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Nonarchetypal Type II-like and atypical strains of *Toxoplasma gondii* infecting marsupials of Australia

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Abstract

Australia is geographically isolated and possesses a remarkable diversity of wildlife species. Marsupials are highly susceptible to infection with the cosmopolitan parasite *Toxoplasma gondii*. Of 46 marsupials screened for *T. gondii* by multilocus PCR-DNA sequencing at polymorphic genes (*B1, SAG3, GRA6, GRA7*), 12 were PCR-positive; the majority (67%; 9/12) were infected by nonarchetypal Type II-like or atypical strains. Six novel alleles were detected at *B1*, indicating greater diversity of genotypes than previously envisaged. Two isolates lethal to marsupials, were avirulent to mice. The data support the conclusion that Australia's isolation may have favoured the persistence of nonarchetypal ancestral genotypes.

Keywords

Toxoplasma gondii; Nonarchetypal; Genotype; Australia; Wildlife; Marsupials

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A myriad of remarkable wildlife species are indigenous to Australia and breed in relative isolation compared with more common, warm-blooded vertebrates that circulate widely, have fewer geographic restrictions and less apparent ecological barriers. Australian native mammals, including marsupials and birds, are particularly prone to developing severe neurological sequelae or death when infected with the protozoan parasite *Toxoplasma gondii* (Obendorf et al., 1996; Dubey and Crutchley, 2008). Outbreaks of toxoplasmosis causing declines and die-offs in Australian marsupial populations are now recognised as serious threats contributing to the decline of threatened marsupials such as the Eastern Barred Bandicoot and Woylie (Obendorf et al., 1996; Smith et al., 2008). In contrast, infections in people and most other terrestrial mammals are generally subclinical, however serious disease can result during congenital infection and in immune-suppressed populations (Boothroyd and Grigg, 2002). Several factors can be proposed to explain this high susceptibility to *T. gondii* infection in

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marsupials including inbreeding depressions, stresses associated with captivity, overcrowding, parasite strain variation, as well as novel host-parasite interactions implicit to a newly emerging pathogen.

Toxoplasma gondii is a highly prevalent parasite zoonosis that replicates both sexually and asexually in nature, and it is this flexibility that enables it to infect essentially any warm-blooded vertebrate worldwide. Wild and domestic felids, introduced to Australia about 300 years ago, are the only mammals known to serve as definitive hosts. In Europe, North America and Africa, three predominant lineages, referred to as Types I, II and III, have expanded clonally to cause the majority of infections in humans and domestic animals (Howe and Sibley, 1995; Grigg et al., 2001a; Lehmann et al., 2006; Lindstrom et al., 2008). In contrast, an entirely different picture emerges when wildlife is sampled throughout the United States, Central and South America and parts of Africa and Asia. Here, a rich diversity of atypical genotypes are circulating in these niches, presumably products of the *Toxoplasma* sexual cycle (Ajzenberg et al., 2004; Miller et al., 2004; Lehmann et al., 2006). Some of these atypical isolates are clearly related to the three dominant Types; they bear mixtures of archetypal alleles with novel alleles introgressed that possess unique nonarchetypal polymorphisms. Other "exotic" isolates have been identified whereby novel alleles exist at every locus investigated, and in total, 11 haplogroups define the population genetic structure for *T. gondii* (Khan et al., 2007).

Studies documenting a moderate seroprevalence of *T. gondii* in wild, free-ranging Australian marsupials suggest that some wild marsupials survive *T. gondii* infection (Johnson et al., 1988; Parameswaran et al., 2009a; Thompson et al., 2009). To date, no study has determined the genetic "Type" of *T. gondii* causing lethal versus asymptomatic infections in Australian marsupials or whether strain type is a major factor governing marsupial susceptibility. Population genetic data is proving that parasite genotype is an important factor determining the severity of disease in a variety of susceptible vertebrate species. In marine mammals, sea otters, harbor seals and sea lions are susceptible to developing severe encephalitis caused by an atypical clade of Type X strains (Miller et al., 2004). In mice and people with severe ocular toxoplasmosis, Type I strains cause highly virulent infections (Grigg et al., 2001a, 2001b; Boothroyd and Grigg, 2002). To determine the genotypes of *T. gondii* infecting a range of native marsupial species of Australia, 128 tissue samples (typically brain, heart or tongue) derived from 17 domestic and 44 wildlife specimens were tested by direct PCR (Table 1).

