International Journal for Parasitology: Parasites and Wildlife 22 (2023) 75–79

Contents lists available at ScienceDirect

International Journal for Parasitology: Parasites and Wildlife

journal homepage: www.elsevier.com/locate/ijppaw

Presence of IgG antibodies is not a reliable marker of *Toxoplasma gondii* infection in feral mice

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ARTICLE INFO

Keywords:
Serology
Mus musculus
Protozoa
Parasites
Apicomplexan
Antibodies

ABSTRACT

The single-celled parasite *Toxoplasma gondii* uses mice as a vector to reach its definitive host, the cat, where it can accomplish its sexual reproduction and produce oocysts, which will contaminate the environment. In this study, we have captured 103 feral house mice (Mus musculus) on Kangaroo Island, Australia. We have measured the level of exposure to *T.gondii* serologically with the Modified Agglutination Test and conjointly with a *T.gondii* B1 gene PCR. We have included stringent quality control steps in the molecular analysis to reduce the risk of false positivity and false negativity. Our results indicated a low seroprevalence of 0.97%, 95%CI [-0.36; 0.58] associated with the detection of *T.gondii* genetic material in 51.46%, 95%CI [41.93, 60.88] of mice brains. Neither sex nor mice body weight had an effect on the PCR outcome. We postulate that both the transmission route, horizontal or vertical, and natural selection processes could lead to this discordance which has been observed elsewhere in wild mice. The question of the biological mechanisms allowing the chronic infection of wild mice in the absence of a measurable humoral immune response remains. Our findings indicate that serological studies should not be used to measure the level of exposure to *T.gondii* in feral house mice.

1. Introduction

Globally occurring, *Toxoplasma gondii* belongs to the phylum Apicomplexa. This single-celled parasite undergoes sexual reproduction in the small intestine of feline species leading to the shedding of oocysts and environmental contamination. In turn, other warm-blooded animal species become infected after the ingestion of sporulated oocysts. The infection of immunocompetent hosts is usually unnoticed and leads to dormant, life-long persistent tissue cysts. The natural cycle of this small parasite relies on the cat's propensity to eat preys, including mice (*Mus musculus*): would the intermediate host be predated by a cat it would perpetuate the life cycle of *T.gondii*.

Following the ingestion of sporulated oocysts, mice will either die or develop a humoral protective immunity that can be detected after 16 days (Dubey and Frenkel, 1973). Apart from this horizontal contamination, the parasite can be transmitted from the mother mouse to her pups. This is the vertical transmission that can happen not only in naive pregnant mice undergoing a primary *T. gondii* infection but also in chronically infected mice, as shown experimentally (Owen and Trees, 1998) and under field conditions (Marshall et al., 2004).

The global *T.gondii* population traditionally consisted of three main clonal lineages (I, II and III) (Howe and Sibley, 1995) with relatively low genetic diversity. Typing techniques evolved, strains with higher polymorphism were described and six major clades were identified (Su et al., 2012; Lorenzi et al., 2016; Galal et al., 2022). This has phenotypic implications, notably on the virulence level in laboratory mice (Dubey et al., 2012; Shwab et al., 2016) ranging from highly lethal to lower pathogenic and lower cerebral tissue cyst burden (Ingram et al., 2013).

Additional variation in virulence also occurs in different mouse subspecies (Hassan et al., 2019; Mukhopadhyay et al., 2020).

In Australia, *T. gondii* was likely introduced along with cats during European settlement (Hillman et al., 2016). Only the clonal type II has been reported in Australia, and the predominant naturally occurring Restriction Fragment Length Polymorphism subgroup in Australia is ToxoDB genotype #3 (Brennan et al., 2016), but the number of documented *T. gondii* genotypes remains low (Shwab et al., 2014; Amouei et al., 2020). Australian feral house mice (thereafter "mice") are all believed to be western European house mice, *Mus musculus domesticus* (Gabriel et al., 2011).

The objective of our study was to measure the level of exposure to *T.*

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https://doi.org/10.1016/j.ijppaw.2023.09.004
Received 12 May 2023; Received in revised form 5 September 2023; Accepted 8 September 2023
Available online 9 September 2023

gondii in mice on Kangaroo Island, Australia, where a cat eradication program has been initiated.

