Vertical transmission of *Toxoplasma gondii* in Australian marsupials

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SUMMARY

To date, little is known about the dynamics of vertical transmission of *Toxoplasma gondii* in Australian marsupials. Studies in mice demonstrate that vertical transmission of *T. gondii* is common and that chronically infected mice can transmit *T. gondii* to successive generations. In this study, PCR and immunohistochemistry were used to detect *T. gondii* in chronically infected marsupial dams and their offspring. *T. gondii* was detected in the unfurred pouch young of 2 out of 10 chronically infected western grey kangaroos (*Macropus fuliginosus*) and in the unfurred pouch young of a brush-tailed bettong (*Bettongia penicillata*). Results of the study suggest that vertical transmission of *T. gondii* can occur in chronically infected Australian marsupials.

Key words: *Toxoplasma gondii*, marsupial, vertical transmission, Australia, congenital, toxoplasmosis.

INTRODUCTION

Vertical (transplacental or transmammary) transmission of *Toxoplasma gondii* and its influence on the maintenance of *T. gondii* in natural populations has been a matter of debate (Johnson, 1997). Although it has long been known that congenital infection with *T. gondii* in mice and guinea pigs can occur while the dam is chronically infected (Remington *et al.* 1961), more recent studies have ignited debate as to whether vertical transmission occurs in other chronically infected animals. Recent studies have verified the high frequency of congenital transmission of *T. gondii* in chronically infected mice and it was proposed that congenital transmission in chronically infected mice can maintain *T. gondii* infection in wild mouse populations (Owen and Trees, 1998; Marshall *et al.* 2004). Recent data also suggest that *T. gondii* can be transmitted via successive vertical transmission within families of sheep (Morley *et al.* 2005).

Evidence for vertical transmission in marsupials to date is anecdotal (Boorman *et al.* 1977; Dubey *et al.* 1988), and the dynamics of vertical transmission in marsupials is poorly understood. However, considering the potential impact of toxoplasmosis in marsupials and the current efforts associated with wildlife conservation, it is important to examine the causes of infection of Australian marsupials with *T. gondii*. Australian marsupials are highly susceptible hosts for *T. gondii* and the parasite causes both chronic and acutely fatal infection (Beveridge, 1993). Information on the occurrence of vertical transmission in chronically infected marsupials may benefit captive breeding programmes of Australian marsupials by ensuring only *T. gondii*-free animals are bred, thereby improving animal health and assisting animal conservation and management.

In order to better understand *T. gondii* transmission in marsupials, we tested the dam and pouch young of western grey kangaroos (*Macropus fuliginosus*) and a woylie (*Bettongia penicillata*) for evidence of *T. gondii* infection. The MAT (modified agglutination test) and DAT (direct agglutination test) were used to test for anti-*T. gondii* IgM in sera of western grey kangaroo dams. Experimental studies in eastern grey kangaroos demonstrate that differences in titre between MAT and DAT are indicative of an IgM response and acute *T. gondii* infection (Johnson *et al.* 1989). Subsequent studies in a range of marsupial species have successfully used the MAT and DAT to diagnose acute *T. gondii* infection (Lynch *et al.* 1993; Skerratt *et al.* 1997; Bettiol *et al.* 2000; Hartley, 2006). All pouch young

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Table 1. Results of various PCRs for western grey kangaroo dams and their pouch young