Wildlife specimens exhibiting neurological signs or sudden death were specifically identified to address whether *Toxoplasma* genetic type is a contributing factor in marsupial susceptibility. These included heart and skeletal muscle samples from a western ringtail possum (*Pseudocheirus occidentalis*) with a history of hindlimb paralysis; brain and heart from four wild woylies (*Bettongia penicillata*), one of which (A1) exhibited neurological signs prior to death in the field; and brain samples from three meerkats (*Suricata suricatta*) that died exhibiting neurological signs during an outbreak of suspected toxoplasmosis at the Perth Zoo (Western Australia). Horsemeat and mice fed as part of the meerkat diet were also tested as potential sources for *T. gondii* infection.

Tissue samples from asymptomatic Western grey kangaroos and their pouch young were obtained from animals culled on a large reserve due to overpopulation by the Department of Environment and Conservation (DEC) in Perth, Western Australia. Brain and tongue samples were collected from adult animals and brain, heart, skeletal muscle, liver, lung, small intestine, kidney and spleen specimens were obtained from pouch young. All tissue specimens were stored at −20°C prior to DNA extraction. Sterile water was used as a DNA extraction negative control.

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Eighteen packets of meat mince (Table 1) were bought from local supermarkets in Perth, Western Australia and stored at 4°C until processed. Bradyzoites were purified from 100 g of mince using pepsin/HCl digestion followed by percoll gradient centrifugation, as described previously (Gajadhar and Marquardt, 1992). The resulting suspension was visualised under the microscope to check for bradyzoites and stored at −20°C prior to DNA extraction.

Five *T. gondii* isolates were obtained from archived Tasmanian (Australia) samples: one cat, two goats and two marsupials; a wallaby (*Macropus rufogriseus*) and a wombat (*Vombatus ursinus*). The latter four animals exhibited neurological symptoms prior to death. Isolates were propagated in human fibroblasts prior to DNA extraction.

DNA was extracted using either: i) a MasterPure DNA purification kit (Epicentre Biotechnologies, Madison, USA); ii) a method of phenol-chloroform DNA extraction (Miller et al., 2004); and/or iii) extraction using the QIAamp DNA MiniKit (QIAGEN Hilden, Germany). For each DNA extraction either 25 mg of infected tissue or 1×10^8 parasites were used. All samples were processed by at least two methods for PCR analysis. DNA extracted directly from tissue samples underwent nested PCR amplification using *T. gondii*-specific primers at *B1, SAG3, GRA6* and *GRA7* genes (Grigg and Boothroyd, 2001; Grigg et al., 2001a; Bottos et al., 2009). DNA extracted from the *T. gondii* isolates were PCR amplified as above, with the addition of primers against *SAG1, SAG2, SAG4, SRS2, BSR4, B-TUB, ROP18, BAG1, PK1, L358, c22-8, c-29-2* (Su et al., 2006). DNA from the RH strain was used as a PCR positive control and distilled water was used as a negative control. Reaction mixtures and amplification conditions were according to published methods (Bottos et al., 2009). PCR products were visualized using 0.8% agarose gels stained with gel red. A 100 bp DNA ladder (Promega, Madison, USA) was included in each agarose gel.

DNA sequencing reactions were performed on the PCR population using both forward and reverse primers (BigDye Terminator v3.1 Cycle Sequencing Kit, Applied Biosystems, Scoresby, Australia). Reactions were electrophoresed through an ABI 3730 automatic sequencer and sequencing profiles analysed using FinchTV version 1.4 (Geospiza, Seattle, USA). Bioedit version 7.0.0 (Ibis Biosciences, Carlsbad, USA) was used to align the sequences for comparison. All positive PCR reactions were repeated once, to check the reproducibility of sequencing results.

To detect *T. gondii* DNA present in each tissue specimen, highly sensitive, nested PCR primers that amplify across the internal transcribed spacer-1 (ITS1) locus in the small subunit ribosomal gene array were applied (Parameswaran et al., 2009a). A single PCR band was detected in 19 tissue samples, comprised of 16 animals and three meat samples (Table 1). No amplicon was produced using DNA extracted from uninfected mouse tissue or from a water only PCR reagent control. DNA sequencing confirmed that the PCR products were that of *T. gondii* (data not shown).