2. Material and methods

2.1. Mice

We baited live folding traps (medium Elliott traps, Elliott Scientific, Australia) with peanut butter and rolled oats. We set them on four private properties located on Kangaroo Island during six different trapping campaigns in August and in December 2019. Each campaign was on three consecutive nights. After every night of capture, we placed the trapped mice in holding bags until euthanized by neck dislocation. We measured their body weight at the nearest ¼ g with a 50g precision spring scale (Pesola, Switzerland) with a ± 0.3% precision. We recorded the sex for each mouse based on genitalia observation. We collected whole blood with 1 ml insulin syringe and we placed it in screw cap EDTA tube (Microvette Sarsted, Germany). We dissected each mouse aseptically and placed the brain in a 2 ml centrifuge tube (Eppendorf, Germany). We kept the brains under controlled temperature at 6 °C, then chilled at −20 °C usually from 5 days post-capture and until further analysis.

2.2. Serological tests

On the day following a three-night capture campaign, we centrifuged (1000×*g* for 5 min) all the blood samples collected and stored at 6 °C. We separated the plasma and we placed it in a cryovial for storage at −20 °C. We performed a Modified Agglutination Test (MAT) (*Toxo-Screen* DA, bioMerieux, France) at the University of Adelaide - Australia according to the manufacturer's recommendation and protocol published elsewhere (Dubey, 2009). We used 5 µl of thawed plasma and the same volume of both the positive and the negative controls included in the testing kit in two dilutions (1/40 and 1/4000). After overnight incubation, we assessed visually each tested sample for evidence of agglutination as per the manufacturer's guidelines. Whenever the reaction was doubtful, we observed under a dissecting microscope at 10x. We considered the result as positive anytime we observed signs of agglutination. This test specifically detects anti-*T.gondii* IgG.

2.3. Molecular biology

2.3.1. Extraction of DNA

Genomic DNA was extracted at the Nanyang Technological University – Singapore from the mice cerebral tissue using commercial kits (DNeasy Blood & Tissue Kits, Qiagen, Germany) according to the manufacturer's instructions. Briefly, 20 mg of brain tissue was dissected from each sample. Tissues were then digested by adding 180 µl Qiagen Buffer ATL and 20 µl proteinase K, followed by overnight incubation at 56 °C before purification via Qiagen DNA purification spin columns. Genomic DNA was eluted from the spin columns with 50 µl of sterile water and subsequently used as the template in the PCR experiments.

2.3.2. Detection of toxoplasma B1 DNA by PCR and sequencing

Obtained genomic DNA was probed for the presence of *Toxoplasma* B1 gene (NCBI Accession Number: AF179871.1) using PCR (forward primer: 5′ -GTG GGA ATG AAA GAG ACG CTA ATG TGT TTG CAT AGG TTG CAG- 3′; reverse primer: 5′-CGT CAC CAT CAG ACG AAT CAA CGG AAC TGT AAT GTG ATA CTG- 3′). Amplification was carried out under the following conditions: 95 °C for 5 min, 40 cycles of 95 °C for 1 min, 57 °C for 1 min, and 68 °C for 1 min, then a final extension of 68 °C for 10 min. Uracil-DNA Glycosylase-based amplification was used to eliminate cross-contamination carried over during successive experimental runs. The size of the expected amplified product based on B1 primers corresponds to 475 bp. Genomic DNA obtained from laboratory-grown *Toxoplasma* type II Prugniaud PA7 strain served as a positive control. Three replicates were employed for each sample. DNA Sanger sequencing was carried out on the extracted B1 DNA band to confirm its identity.

2.3.3. Determination of toxoplasma genotype through SAG2 PCR and restriction sites

Genomic DNA samples were analyzed at the 5′ and 3′ ends of the *Toxoplasma* SAG2 locus separately by adopting a modified nested PCR approach replicated from a previously published paper (Howe et al., 1997). The 5' end of the locus was first amplified by standard PCR (forward primer: 5′ -CTG CTG AAT GTG CTA CCT CGA ACA GGA ACA C- 3′; reverse primer: 5′ -GAG GGT GCA TCA ACA GTC TTC GTT GCG CCG GC- 3′). A second amplification of 40 cycles was then performed with the internal primers by using 5 µl of the resulting PCR amplicon as the DNA template (forward primer: 5′ -GAA CAG AAA TGT TTC AGG TTG CTG CAG TGA C- 3′; reverse primer: 5′ -GGT GGA CGC AAG AGC GAA CTT GAA CAC AAC- 3′).