				PCR Dams		PCR PY							
Dam ID	ELISA Dam	ELISA PY	Age PY (days)	Brain	Tongue	Brain	Heart	Sk musc	Lung	Liver	Kidney	Spleen	Sm Intest
C14	Positive	Positive	145	**B1, ITS1**	N	N	***B1***	N	N	N	nd	N	N
C9	Positive	Positive	114	**B1, ITS1**	**ITS1**	N	N	N	N	N	nd	N	N
J6	Positive	Positive	124	**B1, ITS1**	**ITS1**	N	N	N	nd	nd	nd	nd	N
J10	Positive	Positive	125	nd	**B1, ITS1**	N	N	N	N	N	N	N	N
R7	Positive	Positive	90	**B1, ITS1**	N	N	N	N	nd	nd	nd	nd	nd
Q1	Positive	Positive	154	N	**B1, ITS1**	N	N	N	nd	nd	nd	nd	nd
G21	Positive	Negative	58	***B1***	**ITS1**	N	N	N	nd	nd	nd	nd	nd
F19	Positive	Negative	132	**ITS1**	N	N	N	N	nd	nd	nd	nd	nd
R19	Positive	Negative	142	**ITS1**	N	N	**B1**	N	nd	nd	nd	nd	nd
15B1	Positive	Positive	246	nd	nd	N	N	N	N	N	N	N	N
R4	Negative	Negative	89	nd	nd	N	N	nd	nd	nd	nd	nd	nd
F8	Negative	Negative	98	N	N	N	N	nd	nd	nd	nd	nd	nd
H14	Negative	Negative	84	N	N	N	N	N	nd	nd	nd	nd	nd
I14	Negative	Negative	75	N	N	N	N	nd	nd	nd	nd	nd	nd
Q20	Negative	Negative	129	nd	nd	N	N	nd	nd	nd	nd	nd	nd
15B2	Negative	Negative	233	nd	nd	N	N	N	N	N	N	N	N

B1 – Positive B1 PCR (Bretagne *et al*. 1993).
B1 – Positive B1 PCR (Grigg and Boothroyd, 2001).
ITS1 – Positive ITS1 PCR (Miller *et al*. 2001).
N – Negative on all PCRs.
nd – No tests undertaken.
Sk musc – Skeletal muscle.
Sm intest – Small intestine.

were tested before the time of first pouch exit. While within the pouch, young are protected from the external environment and so are extremely unlikely to be exposed to *T. gondii* oocysts. Marsupial young are born at a very immature state (less than 1 g neonatal weight) at which time they enter the pouch. Young first exit the pouch after a long period of permanent residence (Tyndale-Biscoe and Renfree, 1987). Immunohistochemistry and PCR were used to detect *T. gondii* in the tissue of a woylie dam and western grey kangaroo dams and their corresponding pouch young.

MATERIALS AND METHODS

Western grey kangaroo sera and tissues were obtained from kangaroos culled during Department of Environment and Conservation (DEC) population control programmes in Perth, Western Australia. Kangaroos were culled from a large reserve due to overpopulation. Sixty two dams were obtained, from which samples of blood, tongue and brain were collected. Blood was collected using intracardiac puncture. Each dam's head was removed and labelled. Brain and tongue samples were collected after heads were transported to a laboratory and stored at 4 °C for a maximum of 3 days prior to processing. Samples of brain and tongue were dissected, placed in sterile containers and frozen at −20 °C. The young present in each dam's pouch were killed in line with population control measures, via blunt trauma to the head in the field. Pouch young were labelled and stored at 4 °C for a maximum of 3 days prior to processing in the laboratory. Each pouch young was weighed and measured to estimate its age (Poole *et al*. 1982) after which blood was collected via intracardiac puncture. All sera collected were separated via centrifugation and stored at −20 °C. Pouch young were dissected under sterile conditions to obtain samples of brain, heart, skeletal muscle, liver, lung, small intestine, kidney and spleen. Some portions of tissues were frozen at −20 °C for DNA extraction while other portions were placed in 10% buffered formalin for histology.

A female woylie with young in pouch from a wild population near Manjimup, Western Australia, was submitted to Murdoch University Veterinary Hospital for necropsy with a history of neurological signs. Tissue samples were placed in 10% buffered formalin and consisted of brain, heart, skeletal muscle, lung, liver, spleen and mammary tissue. In addition, brain, heart and mammary gland tissue samples were set aside in 70% ethanol for DNA extraction. The furless pouch young present in the pouch of the necropsied woylie had samples of brain, heart, skeletal muscle, lung and liver removed and placed in 70% ethanol for DNA extraction. The lack of fur in the woylie pouch young sampled indicated that it was too immature to have ever left the pouch.