Multi-locus PCR-DNA sequencing was next performed using primers against diagnostic *B1, SAG3, GRA6* and *GRA7* loci to genotype the *T. gondii* parasites infecting the 19 ITS1-positive tissue samples. Polymorphisms within the *B1* 35 copy tandem gene array can be used to distinguish Type I from Types II or III (which share the same allele) from atypical strains. Of the 19 tissue samples tested, 13 were *B1* PCR positive. DNA sequencing identified five specimens with a type I allele, two with a type II or III allele and seven possessed one or other of five new alleles designated U-1 to U-5, each with novel polymorphisms (SNPs) (Table 2). Two different sequences were identified for sample C9, one type II/III allele and the other U-2. All SNPs were reproducible when re-tested at *B1* by PCR-DNA sequencing (Table 2).

For the single copy genes tested, seven samples were PCR positive at *SAG3*, five at *GRA6*, and two at *GRA7* (Table 1). At *SAG3*, five of the seven samples had a type II allele, one had a type

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I allele and the other, C9, possessed only dinucleotide polymorphisms indicating that the kangaroo was multiply infected with more than one strain. This was confirmed at *B1*, in which two separate alleles were amplified (Table 2). At *GRA6*, four samples had a type II allele whereas one sample, A8, had a type I allele. At *GRA7*, two samples had type II alleles (Table 3).

Of the 13 *T. gondii* PCR-positive specimens using the polymorphic genotyping markers, only three animals were found to be infected with strains bearing archetypal Type I (kangaroo PYR19 and woylie A1) or II (kangaroo Q1) alleles (Table 3). Three samples (A7, A8, A1Y) were infected with atypical strains possessing archetypal alleles but reshuffled in a novel, apparently recombinant inheritance pattern. The remaining seven specimens were infected with strains bearing novel alleles at the *B1* locus (Table 3). *B1* sequencing of *T. gondii* isolates derived from a goat, wombat and wallaby from Tasmania likewise identified the presence of the U-2, as well as an additional unique U-6 *B1* allele, confirming the PCR results obtained against infected marsupial tissue specimens (Table 3). Molecular typing of the Tasmanian isolates at an additional 12 genotyping loci identified either i) Type I alleles (cat, goat); or ii) a mixture of unique alleles, identified at *SRS2* and *BAG1*, and Type II alleles (goat (Tasmania), wombat and wallaby), thus confirming the existence of nonarchetypal strains circulating in Australia that possessed new combinations of Type II, and genetically drifted Type II-like, alleles (Table 3).

Strains bearing archetypal Type I or *B1* U-2 or U-6 alleles were shown to cause infections that resulted in neurological disease (Table 3), suggesting that strain type may predict disease severity. To test whether infections with the wallaby (U-2 allele at *B1*) or wombat (U-6 allele at *B1*) strains were mouse-virulent, these two isolates were infected i.p. alongside Type I and II strains in C57BL/6J mice. All animal research was performed with the approval of the National Institutes of Health (NIH) and National Institute of Allergy and Infectious Diseases (NIAID) Animal Care and Ethics Committee in accordance with government guidelines. The animal study protocol number for the care and maintenance of the mice was LPD-22E. Twenty-five parasites of the Type I RH strain were lethal to mice within 10 days whereas 1,000 parasites of the Type II lineage 76K strain were avirulent and all mice survived infection. Infection with 1,000 tachyzoites of the wallaby and wombat isolates were likewise avirulent; all mice survived (Fig. 1). However, mice infected with the wallaby isolate bearing the U-2 *B1* allele possessed a substantially higher cyst burden (~10-fold) than Type II infected mice. This difference could not be attributed to any measurable difference in the size of the parasite inoculum; all were plated at time of infection and equivalent numbers of parasite plaques were recovered for each of the 76K, wombat and wallaby isolates (data not shown).

This study has identified an unusual abundance of nonarchetypal *T. gondii* strains infecting indigenous Australian wildlife. Nineteen infected specimens and five isolates tested positive. Twelve marsupials possessed sufficient parasite DNA for genotyping by multilocus PCR DNA-sequencing; the majority of these (67%; nine out of 12) were infected with novel, Type II-like or atypical strains. Two additional atypical strains were identified infecting tissues from a horse and a mouse. It is likely that woylie A1 was infected with the same atypical strain identified in A1Y, but typing at only a single locus precluded this designation. DNA sequencing of the two isolates recovered from the wombat and wallaby demonstrated that indigenous *T. gondii* strains circulating in Australia possessed variant Type II alleles (at the *B1*, *BAG1*, and *SRS2* genes) and were genetically distinct from those reported in Europe, North America, and Africa, where >90% of strains typed thus far fall into three distinct clonotypes referred to as Type I, II or III.

