3rd to 4th position in the PIC.

Shire:	AY	BG	BT	CU	DN	EE	GP	HY	JP	KO	LG	MA	MH	MR	MW	NG	PY	WL	WM	WN	WR	YK
Samples:	4	1	4	13	12	7	10	4	7	1	15	1	1	2	2	1	16	3	1	1	4	1

Note that the animals for shire PY consisted of four lambs and 12 sheep which originated from three different properties, with a maximum of eight animals from one property. Similarly, animals from shire LG were all sheep, originating from two properties, with a maximum of 11 animals from any one property.

4.3 Comparison to previous prevalence information

Previous information on *T. gondii* prevalence has been summaries in a Risk Profile "*Toxoplasma gondii* in meat and meat products" prepared for MLA in 2007 (Table 4.4). The relevant information for sheep is reproduced in Table 7 below.

Table 7: Relative seroprevalence of *T. gondii* in sheep in Australia.

State	Test	Animals Tested	Positive	Reference
Queensland	CFT	157	<1	(Cook and Pope, 1959)
NSW	DT	485	6	(Cook, 1961)
	DT	230	88	(Hartley and Moyle, 1968)
Queensland	IFA	1022	14	(Munday, 1970)
Various	DT	48	84	(Plant, Richardson and Moyle, 1974)
Tasmania (ewes)	IFA	144	62	(Munday, 1975)
Tasmania (lambs)	IFA	160	17	(O'Donoghue, Riley and Clarke, 1987)
South Australia	IHAT	1159	7	(Plant, Freeman and Saunders, 1982)
	IgG	1159	9	(Plant et al., 1982)
	ELISA	1159	25	(Plant et al., 1982)
NSW	IgM	5724	9	(Plant et al., 1974)

These results show a large variation in prevalence and because most are not systematic surveys and there are differences in the test methodology they are not directly comparable to the results presented here.

The current survey provides national estimates for lambs and sheep which were not previously available.

4.4 Recent international sero-prevalence estimates

A literature search for post 1990 publications on T. gondii seroprevalence in lambs or sheep was undertaken. The results of the search are displayed in Table 8. From this Table it can be seen that

- The Seroprevalence in lambs has not received much international attention. One exception is the paper by (Dubey et al., 2008) in which the authors report a prevalence of 27.1% in lambs in the US.
- There have been no publications reporting the prevalence in lambs or sheep in New Zealand. This may be due to the availability and use of a vaccine in New Zealand.
- There are few publications on the prevalence in lambs and sheep originating in Europe.
- Most of the published investigations do not report cross-sectional baseline data similar to that reported here.

Table 8. Relative seroprevalence of *T. gondii* in lambs and sheep in countries other than Australia (post 1990)

Country	Area	Sheep/ Lamb	Test	No. Animals Tested	Positive	Reference
USA	Maryland Virginia & West Virginia	Lambs	MAT	383	27.1%	(Dubey et al., 2008)
France	Haute-Vienne	Lambs Ewes	DAT	164 93	22.0% 65.6%	(Dumètre et al., 2006)
Italy	Campania	Sheep	IFAT	1,170	28.5%	(Fusco et al., 2007)
Italy	Sicily	Sheep	ELISA	1,876	49.9%	(Vesco et al., 2007)
Italy	Orobie Alps	Sheep	LAT	1,056	77.7%	(Gaffuri et al., 2006)
Serbia		Ewes	MAT	511	84.5%	(Klun et al., 2006)
Poland		Ewes	IFAT	41	53.65%	(Górecki et al., 2005)
Poland	Olsztyn	Sheep	DAT	20	55%	(Michalski and Platt-Samoraj, 2004)
Lithuania			ELISA	354	42.1%	(Stimbirys et al., 2007)
Brazil	Northeast	Sheep	ELISA	102	29.41%	(Clementino et al., 2007)
Brazil	Guarapuava, Paraná	Sheep	IFAT	305	51.5%	(Romanelli et al., 2007)
Brazil	Guarapuava, Paraná	Sheep	IFAT	157	7%	(Moura et al., 2007)