The 62 western grey kangaroo dams and the corresponding 62 pouch young were all screened for *T. gondii* antibodies using an ELISA (Parameswaran

Table 2. *Toxoplasma gondii* DAT and MAT titres of seropositive western grey kangaroo dams

Dam ID	DAT	MAT
C14	256 000	256 000
C9	256 000	256 000
J6	4096	4096
J10	4096	4096
R7	64 000	64 000
Q1	4096	4096
G21	64 000	64 000
F19	4096	4096
R19	4096	4096
15B1	64 000	64 000

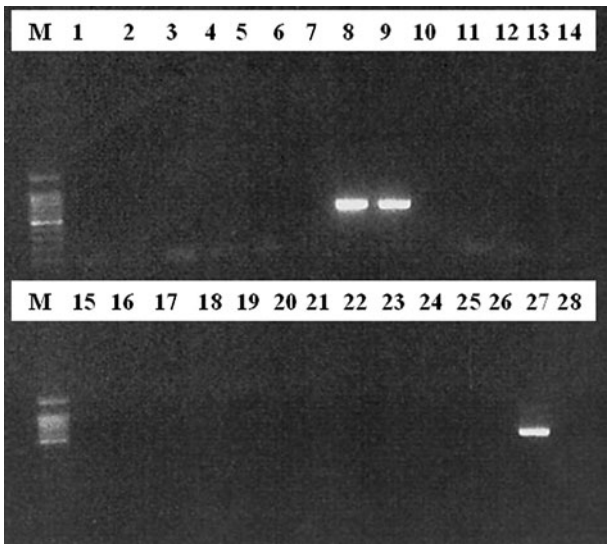

Fig. 1. Agarose gel of nested PCR for the B1 gene (Grigg and Boothroyd, 2001).
Lane M, 100 bp DNA ladder; Lane 1, PYG21, brain; Lane 2, PYG21, heart; Lane 3, PYG21, skeletal muscle; Lane 4, PYF19, brain; Lane 5, PYF19, heart; Lane 6, PYF19, skeletal muscle; Lane 7, PYR19, brain; Lane 8, PYR19, heart; Lane 9, PYR19 heart 1 : 10 dilution; Lane 10, PYR19, skeletal muscle; Lane 11, PY15B1, brain; Lane 12, PY15B1, heart; Lane 13, PY15B1, skeletal muscle; Lane 14, PY15B1, lung; Lane 15, PY15B1, liver; Lane 16, PY15B1, kidney; Lane 17, PY15B1, spleen; Lane 18, PY15B1, small intestine; Lane 19, PYR4, brain; Lane 20, PYR4, heart; Lane 21, PYF8, brain; Lane 22, PYF8 heart; Lane 23, PYH14, brain; Lane 24, PYH14, heart; Lane 25, PYI14, brain; Lane 25, PYI14, heart; Lane 27, *T. gondii* RH strain positive control; Lane 28, *T. gondii* negative control.

et al. 2009) to detect IgG antibodies to *T. gondii* in macropod marsupials. Serum samples from seropositive dams were sent to the Animal Health Laboratory, Tasmania to obtain MAT (modified agglutination test) and DAT (direct agglutination test) titres in order to determine the presence of *T. gondii* IgM. No serum samples were taken from the adult woylie or its pouch young.

The brain and tongue of seropositive and seronegative western grey kangaroo dams and a range of tissues of their offspring underwent DNA extraction. In addition, the brain, heart and mammary gland tissue of a woylie dam and the brain, heart, skeletal muscle, lung and liver of its pouch young underwent DNA extraction. Three methods of DNA extraction were used for each sample. DNA samples were extracted using a MasterPure DNA purification kit (Epicentre Biotechnologies, Madison, USA). A method of phenol-chloroform DNA extraction was also used (Miller *et al*. 2008). In addition, extraction using QIAamp DNA MiniKit (QIAGEN Hilden, Germany) was used. In each DNA extraction 25 mg of homogenized tissue was used. Tissues of seronegative dams and seronegative pouch young were used as DNA extraction negative controls.