The identification of six novel alleles at the *B1* gene established this typing locus as highly polymorphic and a good predictor for identifying nonarchetypal, Type II-like *T. gondii* lines

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indigenous to Australia. This same *B1* typing locus also proved to be an important diagnostic marker for detection of the novel Type X strain known to infect marine mammals off the coast of California (Miller et al., 2004). Detection of the U-2 and U-6 allele in parasite clones isolated from a wallaby and wombat, respectively, argued against the possibility that the novel *B1* alleles were the result of a spurious amplification during direct PCR on DNA extracted from infected tissues, as did successful re-amplification of the same unique alleles upon subsequent PCR on additional, new extractions derived from the same tissue specimens. Hence, the unexpected genetic diversity identified in the limited number of fauna examined underscores how little is known about the distribution, biology and genetic “types” of *T. gondii* native to the Australian sub-continent. Clearly, this report establishes the need for additional studies to dissect the transmission and population genetic structure of *T. gondii* in Australia.

The predominance of atypical and nonarchetypal Type II-like parasites infecting a variety of marsupial populations was markedly different from other human and animal infection studies performed in Europe and North America which previously established archetypal Type II parasites as the primary causal agent of toxoplasmosis. Whether the lack of archetypal Type I, II and III strains identified reflects a relative dearth of domesticated animals that were PCR-positive in this study (five animals) or is the result of Australia’s extreme isolation from the rest of the world due to its strict quarantine protocols, remains to be established. Intriguingly, of the five PCR-positive domesticated animals (cat, two goats, horse, mouse), only two were infected with archetypal parasites, which is conceivably a reflection of the general predominance of atypical strains circulating in Australia. The possibility that Australia’s quarantine protocols have favoured the persistence of independently evolving, genetically drifted Type II-like, as well as atypical, possibly recombinant, ancestral genotypes is a testable hypothesis. Presumably these would have been first introduced 300 years ago during European colonization and the first importation of domestic felids. To investigate whether indigenous Australian strains reflect ancestral parasite genotypes will, however, require DNA sequence analysis at additional loci and the application of more highly resolved microsatellite markers against a larger panel of isolates than are currently available.

It is possible that the unexpected assemblage of nonarchetypal parasite lines infecting Australian marsupials is a direct result of the high genetic diversity in the wild host populations sampled. In effect, natural selection among genetically diverse intermediate hosts may select for parasite lines that maximize transmissibility among only those intermediate hosts that possess a similar genetic background. This likely affords one explanation for why not all macropods are equally susceptible to toxoplasmosis (Dubey and Crutchley, 2008). For example, a parasite strain that has been naturally selected to be asymptomatic in a kangaroo could conceivably cause lethal infection in a wallaby. It could also explain why parasite virulence in mice may have no direct correlation with clinical disease in a marsupial. In this study, neither of the Australian Type II-like isolates recovered from lethal infections in a native wombat or wallaby were virulent in mice (Fig. 1). However, the line recovered from the wallaby bearing the U-2 *B1* allele that was found commonly infecting kangaroos produced an approximately 10-fold higher tissue cyst burden in mice than the archetypal Type II strain, perhaps reflecting this strain’s potential for high transmissibility via scavenging among intermediate hosts.

It is likewise unclear what clinical impact such parasite diversity has on the health of different populations of Australian wildlife. Since the majority of clinical cases reported have been in captive animals, other factors beside parasite genetic type, such as the dose and stage of parasite ingested, the presence of concurrent infections (Smith et al., 2008), and the immune or nutritional status of the host, may exist as important factors governing disease susceptibility. Ultimately, to determine whether parasite genetic type is a critical factor mediating die-offs in native Australian fauna, a study comparing *T. gondii* isolates from wildlife with isolates from

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humans and domestic animals (including felids) endemic to a region afflicted by an outbreak will be necessary to establish the origin, diversification and transmission of pathogenic *T. gondii* strains in Australia. Should such a study determine that felids are infected predominantly with archetypal *T. gondii* strains, then the possibility that a felid-free, sylvatic cycle for *T. gondii* transmission among grazing, indigenous Australian fauna will need to be considered. Examples might include vertical transmission (Parameswaran et al., 2009b), tissue cyst transmission via scavenging, or the possibility that non-felid Australian native species possess an ability to shed *T. gondii* oocysts.