The 3' end of the locus was similarly analyzed by initial amplification via standard PCR (forward primer: 5′ -GCC GCA GTT CTG TTC TCC GAA GTG ACT CCA GG- 3′; reverse primer: 5′ -GCG GTA TTC AAA GCG TGC ATT ATC GCG TCT C- 3′). A second amplification of 40 cycles was similarly performed with the internal primers by using 5 µl of the resulting PCR amplicon as the DNA template (forward primer: 5′ -CGA CCC TGC AAT TCT CAT GCC TCC GCT TCG- 3′; reverse primer: 5′ -GTA GAA CGT TTC ACG AAG GCA CAC CCG CCG G- 3′).

DNA Sanger sequencing was then carried out on the purified SAG2:5′ and SAG2:3′ DNA band to analyse for the presence of restriction enzyme sites, Sau3AI and Hhal respectively. Presence of Sau3AI in the SAG2:5' DNA sequence indicates the *Toxoplasma* to be of a type III strain (Howe et al., 1997).

2.4. Statistical analysis

We used a *t*-test to compare the mean body weight of male and female mice and to compare the mean body weight of mice being either PCR positive or negative. We used a Chi-squared test to compare the PCR outcome in the two sexes. We used Prism 9 (Graphpad, USA) for all statistical work.

Confidence intervals were calculated using the modified Wald (Agresti-Coull) method.

All *p*-values inferior to 0.05 were considered significant.

3. Results

3.1. Total number of captured mice and body weight

We have captured a total of 103 mice in year 2019 from four sites on KI. They comprised 54 females and 49 males. The mean body weight was 14.78 ± 0.37g, ranging from 4.75 to 26.5g. Males were slightly heavier (M = 15.01, SD = 2.82) than females (M = 14.54, SD = 4.40) but this difference was not significant, $t(101) = 0.7243$, $p = 0.4705$.

3.2. Toxoplasma gondii seroprevalence in mice

We tested for the presence of anti-*T. gondii* IgG on all 103 mice. All positive and negative controls did hold. Only one mouse elicited a positive reaction and the seroprevalence was therefore 0.97%, 95%CI [0; 0.58].

3.3. Toxoplasma B1 gene PCR

3.3.1. Mice brain samples contained DNA derived from the toxoplasma genome

The *Toxoplasma* genome contains B1, a gene that is specific to the parasite and which is found in multiple copies within the genome (Burg et al., 1989). We used a PCR to amplify this gene, using genomic DNA

isolated from the brain samples as the template (Supplementary Material 1A).

We isolated the genomic DNA from brain samples of 103 mice, of which only 1 was determined to be seropositive for anti-*Toxoplasma* IgG antibodies, and 102 were seronegative. Experimenters were blind to the serostatus during DNA isolation and subsequent molecular analysis. The presence of B1 was detected in brain tissue of the sole IgG positive animal. Surprisingly, an additional 52 animals were found to contain *Toxoplasma* B1 gene within brain tissue, indicating the presence of *Toxoplasma* genetic material.

The prevalence based on PCR was therefore 51.46%, 95%CI [41.93; 60.88]

The 50 animals were negative for *Toxoplasma* B1 (Supplementary Material 1B). PCR products from all 53 B1-positive samples were DNA sequenced and confirmed to correspond with the expected B1 sequence (Supplementary Material 1C). The target gene was successfully amplified from the laboratory-grown *Toxoplasma* type II Prugniaud PA7 strain, which serves as the positive control. Negative controls did not exhibit B1 amplification throughout the experiment.

3.3.2. *Toxoplasma DNA detected in mice brain samples were from a type II strain*

Animals with positive determination of *Toxoplasma* B1 were further tested for the presence of restriction enzyme sites, Sau3AI and Hhal, at 2 opposite ends of the *Toxoplasma* SAG2 locus, SAG2:5′ and SAG2:3′, respectively. Presence of Sau3AI in the SAG2:5′ DNA sequence indicates the *Toxoplasma* to be of a type III strain. Presence of Hhal in the SAG2:3' DNA sequence indicates the *Toxoplasma* to be of a type II strain. An absence of both restriction enzyme sites indicates the *Toxoplasma* to be of a type I strain. This process allows unambiguous determination of which *Toxoplasma* strain were the mice infected with. The process was carried out via a previously described nested PCR approach but with modifications to complement to our wildlife mice samples.

