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## Accepted Manuscript

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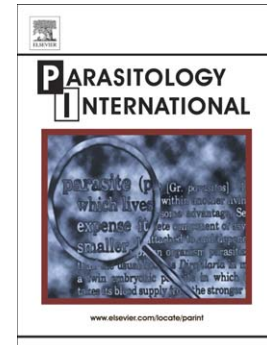
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**Seroprevalence of *Toxoplasma gondii* in wild kangaroos using an ELISA**

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**Abstract**

Infection with *Toxoplasma gondii* is a significant problem in Australian marsupials, and can lead to devastating disease and predispose animals to predation. *T. gondii* infection in kangaroos is also of public health significance due to the kangaroo meat trade. A moderate seroprevalence of *T. gondii* was observed in a study of western grey kangaroos located in the Perth metropolitan area in Western Australia. Of 219 kangaroos tested, 15.5% (95%CI: 10.7-20.3) were positive for *T. gondii* antibodies using an ELISA developed to detect *T. gondii* IgG in macropod marsupials. When compared with the commercially available MAT (modified agglutination test), the ELISA developed was in absolute agreement and yielded a  $\kappa$  coefficient of 1.00. Of 18 kangaroos tested for the presence of *T. gondii* DNA by PCR, the 9 ELISA positive kangaroos tested PCR positive and the 9 ELISA negative kangaroos tested PCR negative indicating the ELISA protocol was both highly specific and sensitive and correlated 100% with the more labour intensive PCR assay.

**Keywords:** *Toxoplasma gondii*; Marsupial; Serology; ELISA; Prevalence; Australia

**1. Introduction**

Australian marsupials are among the most susceptible hosts for *Toxoplasma gondii* and the parasite is known to cause both chronic and acute infection [1, 2]. Infection in marsupials is not always fatal and can result in long-term latent infection which may be reactivated during times of stress [3]. *T. gondii* infection may make a marsupial more prone to predation by affecting its movement, coordination and sight [4, 5]. Not only is infection with *T. gondii* attributed to causing declines in marsupial populations in the wild [6, 7], toxoplasmosis is associated with widespread pathology and death in several collections of captive marsupials [8-16]. Captivity is a stressor and

therefore thought to increase the chance of reactivated *T. gondii* infection [1, 3, 17]. Clinical signs of toxoplasmosis in Australian marsupials vary and include diarrhoea, respiratory distress, weight loss, blindness, neurological deficits and sudden death [18]. Common histopathological findings include myocardial, skeletal and smooth muscle necrosis, with *T. gondii* cysts and tachyzoites in areas of necrosis and interstitial pneumonia of the lungs [8]. Due to the dynamics of *T. gondii* infection in marsupials, knowledge of the *T. gondii* serological status of marsupials is of immense benefit to their management in captivity and in the wild. Although a number of cases of toxoplasmosis are described in captive marsupials, there is little recent data on the prevalence and distribution of *T. gondii* infection in wild marsupials. *T. gondii* seroprevalence in free ranging marsupials was 3.3% in Bennett's wallabies and 17.7% in Tasmanian pademelons using an ELISA [19], and 15% in bridled nailtail wallabies using a latex agglutination test [20]. In addition, *T. gondii* seroprevalence levels of 6.7% in eastern barred bandicoots [6] and 6.3% in the common brushtail possum [7] were observed using the MAT. Not only is the prevalence of *T. gondii* in wild marsupials of importance in terms of conservation, the presence of infection in wild kangaroos in particular is of public health significance due to the kangaroo meat trade.

Infection with *T. gondii* can be diagnosed in a number of ways. Diagnosis using histology and bioassay detect *T. gondii* organisms themselves but require tissue from dead animals. Furthermore, during chronic infection, *T. gondii* is spread sparsely within tissues and is often difficult to detect with histology [21]. Bioassays, although highly sensitive and specific at detecting *T. gondii* infection, are expensive and labour intensive [22]. PCR detection of *T. gondii* DNA also necessitates invasive sampling techniques or necropsy. In contrast, serology identifies serum antibodies, which are easy to detect during routine blood screening. One limitation of serology is that cross-reactive antibodies in animals infected with related coccidian parasites may give false positive results.