At this stage it is unknown whether the non-archetypal strains found in this study are more commonly associated with certain *T. gondii* related diseases. The result that nine out of 12 *T. gondii* strains identified infecting native Australian animals were non-archetypal coincides with the findings of Ajzenberg et al. (2004) who proposed that genetic diversity of *T. gondii* in wildlife and from geographically isolated areas is underestimated. Further studies in Australia are necessary to determine the prevalence of atypical strains among indigenous fauna and to test whether nonarchetypal strains infecting marsupials are causally linked to clinical disease. Because this study identified a large number of atypical strains that possessed genealogies relating them back to the Type II lineage, our data support the conclusion that the Type II lineage expanded from the "old world" (Lehmann et al., 2006).

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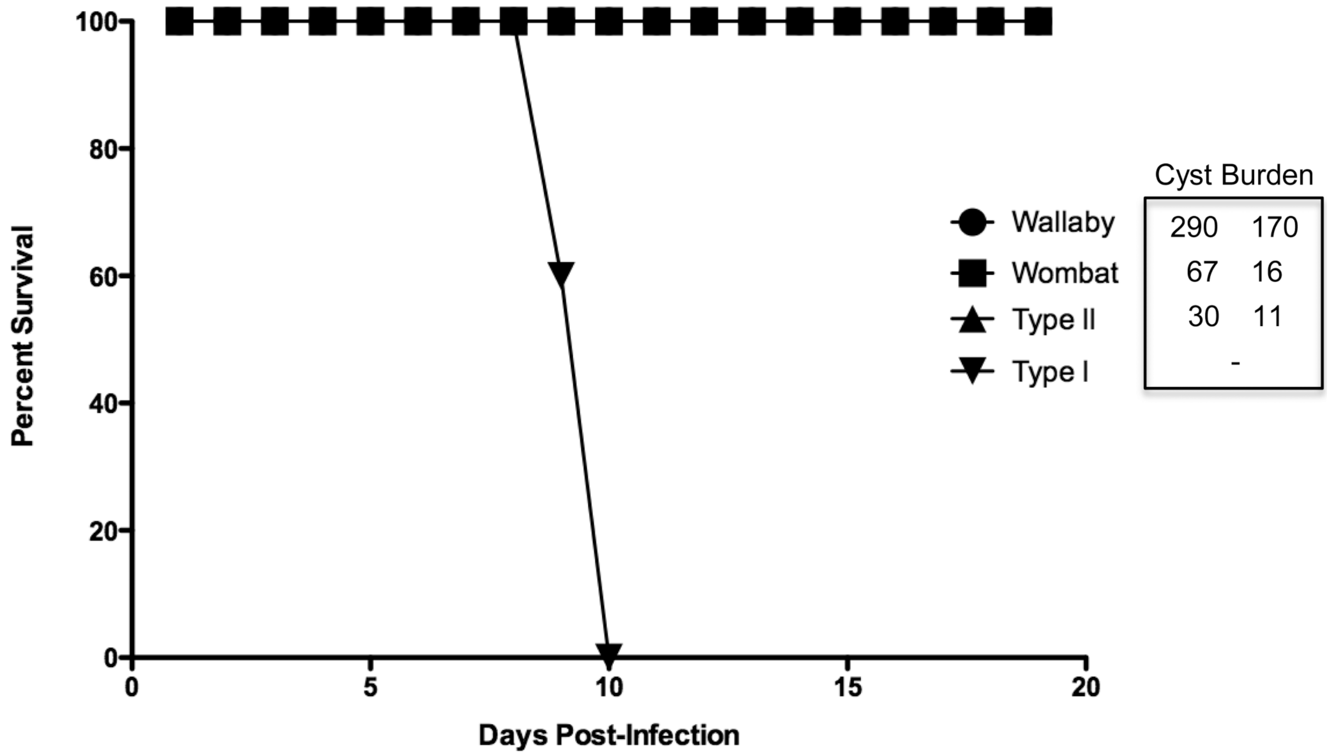

Fig 1.

Australian isolates of *Toxoplasma gondii* are avirulent in C57BL/6 mice. Lethal dose kinetics in seropositive C57BL/6 mice infected with different strains of *T. gondii*. Five mice each were injected with either: i) 25 tachyzoites of the Type I virulent RH strain; ii) 1,000 tachyzoites of the Type II avirulent 76K strain; or iii) 1,000 tachyzoites of the Australian *T. gondii* isolates recovered from a wombat or wallaby. Seropositivity at day 28 p.i. indicated productive infection. Cyst burden was determined 2 months p.i. and recorded as the mean and S.D. of cyst number recovered from each brain from five infected mice.