To test the sensitivity and accuracy of this genotyping approach, laboratory-grown *Toxoplasma* type II Prugniaud PA7 strain served as positive control during the nested PCR amplifications. SAG2 was readily detected from *Toxoplasma* type II Prugniaud PA7 and analysis by DNA Sanger sequencing revealed an absence of the Sau3AI restriction enzyme sites at the SAG2:5′ locus and a presence of Hhal in the SAG2:3' locus DNA sequence. This indicates the *Toxoplasma* to be of a type II strain, confirming the accuracy of this genotyping approach. No PCR product was detected from the non-template sterile water negative control (Supplementary Material 2A and 2B). DNA Sanger sequencing was similarly carried out on laboratory-grown *Toxoplasma* type I RH, and there was an absence of both Sau3Al and Hhal restriction enzyme sites (Supplementary Material 2C). These analysis proved that the nested PCR approach gives consistent genotyping results.

31 *Toxoplasma* B1-positive mice were then taken at random to establish the *Toxoplasma* strains these animals were infected with. The same genotyping approach found all 31 mice to be infected with a type II *Toxoplasma* strain (Supplementary Material 2D). There was no detection of mixed infections. In each amplification process, the target genes were successfully amplified from the laboratory-grown *Toxoplasma* type II Prugniaud PA7 strain, which serves as the positive control. Negative controls did not exhibit B1 amplification throughout the experiment.

3.4. *Relationship of sex and body weight with PCR result*

Out of the 103 mice captured, there were 26 females and 24 males among the 50 PCR -negative mice, and there were 28 females and 25 males among the 53 PCR-positive mice.

The mean body weight was 15.25g, SD = 3.63 and 14.34g, SD = 3.79 in PCR-negative and in PCR-positive mice, respectively (see Fig. 1).

Those differences were not significant and the likelihood of being PCR positive was not affected either by the sex (χ^2 (1, N = 103) = 0.007110, $p = 0.9328$) or the body weight t(101) = 1.243, $p = 0.2166$.

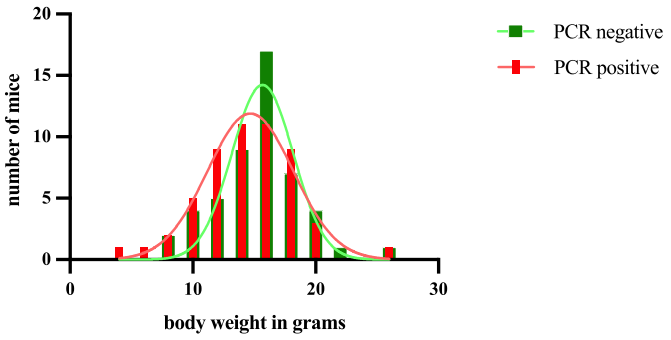

Fig. 1. Frequency distribution of body weight in *Toxoplasma gondii* B1 PCR-negative (in green) and positive mice (in red). (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

4. Discussion and conclusion

The low seroprevalence of 0.97%, 95%CI [−0.36; 0.58] observed in this study is consistent with another study performed in the UK (Murphy et al., 2008), but lower than, for instance, 4.8% in Senegal (Galal et al., 2019), 12.3% in the Canary Islands, Spain (Foronda et al., 2015) or 14.29% in Corsica, France (Izquierdo-Rodríguez et al., 2019).

This serological result contrasts greatly with the 51.46%, 95%CI [41.93, 60.88] *T. gondii* prevalence based on B1 gene PCR that we observed. The quality control steps limited false negative PCR reactions: no amplification occurred with any of the negative controls and the DNA integrity was preserved as demonstrated by the presence of the IGR gene. False positive results were also addressed: all positive controls elicited a positive reaction; the amplicons' band size was compatible with the expected size of the *T. gondii* B1 gene fragment indicating an absence of non-specific reaction. Furthermore, all samples that were typed indicated a *T. gondii* type II, and the sequencing results indicated a polymorphism with the strain used as a positive control in the laboratory.