During studies which involved the screening of western grey kangaroos for *T. gondii* antibodies, the modified agglutination test was used. The MAT (Toxo-Screen DA, bioMerieux, France) was chosen to screen initial sera samples because it is the most commonly used test for serodiagnosis of *T. gondii* infection in Australian marsupials [10, 18, 23-26] and is the only test routinely used to screen marsupials for *T. gondii* infection in zoos throughout Australia. Published studies have shown a good correlation between MAT positivity in marsupials and infection with *T. gondii* [6, 27]. The popularity of the MAT in marsupials stems from the test not requiring a species-specific secondary reagent to detect the *T. gondii* antibodies circulating in infected animals, so enabling it to be used on a range of marsupial species. In addition, the MAT has been used extensively for the diagnosis of toxoplasmosis in a range of other species [28] and is used as a sensitive and specific test to detect *T. gondii* IgG antibodies in humans [29], mice [30], pigs [31], sheep [32] and felids [33, 34]. For routine screening of western grey kangaroos for *T. gondii* antibodies using the MAT, however, we found the test to be cost prohibitive. We therefore developed an ELISA to detect *T. gondii* IgG in macropod marsupials. This ELISA was found to be in absolute agreement with the MAT. The ELISA was then used to determine the seroprevalence of *T. gondii* in wild macropods within the Perth metropolitan area. The ELISA test was subsequently validated using a highly-specific PCR approach to confirm the presence of infection with *T. gondii* DNA in a cohort of seropositive animals.

## 2. Materials and Methods

### 2.1. Modified agglutination test

Sera from 52 western grey kangaroos (*Macropus fuliginosus*) were tested using the commercially available MAT. Sera were tested at two different sera dilutions; 1:40 and 1:4000,

according to the manufacturer's instructions. The positive and negative control sera included in the kit were used in each round of samples tested, in addition to an antigen control comprised of PBS. A serum sample was determined to be *T. gondii* positive when an agglutination reaction was observed at a serum dilution of at least 1:40, based on the manufacturer's directions.

## 2.2 ELISA development

MAT tested sera were used to optimise the in-house ELISA. Antigen for the ELISA was prepared from the Type I RH strain of *T. gondii* tachyzoites grown in Vero cell culture adapted from [35]. After infected cell cultures were harvested, tachyzoites were purified from the Vero cells by shearing through a 30G needle then filtration with a 5µm syringe filter. Purified tachyzoites were washed twice in PBS pH 7.2. The suspension of tachyzoites in PBS was then sonicated for 3 periods of 1 minute at a power level of 5 (Sonicator<sup>R</sup> Ultrasonic Processor, Misonix incorporated, Farmingdale, USA). Nuclei and membranes were spun out at 10,000g for 30 minutes to produce a soluble tachyzoite antigen preparation. A Bradford protein assay (Quick Start<sup>TM</sup> Bradford Dye Reagent, Biorad Laboratories, Gladesville, Australia) was undertaken and the antigen concentration adjusted with a volume of PBS to produce a 1000µg/ml protein preparation.

The optimum concentrations of antigen, serum and reagents for the ELISA were determined using a checker board system with antigen diluted in one direction and a series of different sera and reagent concentrations diluted in opposite directions. Four dilutions of antigen, 1 µg/ml, 10 µg/ml, 25 µg/ml, 50 µg/ml, were tested, with two MAT positive and two MAT negative sera samples diluted at 1:100, 1:200 and 1:400. The dilution of serum and antigen with the highest difference between positive and negative were then selected for use in assays to determine the optimum concentration of secondary and tertiary reagent. Three dilutions of secondary reagent, 1:500, 1:1000 and 1:2000, were tested against three dilutions of tertiary reagent, which were