Country	Area	Sheep/ Lamb	Test	No. Animals Tested	Positive	Reference
Brazil	Bahia State	Sheep	LAT	240	18.75%	(Pita Gondim et al., 1999)
South Africa		Sheep	IFAT ELISA	600	5.6% 4.3%	(Samra et al., 2007)
Morocco	Marrakech	Sheep	ELISA	261	27.6%	(Sawadogo et al., 2005)
Ethiopia	Nazareth	Sheep	MDAT ELISA	116	52.6% 56%	(Negash et al., 2004)
Turkey	Diyarbakir	Sheep	SFDT		65%	(Yagci et al., 2007)
Turkey	Istanbul		ELISA	181	31%	(Oncel and Vural, 2006)
Turkey	Sanliurfa	Sheep	SFDT	300	55.66%	(Sevgili et al., 2005)
Turkey	Nigde	Sheep	SFDT	110	50.9%	(Karatepe et al., 2004)
Turkey	Aydin	Sheep	SFDT	100	72%	(Pasa et al., 2004)
Turkey	Van	Sheep	IHAT	150	34.6%	(Tutuncu et al., 2003)
Turkey	Yozgat	Sheep	SFDT	152	45.4%	(Babür et al., 2001)
Iran	Chaharmhal & Bakhtiari	Sheep	IFAT	1,000	29.1%	(Bonyadian et al., 2007)
Iran	Mazandaran	Sheep	IFA	588	35%	(Sharif et al., 2007)
Saudi Arabia	Tabouk	Sheep	LAT IHAT IFAT	397	23.4% 41.8% 52.2%	(Sanad and Al-Ghabban, 2007)
Syria		Sheep		810	44.56%	(El-Moukdad, 2002)

DAT Direct Agglutination Test
IFAT Indirect Flurorescent Antibody Test
IHAT Indirect Haemaglutination Test
LAT Latex Agglutination Test
MAT Modified Agglutination Test
SFDT Sabin-Feldman Dye Test

4.5 Recent literature on genotyping of *T. gondii*

A literature search was undertaken on Scopus and ISI Web of Knowledge on 15 April 2008 to find articles related to *T. gondii* and genotype/genotyping. The search criteria and results are given in Appendix 4 – Literatur Search Results a total of 49 articles were identified.

The majority of articles (35) related to the isolation and genetic characterisation of *T. gondii* in species such as dogs, dolphins, cougar, etc., three articles were related to other parasites, two articles investigated virulence of *T. gondii* in mice, six articles related to human infections and outbreaks.

The remaining three articles relate to methodology of genotyping *T. gondii;* their details are provided below.

- Edvinsson, B., M. L. Darde, et al. (2007). "Rapid genotyping of *Toxoplasma gondii* by pyrosequencing." Clinical Microbiology and Infection 13(4): 424-429.

  Abstract: Most human infections with the protozoan parasite *Toxoplasma gondii* are asymptomatic, but severe symptoms can occur in immunocompromised patients, in developing foetuses, and in ocular infections in immunocompetent individuals. The majority of *T. gondii* strains can be divided into three main lineages, denoted types I, II and III, which are known to cause different clinical presentations. Simple molecular methods with the capacity to discriminate rapidly among strains may help to predict the course of infection and influence the choice of treatment. In the present study, real-time PCR followed by pyrosequencing was used to discriminate among types I, II and III by analysis of two single nucleotide polymorphisms in the GRA6 gene. Twenty-one isolates of *T. gondii* characterised previously were analysed. Three different GRA6 alleles detected by the pyrosequencing technique identified types I, II and III isolates correctly, while four atypical isolates possessed either the GRA6 allele 1 or the GRA6 allele 3. Reproducibility was 100%, and typeability, when including atypical strains, was 81%. It was also possible to discriminate a mixture of two genotypes. The method was used to identify GRA6 type II in blood and lung tissue from an allogeneic transplant recipient with toxoplasmosis.

- Fazaeli, A. and A. Ebrahimzadeh (2007). "A new perspective on and re-assessment of SAG2 locus as the tool for genetic analysis of *Toxoplasma gondii* isolates." Parasitology Research 101(1): 99-104.

