

Lamb as a potential source of *Toxoplasma gondii* infection for Australians

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Toxoplasmosis is the human infectious disease caused by the protozoan parasite, *Toxoplasma gondii*,¹ which is not restricted geographically and exists as clonal or genetically diverse strains in different regions.² Approximately one-third of the world population is infected with *T. gondii*, although rates of infection – typically detected by serology – vary substantially between countries.¹ In Australia, where toxoplasmosis is not a notifiable disease, seroprevalence has not been documented comprehensively, but probably reflects the global average. A 1979 review of previous epidemiological surveys, conducted across five Australian states, estimated 30% mean population prevalence of *T. gondii* serum antibody, with 24% to 44% range.³ One more recent study, conducted between 1998 and 1999 at the Melbourne Royal Women's Hospital antenatal clinic, identified *T. gondii* antibodies in 23% of blood samples collected from 308 women of child-bearing age.⁴

The most common presentation of toxoplasmosis is eye infection and inflammation, which is based in the retina and termed 'ocular toxoplasmosis'.⁵ Destruction of retinal tissue, which progresses with recurrent attacks of retinitis – as well as complications that include retinal detachment, retinal vascular occlusions and cataract – often result in visual impairment. Ocular toxoplasmosis was identified as one of the leading causes of uveitis in a current audit of the largest tertiary inflammatory eye disease services in New South Wales, published in 2017.⁶ This condition is the most frequently identified

Abstract

Objective: Toxoplasmosis may follow consumption of undercooked meat containing *Toxoplasma gondii* cysts. Lamb is considered to pose the highest risk for contamination across meats. Red meat is often served undercooked, yet there are no current data on *T. gondii* contamination of Australian sourced and retailed lamb. We sought to address this gap in public health knowledge.

Methods: Lamb mincemeat was purchased at the supermarket counter three times weekly for six months. *T. gondii* was detected by real-time polymerase chain reaction (PCR) of DNA extracted from the meat following homogenisation. Purchases were also tested for common foodborne bacterial pathogens.

Results: Conservative interpretation of PCR testing (i.e. parasite DNA detected in three of four tests) gave a probability of 43% (95% confidence interval, 32%–54%) that lamb mincemeat was contaminated with *T. gondii*. None of the purchases were contaminated with *Campylobacter jejuni*, *Salmonella* species or *S. enterica* serovar Typhimurium, indicating sanitary meat processing.

Conclusions: Australian lamb is commonly contaminated with *T. gondii*. Future studies should be directed at testing a range of red meats and meat cuts.

Implications for public health: Consuming undercooked Australian lamb has potential to result in toxoplasmosis. There may be value in health education around this risk.

Key words: *Toxoplasma*, meat, lamb, PCR

form of uveitis among Indigenous Australians living in Central Australia.⁷ While ocular toxoplasmosis usually presents in otherwise healthy adults, the disease is more common and severe in certain groups: congenitally infected neonates, the aged and persons with co-morbidities that impact immune function.^{8–10} These individuals also are particularly susceptible to expanded central nervous system and/or systemic pathology. A 2014 Australian Department of Health-funded report identified toxoplasmosis as one of the foodborne pathogens that continues to cause deaths in Australia.¹¹

Cat species act as primary hosts for *T. gondii*. Humans are secondary hosts of the parasite, and become infected by two main routes: ingestion of material – often water – contaminated with cat faeces containing sporozoites in oocysts; and consumption of raw or undercooked meat harbouring bradyzoites in tissue cysts.¹ Livestock may be exposed to *T. gondii* when grazing in pastures where infected cats have defaecated.¹² Much less commonly, if a woman contracts *T. gondii* during pregnancy, blood-borne tachyzoites may infect her unborn child.¹ Control of feline carriers remains a challenging problem,

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but adequate cooking of meat is a simple measure that prevents infection. Tissue cysts are rendered non-viable by cooking meat to an internal temperature of 66°C or at least 'medium'.¹³ However, while thorough cooking is common practice in preparing white meats for consumption, red meats are often served and eaten 'rare' in Australia.

From an international perspective, meat sourced from sheep poses the highest risk among livestock for human infection with *T. gondii*.¹⁴ A new microbial risk assessment focused on consumption of lamb in the United States suggested an increased risk for contracting *T. gondii* from eating Australian product, compared with American-sourced meat, due to open pasture farming.¹⁵ However, there have been no studies of *T. gondii* contamination of sheep meat products in Australia for more than 30 years. To begin to address this gap in health knowledge, we tested lamb mincemeat purchased at the supermarket counter in South Australia over a period of six months for *T. gondii*. We studied mincemeat in preference to other meat cuts because mincemeat is sourced from multiple animals and thus affords a superior indicator for contamination. Testing was performed by real-time polymerase chain reaction (PCR) on DNA extracted from the mincemeat, which was examined in parallel for common foodborne bacterial pathogens.