Samples of DNA extracted from tissue specimens underwent nested PCR amplification for *T. gondii* using nested primers at the ITS1 locus (Miller *et al*. 2001) and B1 gene, (Bretagne *et al*. 1993; Grigg and Boothroyd 2001). Each sample of DNA was tested once using each set of primers. DNA from the *T. gondii* RH strain (Type I strain) was used as a positive PCR control and PCR negative controls consisted of distilled water. PCR products were visualized using 0·8% agarose gels stained with ethidium bromide. To confirm infection with *T. gondii*, ITS1 and B1 PCR products were gel-purified from agarose gels using the UltraClean GelSpin DNA Extraction Kit (MO BIO Laboratories Inc, Carlsbad, USA) prior to DNA sequencing. Sequencing reactions were performed using a BigDye Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems, Scoresby, Australia) according to the manufacturer's directions using the internal primers used to PCR amplify the ITS1 and B1 gene sequences. Reactions were electrophoresed through an ABI 3730 automatic sequencer and sequencing profiles analysed using FinchTV version 1.4 (Geospiza, Seattle, USA). Sequences were subjected to BlastN analysis using the GenBank nr-nt database at NCBI to confirm that the PCR-amplified sequences were that of *T. gondii*.

Formalin-fixed tissue samples were trimmed and processed before being embedded in paraffin wax, sectioned and stained with haematoxylin and eosin. Paraffin-embedded tissues were also sectioned and immunohistochemically stained with rabbit polyclonal antibodies to *T. gondii*, as previously described by Lindsay and Dubey (1989). Stored paraffin-embedded brain tissue from a *T. gondii*-infected cat was used as a positive control for immunohistochemistry. Brain tissue was not included in histological analysis of western grey kangaroo pouch young due to autolysis, associated with post-mortem changes.

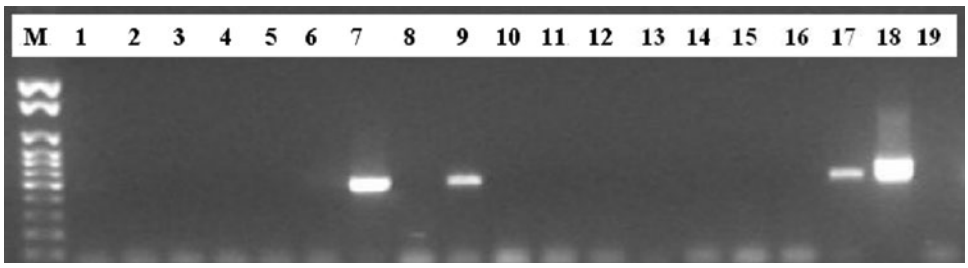

Fig. 2. Agarose gel of nested PCR for the ITS1 locus (Miller *et al*. 2001).
Lane M, 100 bp DNA ladder; Lane 1, PYR4, brain. Lane 2, PYR4, heart. Lane 3, PYF8, brain. Lane 4, PYF8, heart. Lane 5, PYH14, brain. Lane 6, PYH14, heart. Lane 7, R7, brain. Lane 8, R7, tongue. Lane 9, Q1, brain. Lane 10, Q1, tongue. Lane 11, PYI14, brain. Lane 12, PYI14, heart. Lane 13, PYQ20, brain. Lane 14, PYQ20, heart, Lane 15, PY15B2, brain, Lane 16, PY15B2, heart. Lane 17, C14, brain. Lane 18, *T. gondii* positive control. Lane 19, *T. gondii* negative control.

RESULTS AND DISCUSSION

Of 62 Western Kangaroo dams that were screened for *T. gondii* antibodies using an ELISA, 10 dams were seropositive for *T. gondii* and 7 of these dams had corresponding seropositive pouch young (Table 1). The remaining 52 seronegative dams all had corresponding seronegative pouch young. All 10 ELISA seropositive dams were also MAT and DAT positive. MAT and DAT titres were identical in all western grey kangaroo dams, which indicated a lack of IgM in all dam serum samples (Table 2). The lack of IgM in the serum samples is suggestive of chronic *T. gondii* infection in the western grey kangaroos.