1:1000, 1:2000 and 1:4000 and a serum dilution of 1:400 was used with an antigen concentration of 1 µg/ml. Two MAT positive and two MAT negative western grey kangaroo sera were used in the initial optimisation assays. After the optimal antigen and reagent concentrations were identified, 1 positive and 5 negative sera samples were tested at 8 sequential dilutions from 1:400 to 1:51200, using a secondary and tertiary reagent concentration of 1:1000 each. The serum dilution with the highest difference between positive and negative was chosen as the serum dilution for use in the ELISA; this was a serum dilution of 1:800. After the concentration of antigen, reagents and sera were optimised, a total of 54 MAT tested macropod serum samples were used to validate the ELISA and establish a positive cut-off point for optical density. The cut-off point was determined by calculating the mean optical density (OD) plus 3 standard deviations (SD) [36, 37] of MAT negative sera, this was 0.636. A sera sample with an OD reading equal to the mean plus 3 SDs of MAT negative sera was used as a reference positive control. For control of plate to plate variation, 2 negative and 2 positive control sera, including the reference positive control, were included on every plate. A serum sample was considered to be positive when its OD value was greater than that of the reference positive control, as described in [38].

The final protocol for the ELISA commenced with *T. gondii* antigen diluted in 50mM carbonate buffer, pH 9.6, to a concentration of 1 µg/ml. One hundred microliters of diluted antigen was then added to every well of a 96 well ELISA plate (Microton 600, Greiner Bio-one, Frickenhausen, Germany). The 96 well plate was incubated for 1 hour at 37°C. A washing cycle followed which consisted of rinsing in PBS with 0.05% Tween 20 for three periods of 3 minutes. The ELISA plate was blocked for 1 hour using 150 µl of 5% skim milk powder in PBS 0.05% Tween 20 and washed. Duplicates of test sera samples diluted in 5% skim milk in PBS were added at a volume of 100 µl and concentration of 1:800. Two MAT seropositive and two MAT seronegative sera samples were included in every 96 well plate tested. Sera was incubated for 90 minutes at 37°C and the plate washed prior to the addition of 100µl of commercially available



unconjugated Rabbit anti-kangaroo IgG (Kangaroo IgG (h&I) Antiserum, Bethyl Laboratories Inc, Montgomery, USA) at a concentration of 1:1000. The ELISA plate was then incubated for a further 60 minutes and washed after which 100 µl of Horseradish Peroxidase (HRP)-conjugated anti-Rabbit antibody (Donkey Anti-Rabbit: HRP, Affinity Bioreagents<sup>TM</sup>, Golden, USA) was added at a concentration of 1:1000. After the final incubation period of 60 minutes, the plate was washed and 200 µl OPD (o-phenylenediamine Dihydrochloride) Substrate Solution (Sigma Fast<sup>TM</sup> OPD, SIGMA, Saint Louis, USA) added. The OPD was left in the dark at room temperature for 15 minutes before the reaction was stopped with 50 µl of 2M H<sub>2</sub>SO<sub>4</sub>. The optical density was then read at 450nm using a spectrophotometer. Serum samples tested using the MAT, and used to optimize the ELISA, were retested using the final ELISA protocol (Table 1).

### *2.3 Seroprevalence study*

Two hundred and twenty western grey kangaroo sera were obtained from 7 different locations on the outskirts of the Perth metropolitan area over an 18 month period. Kangaroos were culled during Department of Environment and Conservation (DEC) population control programmes in areas such as parks, reserves, golf courses and farms due to overpopulation. During culling programmes an ID was allocated to each animal and the sex of the kangaroo noted. Blood was collected by needle aspiration of the heart within 4 hours of death of the kangaroo. Sera was separated by centrifugation and stored at -20°C. Fifty four of these sera samples were tested using both the MAT and ELISA, while the remaining were tested using the ELISA only.

Brain and tongue tissue from 62 of the 220 adult kangaroos were collected during the kangaroo culling programmes in Perth. The head of each kangaroo was removed in the field and transported to our laboratory for processing. Samples of brain and tongue were then removed from the head of adult kangaroos, placed in sterile containers and frozen at -20°C. The pouch young of

all 62 kangaroos were also killed in line with DEC population control measures, via blunt trauma to the head. Samples of brain and heart were removed from the pouch young once transported to our laboratory. Tissue samples were placed in sterile containers and frozen at -20°C.