Methods

Sourcing of lamb mincemeat

Lamb mincemeat parcels (approximately 500 g) were purchased from a large supermarket serving the southern region of Adelaide, South Australia. This product is prepared from primary and secondary lamb cuts taken from between 15 and 20 animals, and it represents a combination of meat from organically farmed South Australian livestock and conventionally farmed countrywide livestock. In line with standard practice, mincemeat does not contain preservatives and is not frozen prior to presentation for sale. Following purchase, the mincemeat was stored at -20°C ahead of testing.

Based on the National Health and Medical Research Council 'Eat for Health' 2013 Australian Dietary Guidelines – recommending weekly red meat, taken as several small servings totalling 455 g – lamb mincemeat parcels were purchased three times weekly for testing. Parcels were bought between

April 2017 and September 2017, giving a total of 79 samples. Food expiration dates on the parcels were used to track samples and confirm that each parcel represented an independent mincemeat preparation.

Preparation of mincemeat for testing

Fifty grams of each lamb mincemeat sample was homogenized using the Stomacher 400 (Seward, Worthing, United Kingdom). Stomaching was performed in Strainer Stomacher Bags (Seward) with 125 mL of 0.1 M Tris-0.01 M EDTA buffer, separating the homogenate from the filtrate, for collection and storage at -20°C. DNA was extracted from 250 mg of each mincemeat homogenate using the PureLink Genomic DNA Mini Kit (Thermo Fisher Scientific–Invitrogen, Carlsbad, CA). Purity and concentration of DNA extracts were read on the NanoDrop 2000 Spectrophotometer (Thermo Fisher Scientific, Waltham, MA), and extracts were stored at -20°C prior to PCR testing.

Testing of mincemeat for parasitic and bacterial contamination

Real-time PCR detection of *T. gondii* was performed on the CFX Connect Real-Time PCR Detection System, including CFX Manager v3.1 software (Bio-Rad, Hercules, CA). In the first stage of PCR testing, DNA extracts from the lamb mincemeat were tested for the presence of parasite B1 gene. Each extract – representing one lamb mincemeat parcel – was tested in quadruplicate. In the second stage of PCR testing, confirmation of the presence of the parasite B1 gene in amplicons obtained in the first stage was performed by nested PCR.

Meat DNA extracts were diluted to 10 ng/ μ L in nuclease-free water. Polymerase chain reaction products from the first stage PCR were diluted 1:100 in nuclease-free water. Reaction mixtures included: 4 μ L (first stage PCR) or 1 μ L (second stage, nested PCR) DNA; 4 μ L iQ SYBRGreen Supermix (Bio-Rad), 1.5 μ L each of 10 μ M forward and reverse primers (Sigma-Aldrich, St. Louis, MO; Supplementary Table 1); and 9 μ L (first stage PCR) or 12 μ L (second stage, nested PCR) of nuclease-free water, giving a final volume of 20 μ L. Amplification for both first and second stage PCRs consisted of: a pre-cycling incubation at 95°C for 5 minutes; 40 cycles of denaturation for 30 seconds at 95°C; annealing for 30 seconds at 61°C; extension for 30 seconds at 72°C; and post-extension at 75°C for 30

seconds. Melt curves were generated by one-second readings taken every 0.5°C from 65°C to 95°C.

In addition to DNA extracts from the mincemeat, all runs included PCRs of nuclease-free water in place of DNA as negative control, and DNA extracted from lamb mincemeat spiked with *T. gondii* (GT-1 strain, originally isolated from a goat¹⁶ and maintained in-house by serial passage in human fibroblasts, gifted by L. David Sibley, PhD, Washington University, St. Louis, MI) at 1 \times 10⁵ parasites/g as positive control, as well as PCRs for lamb glyceraldehyde 3-phosphate dehydrogenase (GAPDH) to confirm DNA amplification.