  Abstract: SAG2 locus, the coding gene of the P22 protein, has been widely used for the molecular epidemiology of *Toxoplasma gondii* and characterization of the parasite isolates with two separate polymerase chain reaction (PCR)-restriction fragment length polymorphism (RFLP) processes. To re-assess the resolution power and suitability of this genetic marker for molecular characterization of the parasite isolates, a number of 27 Toxoplasma strains from different zymodeme patterns were used in the present study. Both codon and non-codon regions of the SAG2 locus of all 27 strains were amplified and subjected to sequencing and nucleotide alignment. Nucleotide variations clustered the three major genotypes (I, II and III). Some minor genotypes, unidentifiable by SAG2-RFLP, could be identified by sequence comparison. However, there were other genotypes that could not be differentiated from the major types due to having identical sequences. This suggests that a remarkable number of field isolates representing several minor types will be miss-clustered with the major types by using the traditional SAG2-PCR-RFLP method. It was concluded that this technique seems not to be suitable for Toxoplasma population study. Thus, the utilization of more variable markers and other discriminatory methods are also recommended.

- Zhang, H., S. Liao, et al. (2007). "Advances in the molecular diagnosis and genotyping of *Toxoplasma gondii*" Journal of Tropical Medicine (Guangzhou) 7(3): 292-295.

  Abstract (translated from Chinese using Google): Toxoplasmosis is a worldwide distribution of a total of animal suffering from parasitic diseases, serious harm to human health and animal husbandry around the world causing huge economic losses. Toxoplasmosis However, the lack of specificity of clinical symptoms and signs, diagnosis more difficult. Detection of traditional diagnostic methods such as the etiology, immunological testing, time-consuming and is not stable enough. while the nucleic acid amplification technology as the foundation of molecular biology to the rapid development of technology, such as real-time quantitative PCR (RTPCR), nested PCR and PCR-RFLP,

and other applications, not only for diagnosis of toxoplasmosis in a rapid, sensitive, specific and stable detection methods, but also through the analysis of the pathogen genotypes identified as *Toxoplasma gondii* groups biology, epidemiology, vaccine research and genotype and disease patterns between the potential relevance of research provides an important basis.

These articles, especially the first two, highlight the need for sequencing *T. gondii* genes to see the variety of strains, over and above the previously identified archetypal genotypes I, II and III. Of course, this genetic characterisation work is not new – over 36 of the articles identified used some form.

However, Edvinsson et al uses 'pyrosequencing' which is a recent sequencing technology (ca. 1 year on the market). This technology is very powerful in the sense of being able to provide high throughput at low cost, but does the same thing as the PCR-RFLP sequencing method, which is probably the most widely used method as it results in good, long sequence reads.

5 Success in Achieving Objectives

The objectives of the work have been achieved as follows:

- A national sero-prevalence survey for *T. gondii* in lambs and sheep has been undertaken.
- All serum and tongue samples are stored in a -80ºC freezer – tongues from seropositive animals can be subjected to genotyping at a later stage.
- Recently published literature indicates that there have been view methodological advances in relation to genotyping, with the exception of pyrosequencing which may be particularly useful in high throughput situations.

Consequently, a significant data gap, previously identified in the risk profile for *T. gondii*, has now been addressed.

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

Prior to this project there was no information national prevalence of *T. gondii* in lambs and sheep and the information that was available was 25 years out-of-date.

With this up-to-date information the industry is now in a better position to assess the level of risk posed to consumers when considering various end-us-pathways.

In addition, the establishment of a tongue bank which can potentially be used later to genotype tissues cysts if present in tongues. The results can then be used to compare genotypes founding lambs and sheep with those isolated from human cases.

7 Conclusions and Recommendations

The results of this survey indicate that

- The national prevalence of lambs is 16.4% (approx 95% CI: 9.8% to 22.9%)
- The national prevalence of sheep is 31.9% (approx 95% CI: 26.5% to 37.3%)
- The lower prevalence observed in lambs compared to sheep is consistent with previous research.
- Potentially large, but not statistically significant, differences in prevalence between states (not of primary interest in this survey).

Consequently, it is recommended that further work include

- A review of methods useful for detection of *T. gondii* tissue cysts in frozen tongue samples collected as part of the survey. A useful method may be difficult to find as tissues cysts will no longer be viable in the frozen tongue samples and thus cannot be multiplied in the same way that viable cysts can (feeding to cats/mice or tissue cultures). Consequently, detection may depend on the concentration of cysts in the tongue.
- Isolation and genotyping of *T. gondii* from frozen tongue samples and comparison of genotypes to those obtained from human cases.
- Identification of end-use-pathways of lamb and sheep meat and subsequent assessment of risk to consumers.