#### 2.4 DNA extraction and PCR

The B1 gene [39] and ITS1 locus [40] were amplified using nested PCR to test for *T. gondii* DNA in 9 ELISA positive and 9 ELISA negative western grey kangaroos in which both sera samples and tissues were obtained. B1 gene primers used for nested PCR were: Pml/S1, 5'-TGTTCTGTCCTATCGCAACG; Pml/S2, 5'-TCTTCCCAGACGTGGATTTC; Pml/AS1, 5'-ACGGATGCAGTTCCTTTCTG; Pml/AS2, 5'-CTCGACAATACGCTGCTTGA. ITS1 locus primers used for hemi-nested PCR were: 22/FWD, 5'-GGGAAGTTTTGTGAACCT; ITS-5, 5'-GGAAGTAAAAGTCGTAACAAGG; ITS-2, 5'-GCTGCGTTCCTTCATCGATGC. Tissue samples tested included brain, tongue and heart samples. DNA was extracted using a QIAamp DNA MiniKit (QIAGEN, Hilden, Germany). For PCR, 1µl of template DNA was added to a total reaction volume of 50µl, which consisted of 5µl of 10x PCR buffer (SIGMA PCR Buffer 10x containing 15mM MgCl<sub>2</sub>, SIGMA, Saint Louis, USA), 5µl of 2mM dNTPs, 0.4µl of 50pmol forward primer, 0.4µl of 50 pmol reverse primer and 1.25 U of Taq Polymerase (SIGMA taq DNA polymerase A, SIGMA, Saint Louis, USA). DNA from the RH strain of *T. gondii* was used as a positive PCR control and PCR negative controls consisted of distilled water. Amplification consisted of denaturing at 94°C for 5 minutes followed by 30 cycles of 94°C for 40 seconds, 60°C for 40 seconds and 72°C for 90 seconds, after which there was an extension period of 10 minutes at 72°C. PCR products were visualized using 0.8% agarose gels stained with ethidium bromide. PCR products from the ITS1 PCR were sequenced to identify a *T. gondii* specific DNA sequence.

## 2.5 Statistics

Agreement between the MAT and ELISA was estimated by  $\kappa$  coefficient [41]. The Pearson  $p$  value of the Chi squared test [42] and odds ratios [43] were utilized to compare the seroprevalence results of male and female western grey kangaroos. A  $p$  value of less than 0.05 was considered statistically significant. Odds ratios were calculated with 95% confidence intervals.

## 3. Results

The in-house ELISA was in absolute agreement with the MAT as illustrated in Table 1 and yielded a  $\kappa$  coefficient of 1. All 47 MAT negative sera samples were ELISA negative and all 7 MAT positive sera samples were ELISA positive (Table 1).

The optimised ELISA protocol was used consistently in kangaroo samples tested in the *T. gondii* seroprevalence study (Table 2). Of the 219 western grey kangaroos sampled within the Perth metropolitan area, 15.5% (95%CI: 10.7-20.3), were seropositive for *T. gondii* using the in-house ELISA. Male kangaroos had an overall seroprevalence of 10.9% compared to 21.5% for females. This difference was statistically significant ( $p = 0.038$ ; OR = 0.45, CI: 0.21, 0.97).

The results of the PCR were consistent with the ELISA results (Table 3). *T. gondii* specific DNA was detected in all nine animals that had sera which was ELISA positive. In addition, all tissue samples from ELISA negative kangaroos were PCR negative for *T. gondii* DNA using primers for both the B1 gene [39] and ITS1 locus [40]. Clean PCR amplification products were observed for both the B1 gene (data not shown) and ITS1 locus (Figure 1). All ITS1 positive PCR samples were sequenced, and BlastN analysis of the DNA sequences revealed that the amplicons were specific for *T. gondii* (data not shown).