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Table 1

Tissue samples tested for *Toxoplasma gondii* DNA using direct PCR for *ITS1*, *B1*, *SAG3*, *GRA6* and *GRA7*.

Specimen ID	Specimen	Species	Location	*ITS1*	*B1*	*SAG3*	*GRA6*	*GRA7*
B1	Kangaroo mince	Unknown	Supermarket, Perth	Negative	Negative	nd	nd	nd
B2	Kangaroo mince	Unknown	Supermarket, Perth	Negative	Negative	nd	nd	nd
HK6	Kangaroo mince	Unknown	Supermarket, Perth	Negative	Negative	nd	nd	nd
PK6	Kangaroo mince	Unknown	Supermarket, Perth	Negative	Negative	nd	nd	nd
K1.8	Kangaroo mince	Unknown	Supermarket, Perth	Negative	Negative	nd	nd	nd
K28	Kangaroo mince	Unknown	Supermarket, Perth	Positive	Positive	Negative	Negative	Negative
K12	Kangaroo mince	Unknown	Supermarket, Perth	Negative	Negative	nd	nd	nd
K13	Kangaroo mince	Unknown	Supermarket, Perth	Negative	Negative	nd	nd	nd
HK14	Kangaroo mince	Unknown	Supermarket, Perth	Negative	Negative	nd	nd	nd
PK14	Kangaroo mince	Unknown	Supermarket, Perth	Negative	Negative	nd	nd	nd
B4	Lamb mince	*Ovis aries*	Supermarket, Perth	Negative	Negative	nd	nd	nd
L2.7	Lamb mince	*Ovis aries*	Supermarket, Perth	Negative	Negative	nd	nd	nd
L1.11	Lamb mince	*Ovis aries*	Supermarket, Perth	Negative	Negative	nd	nd	nd
L2.11	Lamb mince	*Ovis aries*	Supermarket, Perth	Negative	Negative	nd	nd	nd
L12	Lamb mince	*Ovis aries*	Supermarket, Perth	Negative	Negative	nd	nd	nd
Mutt13	Mutton mince	*Ovis aries*	Supermarket, Perth	Negative	Negative	nd	nd	nd
B3	Pork mince	Unknown	Supermarket, Perth	Negative	Negative	nd	nd	nd
P8	Pork mince	Unknown	Supermarket, Perth	Negative	Negative	nd	nd	nd
A6	Horse meat	*Equus caballus*	Perth	Positive	Negative	nd	nd	nd
A7	Horse meat	*Equus caballus*	Perth	Positive	Positive	Positive	Positive	Negative
A12	Meerkat	*Suricata suricatta*	Perth Zoo	Negative	Negative	nd	nd	nd
A13	Meerkat	*Suricata suricatta*	Perth Zoo	Positive	Positive	Positive	Positive	Positive
A15	Meerkat	*Suricata suricatta*	Perth Zoo	Positive	Negative	nd	nd	nd
A8	Mouse	*Mus musculus*	Perth Zoo	Positive	Positive	Positive	Positive	Negative
A9	Mouse	*Mus musculus*	Perth Zoo	Negative	Negative	nd	nd	nd
A10	Mouse	*Mus musculus*	Perth Zoo	Negative	Negative	nd	nd	nd
A11	Mouse	*Mus musculus*	Perth Zoo	Negative	Negative	nd	nd	nd
C14	Kangaroo	*Macropus fuliginosus*	Perth Metropolitan Area	Positive	Positive	Negative	Positive	Negative
C9	Kangaroo	*Macropus fuliginosus*	Perth Metropolitan Area	Positive	Positive	Positive	Negative	Negative