To confirm the ELISA results, DNA extracted from 9 kangaroo dam tissues was tested for the presence of *T. gondii* DNA by PCR. One seropositive kangaroo dam was not tested due to the absence of tissue samples. All 9 seropositive western grey kangaroo dams were PCR positive for the presence of *T. gondii* DNA using primers at the ITS1 locus and B1 gene, confirming the serology results (Table 1). Negative controls included DNA extracted from tissues of 3 seronegative dams and 6 pouch young from seronegative dams. All negative controls were PCR negative for *T. gondii*. Serology results in adult kangaroos had a high correlation with PCR results, suggesting the PCR methods used were sensitive and specific.

For the 10 chronically infected western grey kangaroos, *T. gondii* DNA was detected in the heart tissue of 2 pouch young from the seropositive dams. One of the *T. gondii* PCR-positive pouch young was seronegative by the ELISA assay. No *T. gondii* specific DNA was detected in tissues from the remaining 8 pouch young. All ITS1 and B1 PCR bands amplified were sequenced and were confirmed to be that of *T. gondii* (data not shown). As PCR results had a high correlation with serology results in adult kangaroos, it is likely that the 6 seropositive, PCR negative kangaroo pouch young were not infected with *T. gondii* and were only seropositive due to the passive transfer of antibodies from the dam.

PCR using primers for B1 and ITS1 detected *T. gondii* DNA in the mammary gland of the one woylie tested and in the brain of its corresponding pouch young. All other woylie tissue tested was negative for *T. gondii* DNA. Identification of *T. gondii* DNA in the mammary gland of the woylie dam suggests that infection of the woylie pouch young was from suckling breast milk. Clean PCR amplification products were observed for both the B1 gene (Fig. 1) and ITS1 locus (Fig. 2). Both ITS1 and B1 PCR bands sequenced were that of *T. gondii* (data not shown).

It is highly unlikely that the pouch young tested in this study were exposed to *T. gondii* oocysts from the external environment. Marsupial young first exit the pouch after a long period of permanent residence and while within the pouch, young are protected from the external environment (Tyndale-Biscoe and Renfree, 1987). All western grey kangaroo pouch young in this study were tested before the time of first pouch exit, which is approximately 298 days in this species (Tyndale-Biscoe and Renfree, 1987). In addition, the woylie pouch young in this study was unfurred and therefore too young to have left the pouch.

Generalized congestion and oedema of the lung was observed in all western grey kangaroo pouch young. No other significant lesions were observed in histological sections from all other pouch young tested from seropositive and seronegative dams. Upon immunohistochemistry, no *T. gondii* tachyzoites or cysts were found in the pouch young tested. The presence of *T. gondii* was, however, detected in the positive control tissues analysed at the same time. *T. gondii* was not detected upon histology or immunohistochemistry in the adult woylie. Histologically there was acute diffuse pulmonary congestion and oedema, and autolysis of the intestines. In addition, there was a small haematoma and focus of inflammation associated with one mammary gland.

The detection of *T. gondii* DNA in only the heart muscle of the 2 western grey kangaroo pouch young is not surprising; *T. gondii* has been detected previously in the heart muscle of a black-faced kangaroo (*Macropus fuliginosus melanops*) pouch young (Dubey *et al*. 1988) and 2 juvenile common wombats (*Vombatus ursinus*) (Hartley, 2006), and is known to commonly infect the heart of adult macropod marsupials (Canfield *et al*. 1990). However, as no pathology or *T. gondii* organisms were observed upon pathological and immunohistological examination of tissue sections from the 2 PCR-positive pouch young in this study, this suggests that neither of the PCR-positive pouch young had clinical toxoplasmosis.

This is the first evidence of vertical transmission of *T. gondii* in chronically infected marsupials and points to further research regarding the frequency of vertical transmission of *T. gondii* in Australian marsupials. Information on the frequency of vertical transmission of *T. gondii* in chronically infected Australian marsupials would benefit captive breeding programmes of endangered Australian marsupial species and contribute to the management of wild populations.

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