DNA extracts from lamb mincemeat also were tested by real-time PCR for the presence of *Campylobacter jejuni*, *Salmonella* species and *S. enterica* serovar Typhimurium. These PCRs were performed on the Rotor-Gene 3000 (Corbett Research, Mortlake, Australia), singleplex for *C. jejuni*, and multiplex for *Salmonella* species and *S. Typhimurium*. For *C. jejuni*, 20 μ L reaction mixtures included: 100 ng DNA; 1 \times SsoAdvanced Universal Probes Supermix (Bio-Rad); 0.4 μ M forward primer and reverse primer (Bio-Rad; Supplementary Table 1); and 0.1 μ M probe (Bio-Rad). Cycling conditions consisted of: a pre-cycling incubation at 95°C for 3 minutes; 50 cycles of denaturation for 15 seconds at 95°C; and annealing and extension for 30 seconds at 60°C. For *Salmonella* spp and *S. Typhimurium*, 20 μ L reaction mixtures included: 100 ng DNA; 1 \times SsoAdvanced Universal Probes Supermix; 0.2 μ M forward primers and reverse primers (Bio-Rad; Supplementary Table 1); and 0.05 μ M probes (Bio-Rad). The cycling conditions consisted of: a pre-cycling incubation at 95°C for 10 seconds; 45 cycles of denaturation for 10 seconds at 95°C; and annealing and extension for 120 seconds at 64°C.

The PCR runs for *C. jejuni*, *Salmonella* species and *S. Typhimurium* included PCRs of nuclease-free water in place of DNA as negative control, and DNA extracted from lamb mincemeat spiked with *C. jejuni* (American Type Culture Collection [ATCC], Manassas, VA; catalogue number 33291) and *S. Typhimurium* (ATCC; catalogue number 53647) as positive control.

Statistical analysis

Probabilities of detecting lamb mincemeat contaminated with *T. gondii* were calculated

using an exact binomial method in R v3.4.2 software (RStudio, Boston, MA).

Results

A total of 79 lamb mincemeat parcels were purchased from the supermarket over a defined six-month period in 2017. DNA extracted from each parcel was tested for the presence of the *T. gondii* B-1 gene by quantitative PCR, including nested PCR. Results were interpreted alongside PCRs performed with negative and positive controls, and to confirm DNA amplification. The B-1 gene was detected in three or four of four tests for 34 samples, and in all these samples, the presence of the gene was confirmed by the nested PCR. For an additional 21 samples, the B-1 gene was detected in two of four tests, and in 20 of these samples, the presence of the gene was confirmed by the nested PCR. Across positive samples, mean cycle threshold of amplification ranged from 24.3 to 39.4.

The more conservative interpretation of PCR testing (i.e. parasite DNA detected in at least three of four tests) indicated a probability of 43% (99% confidence interval, 29%–58%; 95% confidence interval, 32%–54%) that lamb mincemeat was contaminated with *T. gondii*. The more liberal interpretation of PCR testing (i.e. parasite DNA detected in at least two of four tests) indicated a probability of 68% (99% confidence interval, 53%–80%; 95% confidence interval, 57%–78%) that mincemeat was contaminated. By comparing the positive PCR results with those obtained for lamb mincemeat that had been spiked with known numbers of *T. gondii* tachyzoites, the level of contamination in the samples was approximated at between 260 and 2.9×10^5 parasites/g of meat. These results indicate lamb that is sourced and retailed in Australia is often contaminated with *T. gondii* at variable levels.

Quantitative PCR for the common Australian foodborne bacterial pathogens that occur in meat – *C. jejuni*, and *Salmonella* species including *S. Typhimurium* – was also performed on the DNA extracted from the lamb mincemeat. These pathogens were not detected in any of the 79 parcels, although PCR using DNA isolated from meat spiked with bacterial species was positive. This observation supports the concept that meat preparation is sanitary, and *T. gondii* DNA detected in the meat parcels reflects infection

of livestock in the pasture, rather than introduction during processing.

Discussion

Consuming raw or undercooked meat is a common route for individuals to contract toxoplasmosis, and meat sourced from sheep, in particular, has the potential to harbour *T. gondii*. Lamb is a staple meat in Australia, where it typically is marketed without freezing and often is served 'rare'. Surprisingly, however, there are no current data on *T. gondii* contamination of locally produced and marketed lamb. We tested lamb mincemeat, purchased from the supermarket three times weekly for six months, for *T. gondii* by PCR, and identified parasite DNA in a substantial number of purchases. On the basis of four PCR tests per parcel, probability of *T. gondii* contamination of the meat product was conservatively estimated (i.e. at least three PCR tests positive) at 43%. Although the meat was purchased at one supermarket, located in South Australia, our findings are likely to be generalisable, because Australian supermarket retailers purchase their lamb across a range of farms throughout the country. None of the same purchases were contaminated with *C. jejuni* or *Salmonella* species – common foodborne bacterial pathogens in Australia. Bacterial contamination of meat typically occurs at the abattoir, through contact with faeces on the hide or internal organs,¹⁷ and its absence suggests that either the livestock were not carriers of these pathogens, or the pathogens were removed by post-slaughter decontamination processes. Unlike the bacteria, *T. gondii* exists within the musculature of the animal and is not removed by washing.