Following the ingestion of oocysts by mice, the first single tachyzoites are observed in the brain as soon as day 7 (Ferguson et al., 1991) and seroconversion happens at day 16 (Dubey and Frenkel, 1973). Due to the brevity between the two events (the presence of the organism in cerebral tissue and the presence of anti-*T.gondii* immunoglobulins), it would be surprising and unlikely to find, at any given moment, a large proportion of mice in this situation.

While our results show the discordance between serological and molecular detection methods, reasons underlying this discordance in wild mice remain unclear, although a few plausible mechanisms can be postulated such as the proportion of mice that are congenitally affected, the proportion of mice trophically infected and natural selection processes that might have different weight on each population.

An experiment demonstrated that vertical transmission of *T.gondii* in mice is rather common and also leads to a situation that could partially explain our results. In this experiment, captive female mice were chronically infected each with 50 oocysts (Owen and Trees, 1998). They were mated and gave birth to pups, which were tested for strict vertical *T. gondii* exposure at six weeks of age. The authors found that 89.85%, 95CI [84.91, 93.61] of the 207 pups vertically infected were *T.gondii* PCR-positive, but only 64.25%, 95CI [57.31, 70.77]) were seropositive.

From this template obtained in the laboratory, we can hypothesize that natural selection processes occurring in the wild might affect the populations of seropositive PCR-positive mice and seronegative PCR-positive mice and modify their respective proportions. A team in the UK reported results similar to ours with 59.00%, 95CI [51.84, 65.89] of 200 wild-caught mice were SAG1 gene PCR-positive (Marshall et al., 2004) but the MAT-seroprevalence was 1.05%, 95CI [0.13, 3.75] of a subset of 190 mice (Murphy et al., 2008). They found evidence of

vertical transmission in the 12 pregnant mice that were SAG1 PCR positive. Each had at least one foetus SAG1 PCR positive, and from all the 63 foetuses the vast majority (74.6%) were SAG1 PCR positive (Marshall et al., 2004).

Mice congenitally affected by *T.gondii* might not develop a serological immune response to the parasite (Beverley, 1959; Jacobs, 1964; reviewed in Hide, 2016). This observation can partially explain the poor concordance between tests which was also highlighted more recently: out of 671 wild mice captured in Senegal 4.8% were positive to MAT against 13.3% positive to B1-PCR (Galal et al., 2019).

It is still unknown if the natural selection processes apply similarly to mice affected either horizontally or vertically. The risk of horizontal transmission increases with time (Afonso et al., 2007). After such exposure, mice can either be killed acutely by the direct pathogenic effects of *T.gondii*, or undergo a dormant infection with detrimental effects on their survival, for instance by potentially increasing their risk of being predated (Vyas et al., 2007; Ingram et al., 2013). Both would result in a survival bias, possibly reducing the *T.gondii* prevalence in the captured mice as they become older.

Our results show that sex has no effect on the probability of being exposed to *T.gondii* and surviving this infection until the mouse is captured. This observation is compatible with both transmission routes.

The body weight can be taken as a coarse estimate for age (Crowcroft and Rowe, 1961). We found that the level of exposure to *T.gondii*, (PCR-positivity) is not influenced by body weight, therefore it is not influenced by age. This observation is compatible with a congenital infection if no survival bias is associated with this condition. Inversely, this observation is also compatible with a vertical transmission if the survival bias counteracts exactly the probability to encounter the parasite.

Our study also poses the question of the persistence of *T.gondii*, a potentially lethal parasite, in wild mice, in the absence of a measurable humoral immune reaction. It has been demonstrated that cellular immunity with the intervention of macrophages and interferon-gamma (IFN-γ) cytokine play a critical role in the protection against this parasite (Innes, 1997; Suzuki et al., 1988; Lieberman and Hunter, 2002). We suggest investigating the cellular immunity in wild-caught mice in further studies.

However, the disagreement between the serological and the molecular biology test results prevent to recommend the use of serological studies for surveying of *T.gondii* exposure in wild mice, as already stated (Galal et al., 2019).

Declaration of competing interest

None.

Ethical statement

This study was performed according to the animal ethics approval 0000021665 (v1) issued by the University of Adelaide Animal Ethics Committee.

Funding

This study was funded by the Human Frontier Science Program (Grant RGP0062/2018).

Acknowledgements

We would like to thank the different landowners on KI for providing access to their property allowing us to perform this study.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.ijppaw.2023.09.004.

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