Specimen ID	Specimen	Species	Location	*ITS1*	*B1*	*SAG3*	*GRA6*	*GRA7*
J6	Kangaroo	*Macropus fuliginosus*	Perth Metropolitan Area	Positive	Positive	Positive	Positive	Positive
J10	Kangaroo	*Macropus fuliginosus*	Perth Metropolitan Area	Positive	Positive	Negative	Negative	Negative
R7	Kangaroo	*Macropus fuliginosus*	Perth Metropolitan Area	Positive	Positive	Negative	Negative	Negative
Q1	Kangaroo	*Macropus fuliginosus*	Perth Metropolitan Area	Positive	Positive	Positive	Negative	Negative
G21	Kangaroo	*Macropus fuliginosus*	Perth Metropolitan Area	Positive	Negative	nd	nd	nd
F19	Kangaroo	*Macropus fuliginosus*	Perth Metropolitan Area	Positive	Negative	nd	nd	nd
R19	Kangaroo	*Macropus fuliginosus*	Perth Metropolitan Area	Positive	Negative	nd	nd	nd
H14	Kangaroo	*Macropus fuliginosus*	Perth Metropolitan Area	Negative	Negative	nd	nd	nd
I14	Kangaroo	*Macropus fuliginosus*	Perth Metropolitan Area	Negative	Negative	nd	nd	nd
F8	Kangaroo	*Macropus fuliginosus*	Perth Metropolitan Area	Negative	Negative	nd	nd	nd
PYC14	Kangaroo	*Macropus fuliginosus*	Perth Metropolitan Area	Positive	Negative	nd	nd	nd
PYC9	Kangaroo	*Macropus fuliginosus*	Perth Metropolitan Area	Negative	Negative	nd	nd	nd
PYJ6	Kangaroo	*Macropus fuliginosus*	Perth Metropolitan Area	Negative	Negative	nd	nd	nd
PYJ6	Kangaroo	*Macropus fuliginosus*	Perth Metropolitan Area	Negative	Negative	nd	nd	nd
PYJ10	Kangaroo	*Macropus fuliginosus*	Perth Metropolitan Area	Negative	Negative	nd	nd	nd
PYR7	Kangaroo	*Macropus fuliginosus*	Perth Metropolitan Area	Negative	Negative	nd	nd	nd
PYQ1	Kangaroo	*Macropus fuliginosus*	Perth Metropolitan Area	Negative	Negative	nd	nd	nd
PYG21	Kangaroo	*Macropus fuliginosus*	Perth Metropolitan Area	Negative	Negative	nd	nd	nd
PYF19	Kangaroo	*Macropus fuliginosus*	Perth Metropolitan Area	Negative	Negative	nd	nd	nd
PYR19	Kangaroo	*Macropus fuliginosus*	Perth Metropolitan Area	Positive	Positive	Negative	Negative	Negative
PY15B1	Kangaroo	*Macropus fuliginosus*	Perth Metropolitan Area	Negative	Negative	nd	nd	nd
PYH14	Kangaroo	*Macropus fuliginosus*	Perth Metropolitan Area	Negative	Negative	nd	nd	nd
PYI14	Kangaroo	*Macropus fuliginosus*	Perth Metropolitan Area	Negative	Negative	nd	nd	nd
PYR4	Kangaroo	*Macropus fuliginosus*	Perth Metropolitan Area	Negative	Negative	nd	nd	nd
PYF8	Kangaroo	*Macropus fuliginosus*	Perth Metropolitan Area	Negative	Negative	nd	nd	nd
PYQ20	Kangaroo	*Macropus fuliginosus*	Perth Metropolitan Area	Negative	Negative	nd	nd	nd
PY15B2	Kangaroo	*Macropus fuliginosus*	Perth Metropolitan Area	Negative	Negative	nd	nd	nd
A14	Western ringtail possum	*Pseudocheirus occidentalis*	Perth Metropolitan Area	Negative	Negative	nd	nd	nd
A1	Woylie	*Bettongia penicillata*	Manjimup reserve	Positive	Positive	Negative	Negative	Negative
A1Y	Woylie	*Bettongia penicillata*	Manjimup reserve	Positive	Positive	Positive	Negative	Negative
O7214	Woylie	*Bettongia penicillata*	Manjimup reserve	Negative	Negative	nd	nd	nd
O7161	Woylie	*Bettongia penicillata*	Manjimup reserve	Negative	Negative	nd	nd	nd

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Specimen ID	Specimen	Species	Location	*ITS1*	*B1*	*SAG3*	*GRA6*	*GRA7*
61 specimens				***19***	***13***	***7***	***5***	***2***

nd, not done.

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ustralian samples.