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9 Appendices

9.1 Appendix 1 – Weighted prevalence estimation

The following is a brief explanation of how geographical (weighted) prevalence estimates were obtained. The following will illustrate the computations with only two strata (states) for simplicity sake, but these equations generalise naturally to more than two strata.

Consider two strata within a population, with stratum 1 and 2 making up 75% of the population, respectively. For each stratum the prevalence estimates $\hat{p}_1$ and $\hat{p}_2$ are obtained from two samples of sizes n_1 and n_2, where x_1 and x_2 sampled units are positive for the characteristic of interest (in our case *T. gondii*).

When sample sizes are proportional to the relative stratum size, then an overall prevalence estimate can be obtained in the usual straight-forward manner, that is, by summing up the total number of positive samples and dividing by the total sample size.

However, when sample sizes are disproportional to the stratum size, then the overall prevalence estimate is obtained as follows:

$$\hat{p} = f_1 \frac{x_1}{n_1} + f_2 \frac{x_2}{n_2} = f_1 \hat{p}_1 + f_2 \hat{p}_2$$

A variance estimate is then obtained as follows:

$$V(\hat{p}) = f_1^2 \frac{\hat{p}_1(1-\hat{p}_1)}{n_1} + f_2^2 \frac{\hat{p}_2(1-\hat{p}_2)}{n_2}$$

However, the assumption is that the sample size is fixed in advance, which is not the case here – it was unknown how many animals from each state would be sampled prior to sample collection. Nevertheless, the prevalence estimate will still hold approximately, while the variance estimate will likely be an underestimate as it is unknown how variable the samples size n_1 and n_2 are.