#### 4. Discussion

The seroprevalence of *T. gondii* in western grey kangaroos in this study was found to be 15.5% (95%CI: 10.7-20.3). This is similar to the 17.7% seroprevalence levels found in wild Tasmanian pademelons [19], and the 15.5% seroprevalence in free ranging bridled nailtail wallabies [20]. The prevalence of *T. gondii* antibodies in Bennett's wallabies was lower at 3.3% and was also lower in smaller sized, non macropod marsupial species such as common brushtail possum and eastern barred bandicoots which were 6.3% [7] and 6.7% [6] respectively. The moderate seroprevalence of *T. gondii* in wild western grey kangaroos confirms kangaroos can survive with *T. gondii* infection in the wild. Thirty four kangaroos in the Perth Metropolitan area had evidence of exposure to *T. gondii*. The source of *T. gondii* for these kangaroos is unknown at this time, as there is no information regarding the presence of infected cats in the areas surveyed. Felids are the only definitive host of *T. gondii* and there are no native Australian felids, leaving domestic and feral cats as the only source of *T. gondii* oocysts. Another possible source of *T. gondii* infection is congenital transmission. Evidence for congenital transmission in marsupials to date is anecdotal [10, 11] however it is well established in a number of species including sheep, mice, rats, cats and humans [44-46]. Kangaroos are herbivorous, therefore *T. gondii* infected animal tissue is an unlikely source of infection in the western grey kangaroos tested.

In this study of western grey kangaroos, the prevalence in males was significantly less than in female kangaroos ( $p < 0.05$ ). A few other studies published have also identified a significantly higher *T. gondii* seroprevalence in female sheep and goats compared to their male counterparts [47, 48]. It is possible that differences in behaviour between male and female western grey kangaroos accounts for their different levels of exposure to *T. gondii*. For example it is thought that female kangaroos are able to crop short grass better than males [49]. Males may then be forced onto other

food and may be seen camped separately from females [50]. Females which graze close to the ground may be thus more likely to be exposed to *T. gondii* oocysts in soil.

Absolute agreement between *T. gondii* ELISA results and the PCR results was found in the 18 kangaroos tested using both ELISA and PCR. Other studies have found PCR to be far less sensitive than serology at detecting *T. gondii* infection in pigs [22, 51] and cattle [52]. The PCR used in this study was found to have a high sensitivity for detecting *T. gondii* DNA in ELISA positive western grey kangaroos, which could indicate that marsupials possess higher tissue burdens of parasites than for instance, pigs and cattle. All ITS1 PCR products were sequenced and the results confirmed that the amplicons were that of *T. gondii* in the tissue samples of seropositive animals. *T. gondii* DNA was not detected in the tissues of 9 seronegative kangaroos, which consisted of 3 adults and 6 pouch young. Although *T. gondii* has been detected in seronegative animals previously [53], this is reported infrequently. PCR results from DNA extracted from seropositive and seronegative kangaroos correlated exactly with the serological results suggesting the ELISA developed is sensitive and specific.

Fifty two MAT-tested serum samples were used to validate and optimize the ELISA developed in this study. This ELISA was found to have a comparable sensitivity and specificity to the MAT, based on its Kappa value of 1. The principle advantage of the ELISA is that it can be used to screen large amounts of sera samples more cost effectively than the commercially available MAT. ELISA results can also be easily interpreted based on the cut off point for optical density, as compared to an IFAT where slides need to be examined by experienced readers. Due to the relatively high serum dilution used in this ELISA protocol, only a small amount of marsupial serum is required to detect *T. gondii* antibodies. This is of particular benefit in wildlife research as only small volumes of sera are usually obtainable in live trapped animals and these are often used to test for multiple conditions. This ELISA could theoretically be easily modified to suit non-macropodine marsupials by changing the secondary reagent.

It is unknown if kangaroos are infected with coccidia other than *T. gondii* and produce cross reactive antibodies. Cross reactive antibodies to *T. gondii* reduce the specificity of serological tests. The specificity of the MAT in marsupials is unknown, however as the MAT is the most commonly used and widely accepted test to screen for anti-*T. gondii* IgG in marsupials, the MAT was used in this study as a reference test to optimise and validate the in-house ELISA protocol. PCR of tissues from ELISA positive and negative kangaroos was used to further validate the ELISA results. The number and range of coccidia that infect Australian marsupials is unknown, therefore the level of cross reactivity that exists in wild kangaroos infected with other coccidia is unknown. Furthermore, the absolute agreement between the MAT and ELISA suggests the ELISA developed has a similar specificity to the MAT and is suitable for use in seroprevalence studies and serodiagnosis. The high agreement between the ELISA results and PCR results also suggest the ELISA developed is sensitive and specific.