Multiple studies from across the globe that have reported on prevalence of *T. gondii* in meat were recently subjected to systemic review and meta-regression.¹⁴ Twenty-nine studies from countries in Africa, Asia, Europe, North and South America and Oceania provided a range for prevalence in lamb meat, from as low as 0.144 in North America, to as high as 0.271 in Asia.¹⁴ Two studies from Oceania included one from Australia: per the report published in 1985, Rothe and colleagues¹⁸ examined 30 lamb chops, collected in two visits to 15 South Australian butcher shops, and did not identify the parasite in any chop. The results of their study reflect the environment and farming practices

of 30 years ago and involve a relatively limited sample size, but several technical differences also may explain the difference from our results. We studied lamb mincemeat, which is sourced from more than one animal – in our study, up to 20 – increasing the likelihood of parasitic contamination. Rothe et al.¹⁸ detected parasites by bioassay, which involved inoculating mice with lamb homogenate, and checking serology and brain histopathology. We tested the meat by real-time PCR, which is the most commonly used method today and is more sensitive to presence of parasite, although practically, a lesser quantity of meat is tested. An independent description of *T. gondii* in meat retailed in Canada included Australian lamb, but did not specify details such as meat cut, source and sampling interval.¹⁹ Several groups have studied the seroprevalence of *T. gondii* in Australian lambs and sheep: in three studies covering multiple decades (1974 to 2007) seropositivity was measured at significant levels 16.4% to 20.7% in lambs and 29.6% to 61.7% of sheep.^{20–22} These seroprevalences are consistent with our findings for meat.

A new development in livestock systems is organic farming. Studies conducted in swine indicate that organic farming increases the risk of *T. gondii* infection of the animals, probably related to increased exposure to cat populations and fields that the felines may have contaminated.²³ Australia is home to approximately 60% of certified organic farmland globally,²⁴ and feral cats are common across much of the country and are abundant in South Australia.²⁵ The lamb mincemeat we tested was produced as meat off-cuts, representing a mixture of nationally sourced non-organic lamb, and organic lamb grazed in northern South Australia.

In reducing the risk of toxoplasmosis, from the communal health perspective, food preparation is more readily tackled than cat control. However, while many of the public know of the link between *T. gondii* infection and cats, there is little general awareness of the association between infection and foodstuffs. For example, current Australian governmental recommendations do not advise on the need to cook fresh red meat thoroughly.²⁶ Tissue cysts are rendered non-viable by cooking meat to an internal temperature of 66°C.¹³ Alternatively, meat may be frozen overnight at -12°C to destroy the cysts.¹³ Specific messaging that is sensitive to consumer cooking preferences

may be helpful to educate the Australian population of the risk related to consuming undercooked lamb, which applies particularly to pregnant women, the elderly and immunocompromised persons.

Our work has some limitations. In working with lamb mincemeat, we have studied *T. gondii* contamination of one type and one cut of meat; since there had been no work in this area for multiple decades, we chose to study one of the potentially highest-risk meat products. Given the increasing popularity for undercooked meat, including pork and chicken, it would be of interest to study a range of red and white meats and meat cuts. Real-time PCR is the most common method used to study meat contamination with *T. gondii* today, but in interpreting the results, it is important to recognise that detection of parasite DNA does not equate with a human infection. The method cannot distinguish between viable and non-viable micro-organisms and does not account for the fact that *T. gondii* occurs as groups within tissue cysts, rather than individual parasites dispersed across the meat. Alternative methods for detecting tissue cysts in meat include Percoll gradient centrifugation or bioassays in laboratory animals, such as the cat or the mouse.^{18,27} In addition, contracting the infection depends on multiple factors, such as parasite load and other food that is consumed contemporaneously, as well as host immune competence. Acknowledging these limitations, our study provides current evidence that consuming undercooked lamb in Australia has the potential to result in toxoplasmosis and suggests there may be value in public education around this risk associated with consuming rare or raw lamb.

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Supporting Information

Additional supporting information may be found in the online version of this article:

Supplementary Table 1: Primer pairs, probes and product sizes.