9.2 Appendix 2 – R code

```
##    ------------------------------------------------------------------------
## Data Import and manipulation:
##    ------------------------------------------------------------------------
toxo <- read.csv("../data/data.csv", header=TRUE, as.is=TRUE)

## Modify raw data
toxo$tongue.lab.num <- NULL
toxo$alt.id <- NULL
toxo$abattoir.state <-
  factor(sapply(toxo$abattoir,
                function(el) {
                  substr(el, 1, nchar(el)-1)}))
toxo$state[toxo$state==""] <- NA
toxo$state <- factor(toxo$state)
toxo$titre[toxo$titre==""] <- NA
toxo$nlis[toxo$nlis==""] <- NA

## Turn 'text' titres into titre & qualifier
## ie. 0=non-detect or "<", while 1=detect
tmp <- lod.ind(toxo$titre, c(1,0))
toxo$titre <- tmp$value

## 1:16 is officially negative
## Only taken titres out to 1:1024
toxo$pos <- tmp$ind
toxo$pos[toxo$titre==16] <- 0
toxo$titre[toxo$pos==0] <- NA

## Check that there is no NLIS entry without a state of animal origin and that
## states correctly correspond to NLIS tags
subset(toxo, is.na(nlis))$state

unique(substr(subset(toxo, state=="NSW")$nlis, 1, 1))
unique(substr(subset(toxo, state=="SA")$nlis, 1, 1))
unique(substr(subset(toxo, state=="TAS")$nlis, 1, 1))
unique(substr(subset(toxo, state=="VIC")$nlis, 1, 1))
unique(substr(subset(toxo, state=="WA")$nlis, 1, 1))

## Subset of only those results that worked.
toxo.good <- subset(toxo, !is.na(pos))

##    ------------------------------------------------------------------------
## Overall prevalence estimates: 0=negative, 1=positive
##    ------------------------------------------------------------------------
with(toxo, table(type)) ## Total sera sampled
with(toxo, table(type, is.na(pos))) ## Sera usable and unusable
tb1 <- with(toxo.good, table(type, pos))               ## Usable sera - pos &
neg
tb1
pt1 <- prop.table(with(toxo.good, table(type, pos)),1) ## Same table with
proportion
pt1[,2]                                                ## Prop +ve
sd1 <- sqrt(pt1[,2]*(1-pt1[,2])/rowSums(tb1))          ## SD of perv
pt1[,2] + qnorm(0.025)*sd1                             ## Lower Conf bound
pt1[,2] + qnorm(0.975)*sd1                             ## Upper Conf bound

## For positive samples, show the titre distribution.
with(subset(toxo.good, pos==1), table(type, titre))

## Sera usable and unusable by state - where were the differences
with(toxo, table(abattoir.state, is.na(pos), type))

##    ------------------------------------------------------------------------
## By state of origin summaries
```

```
##    ---------------------------------------------------------------------
with(toxo.good, table(type, is.na(state)))

with(toxo.good, table(state, abattoir.state, type))  ## Usable sera - pos &
neg
tbl2 <- with(toxo.good, table(state, type))  ## Samples per state & type
tbl2

with(toxo.good, table(state, pos, type))  ## Usable sera - pos & neg
print(100*prop.table(with(toxo.good, table(state, pos, type)),
                 c(1,3)), digits=3) ## Same table with proportion

## For positive samples, show the titre distribution.
with(subset(toxo.good, pos==1), table(state, titre, type))

## Difference in state prevalence: lambs
fisher.test(with(toxo.good, table(state, pos, type))[,,1])
## Difference in state prevalence: sheep
fisher.test(with(toxo.good, table(state, pos, type))[,,2])

## Weighted national estimate based on processing
x.names <- c("NSW", "SA", "TAS", "VIC", "WA")
prior.prop.lamb <- c(0.33, 0.16, 0.03, 0.21, 0.24)
names(prior.prop.lamb) <- x.names

## For lambs
lamb.prev <- prop.table(with(toxo.good, table(state, pos, type)),
c(1,3))[,,1][,2]
lamb.prev[3] <- 0 ## nothing for TAS
lamb.prev         ## State Prevalence estimates
prev.l <- sum(lamb.prev*prior.prop.lamb) ## Weighted estimate
prev.l
sd2l <- sqrt(sum(prior.prop.lamb^2 *
                 lamb.prev*(1-lamb.prev)/tbl2[,1], na.rm=TRUE))
prev.l + qnorm(0.025)*sd2l ## Lower Conf bound
prev.l + qnorm(0.975)*sd2l ## Upper Conf bound

## For sheep
prior.prop.sheep <- c(0.33, 0.12, 0.03, 0.21, 0.25)
names(prior.prop.sheep) <- x.names
sheep.prev <- prop.table(with(toxo.good, table(state, pos, type)),
c(1,3))[,,2][,2]
sheep.prev
prev.s <- sum(sheep.prev*prior.prop.sheep) ## Weighted estimate
prev.s
sd2s <- sqrt(sum(prior.prop.sheep^2 *
                 sheep.prev*(1-sheep.prev)/tbl2[,2], na.rm=TRUE))
prev.s + qnorm(0.025)*sd2s ## Lower Conf bound
prev.s + qnorm(0.975)*sd2s ## Upper Conf bound


##    ---------------------------------------------------------------------
## Geographical distribution
##    ---------------------------------------------------------------------
table(factor(substr(subset(toxo.good,state=="NSW")$nlis,3,4)))
table(subset(toxo.good, state=="NSW" & substr(nlis,3,4)=="02")$nlis)
length(table(subset(toxo.good, state=="NSW" & substr(nlis,3,4)=="02")$nlis))
subset(toxo.good, state=="NSW" & substr(nlis,3,4)=="02")

table(subset(toxo.good, state=="NSW" & substr(nlis,3,4)=="25")$nlis)
length(table(subset(toxo.good, state=="NSW" & substr(nlis,3,4)=="25")$nlis))
subset(toxo.good, state=="NSW" & substr(nlis,3,4)=="25")

## SA
table(substr(subset(toxo.good,state=="SA")$nlis,3,4))
subset(toxo.good, state=="SA" & substr(nlis,3,4)==27)
```

```
table(subset(toxo.good, state=="SA" & substr(nlis,3,4)==27)$nlis)
length(table(subset(toxo.good, state=="SA" & substr(nlis,3,4)==27)$nlis))

subset(toxo.good, state=="SA" & substr(nlis,3,4)==50) ## Kangaroo Island
table(subset(toxo.good, state=="SA" & substr(nlis,3,4)==50)$nlis)
length(table(subset(toxo.good, state=="SA" & substr(nlis,3,4)==50)$nlis))

## TAS
table(substr(subset(toxo.good, state=="TAS")$nlis,3,4))

## VIC
table(substr(subset(toxo.good, state=="VIC")$nlis,2,3))

table(subset(toxo.good, state=="VIC" & substr(nlis,2,3)=="AR")$nlis)
length(table(subset(toxo.good, state=="VIC" & substr(nlis,2,3)=="AR")$nlis))
subset(toxo.good, state=="VIC" & substr(nlis,2,3)=="AR")

table(subset(toxo.good, state=="VIC" & substr(nlis,2,3)=="LN")$nlis)
length(table(subset(toxo.good, state=="VIC" & substr(nlis,2,3)=="LN")$nlis))
subset(toxo.good, state=="VIC" & substr(nlis,2,3)=="LN")

## WA
table(substr(subset(toxo.good, state=="WA")$nlis,3,4))

table(subset(toxo.good, state=="WA" & substr(nlis,3,4)=="PY")$nlis)
length(table(subset(toxo.good, state=="WA" & substr(nlis,3,4)=="PY")$nlis))
subset(toxo.good, state=="WA" & substr(nlis,3,4)=="PY")

table(subset(toxo.good, state=="WA" & substr(nlis,3,4)=="LG")$nlis)
length(table(subset(toxo.good, state=="WA" & substr(nlis,3,4)=="LG")$nlis))
subset(toxo.good, state=="WA" & substr(nlis,3,4)=="LG")
```