In this study we found a moderate seroprevalence of *T. gondii* in wild western grey kangaroos, using an in house ELISA. Infection with *T. gondii* in marsupials has the potential to progress to fulminant disease, alters the way marsupials should be managed in captivity and is a public health issue as kangaroo meat is now commonly consumed by humans and domestic felids [54]. Wild western grey kangaroos are harvested for meat in Australia [55]. Kangaroo meat is commonly enjoyed rare and kangaroo pet meat is regularly served raw. *T. gondii* bradyzoites remain infective when meat is undercooked, making the ingestion of rare or raw kangaroo meat a risk factor in *T. gondii* transmission [54]. *T. gondii* infected kangaroos are not only a source of infection for humans, but also for domestic cats, which may subsequently shed oocysts and perpetuate the life cycle.

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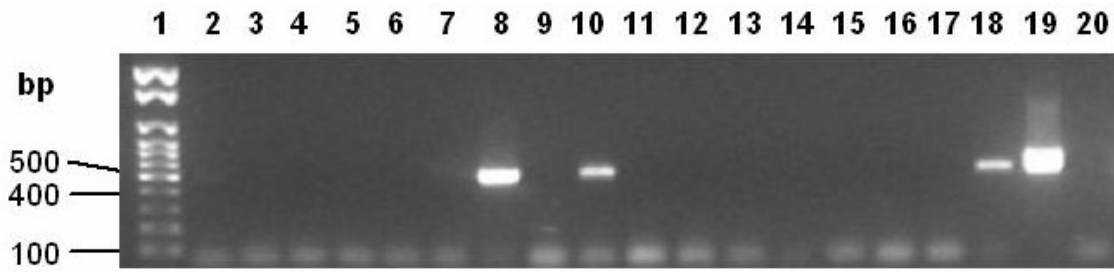
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ACCEPTED MANUSCRIPT



**Figure 1**

ITS1 PCR agarose gel. Lane 1, 100bp DNA ladder. Lane 2, pouch young 1, brain. Lane 3, pouch young 1, heart. Lane 4, pouch young 2, brain. Lane 5, pouch young 2, heart. Lane 6, pouch young 3, brain. Lane 7, pouch young 3, heart. Lane 8, adult 8, brain. Lane 9, adult 8, tongue. Lane 10, adult 9, brain. Lane 11, adult 9, tongue. Lane 12, pouch young 4, brain. Lane 13, pouch young 4, heart. Lane 14, pouch young 5, brain. Lane 15, pouch young 5, heart, Lane 16, pouch young 6 brain, Lane 17, pouch young 6 heart. Lane 18, adult 1, brain. Lane 19, *T. gondii* positive control. Lane 20, *T. gondii* negative control.

**Table 1**

Level of agreement between a commercially available MAT and an ELISA in western grey kangaroos

MAT	ELISA	
	+	-
+	7	0
-	0	47

**Table 2**

Prevalence of *T. gondii* antibodies in western grey kangaroos as determined by ELISA

Sex	Number positive	Number tested	Prevalence
Male	11	101	10.9%
Female	23	107	21.5%
Unknown	0	11	0.0%
TOTAL	34	219	15.5%

**Table 3**

PCR results of ELISA positive and negative western grey kangaroos

<b>Animal</b>	<b>ELISA result</b>	<b>PCR results</b>	
		<b>Brain</b>	<b>Tongue</b>
Adult 1	Positive	B1, ITS1	Negative
Adult 2	Positive	B1, ITS1	ITS1
Adult 3	Positive	B1, ITS1	ITS1
Adult 4	Positive	nd	B1, ITS1
Adult 5	Positive	B1, ITS1	Negative
Adult 6	Positive	Negative	B1, ITS1
Adult 7	Positive	Negative	ITS1
Adult 8	Positive	ITS1	Negative
Adult 9	Positive	ITS1	Negative
Adult 10	Negative	Negative	Negative
Adult 11	Negative	Negative	Negative
Adult 12	Negative	Negative	Negative
		<b>Brain</b>	<b>Heart</b>
Pouch young 1	Negative	Negative	Negative
Pouch young 2	Negative	Negative	Negative
Pouch young 3	Negative	Negative	Negative
Pouch young 4	Negative	Negative	Negative
Pouch young 5	Negative	Negative	Negative
Pouch young 6	Negative	Negative	Negative

nd- not tested