								GRA6									*BAG1*					*SRS2*					
06	**162**	**171**	**304**	**336**	**561**	**576**	**614**	**635– 652**	**654**	**660**	**677**	**690**	**692**	**702**	**711**	**ALLELE**	**626**	**707**	**803**	**823**	**ALLELE**	**547**	**###**	**###**	**###**	**###**	**ALLELE**
C	**G**	**A**	**T**	**T**	**A**	**A**	**G**	**TACGGAGGCAGAGGTGAA**	**G**	**G**	**C**	**A**	**C**	**T**	**A**		**A**	**T**	**G**	**G**		**G**	**T**	**T**	**G**	**G**	
.	.	.	A	.	.	.	.	....	.	C	.	.	.	.	.	I	T	C	-	T	I	A	G	A	.	A	I
.	.	G	.	C	.	G	.	-----------------	A	.	.	G	A	A	T	II	.	.	.	.	II/III	.	.	.	.	.	II/III
T	A	.	.	.	C	.	C	....	.	.	G	.	.	.	.	III	.	.	.	.	II/III	.	.	.	.	.	II/III
.	.	.	A	.	.	.	.	....	.	C	.	.	.	.	.	I	T	C	-	T	I	A	G	A	.	A	I
.	.	.	A	.	.	.	.	....	.	C	.	.	.	.	.	I	T	C	-	T	I	A	G	A	.	A	I
.	.	G	.	C	.	G	.	-----------------	A	.	.	G	A	A	T	II	.	C	.	.	U-1	.	.	.	.	.	II/III
.	.	G	.	C	.	G	.	-----------------	A	.	.	G	A	A	T	II	.	C	.	.	U-1	.	.	.	A	.	U-1
.	.	G	.	C	.	G	.	-----------------	A	.	.	G	A	A	T	II	.	C	.	.	U-1	.	.	.	A	.	U-1
.	.	.	A	.	.	.	.	....	.	C	.	.	.	.	.	I											
.	.	G	.	C	.	G	.	-----------------	A	.	.	G	A	A	T	II											
.	.	G	.	C	.	G	.	-----------------	A	.	.	G	A			II											
.	.	G	.	C	.	G	.	-----------------	A	.	.	G	A	A	T	II											
.	.	G	.	C	.	G	.	-----------------	A	.	.	G	A	A	T	II											

SRS2 and *GRA6* respectively; "**.**"
rain.

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Table 3

Multi-locus *Toxoplasma gondii* genotypes from isolates or infected animal tissues in Australia.

Sample	Animal	Neurologic	Genotype	*B1*	*SAG3*	*GRA6*	*GRA7*	*SAG1*	*SAG2*	*SAG4*	*SRS2*	*BSR4*	*BTB*	*ROP18*	*BAG1*	*PK1*	*L358*	*c22-8*	*c29-2*
Cat	Domestic cat	−	I	I	I	I	I	I	I	I/II	I	I/II	I	I	I	I	I	I	I
Goat	Goat	+	I	I	I	I	I	I	I	I/II	I	−	I	I	I	I	I	I	I
Goat (Tas)	Goat	+	nonarchetypal	U-6	II	II	II	II/III	II	I/II	U-1	I/II	II	II	U-1	II	II	II	II
Wombat	Wombat	+	nonarchetypal	U-6	II	II	II	II/III	II	−	U-1	−	II	II	U-1	II	II	II	II
Wallaby	Wallaby	+	nonarchetypal	U-2	II	II	II	II/III	II	−	II/III	I/II	II	II	U-1	II	II	II	II
A1	Woylie	+	I	I	−	−	−												
A1Y	Woylie	+	recombinant	I	II	−	−												
A7	Horse meat	n/a	recombinant	I	I	II	−												
A8	Mouse	−	recombinant	I	II	I	−												
K28	Kangaroo mince	n/a	nonarchetypal	U-2	−	−	−												
A13	Meerkat	+	nonarchetypal	U-2	II	II	II												
PYR19	Kangaroo	−	I	I	−	−	−												
Q1	Kangaroo	−	II	II/III	II	−	−												
C14	Kangaroo	−	nonarchetypal	U-1	−	II	−												
R7	Kangaroo	−	nonarchetypal	U-3	−	−	−												
J6	Kangaroo	−	nonarchetypal	U-3	II	II	II												
J10	Kangaroo	−	nonarchetypal	U-4	−	−	−												
C9	Kangaroo	−	mixed	U-2+II	I+II	−	−												

Toxoplasma gondii isolates (Cat, Goat, Goat (Tasmania), Wombat, Wallaby) were genotyped by PCR-DNA sequencing at 16 loci "U" indicates a nonarchetpyal allele; I, II or III refers to the archetypal allele from a Type I, II or III strain

− Negative; + Positive; n/a non-applicable.