9.3 Appendix 3 – Literature Search Results

The following references were obtained in relation to a literature search on *T. gondii* (published since 2006).

Scopus:

```
(TITLE-ABS-KEY(toxoplasma OR gondii) AND TITLE-ABS-KEY(genotyp*)) AND
DOCTYPE(ar OR re OR ip) AND PUBYEAR AFT 2006
```

The search is for documents with the following criteria:

- Words “toxoplasma” or “gondii” in the title, abstract or keywords,
- AND the word “genotype*” in the title, abstract or keywords (‘*’ is a wildcard, so the matches include “genotype” and “genotyping”),
- AND the document is an article, review article or an articles in press,
- AND has been published after 2006.

This resulted in 33 articles.

ISI Web of Knowledge:

```
Topic=(toxoplasma or gondii) AND Topic=(genotyp*). Timespan=2007-
2008.  Databases=ABSTRACTS.
```

The search is for documents with the following criteria:

- Words “toxoplasma” or “gondii” in the topic, which includes title, abstract or keywords,
- AND the word “genotype*” in the topic (‘*’ is a wildcard, so the matches include “genotype” and “genotyping”),
- AND all document types,
- AND has been published after 2006.

This resulted in 38 articles.

Duplicate articles were removed, which resulted in the following list:

Belfort-Neto, R., V. Nussenblatt, et al. (2007). "High prevalence of unusual genotypes of Toxoplasma gondii infection in pork meat samples from Erechim, Southern Brazil." Anais da Academia Brasileira de Ciencias **79**(1): 111-114.

Carruthers, V. B. and Y. Suzuki (2007). "Effects of Toxoplasma gondii infection on the brain." Schizophrenia Bulletin **33**(3): 745-751.

Cavalcante, A. C. R., A. M. Ferreira, et al. (2007). "Virulence and molecular characterization of Toxoplasma gondii isolated from goats in Ceara, Brazil." Small Ruminant Research **69**(1/3): 79-82.

Demar, M., D. Ajzenberg, et al. (2007). "Fatal outbreak of human toxoplasmosis along the Maroni River: Epidemiological, clinical, and parasitological aspects." Clinical Infectious Diseases **45**(7).

Demar, M., D. Ajzenberg, et al. (2008). "Atypical Toxoplasma gondii strain from a free-living jaguar (Panthera onca) in French Guiana." American Journal of Tropical Medicine and

Hygiene **78**(2): 195-197.
Dubey, J. P., L. Applewhaite, et al. (2007). "Molecular and biological characterization of Toxoplasma gondii isolates from free-range chickens from Guyana, South America, identified several unique and common parasite genotypes." Parasitology **134**(11): 1559-1565.
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