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Detection of viable *Toxoplasma gondii* in retail venison

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ABSTRACT

Toxoplasma gondii is an important zoonotic pathogen and the consumption of undercooked meat is known to be a significant risk factor for infection. Previously, a small-scale study of retail meat in Scotland identified a high incidence of *T. gondii* in venison products but the risk to public health could not be assessed as parasite viability was not determined. The aim of the present study, therefore, was to build on this work and establish *T. gondii* viability in retail venison. Twenty-three venison products were purchased from farm shops or supermarkets, and 50 g samples were processed for DNA extraction and qPCR. Any samples positive for *T. gondii* by qPCR were assessed for viability in a mouse bioassay. Viable *T. gondii* was isolated from 2 out of 5 (40 %) positive venison samples. Genotyping by RFLP and microsatellite analysis revealed both isolates to be Type II. This is the first study to report the presence of viable *T. gondii* in retail venison and highlights the potential risk to public health if this meat is eaten undercooked. Consumers should freeze venison, or cook it thoroughly, before consumption to reduce the risk of foodborne toxoplasmosis.

1. Introduction

Toxoplasma gondii is a globally important protozoan parasite. Although cats (and other members of the Felidae family) are the only hosts capable of shedding oocysts in their faeces, the parasite can infect all warm-blooded animals, including humans, making it an important zoonotic parasite (Dubey, 2010). Humans can become infected through the consumption of infectious oocysts in contaminated food and water, through the ingestion of tissue cysts in undercooked or raw infected meat, or congenitally during a primary infection in pregnancy. Of these routes, foodborne transmission (tissue cysts and oocysts) is thought to be a major source of *T. gondii* and is attributable to an estimated 40–60 % of infections (EFSA, 2018). Indeed, the consumption of undercooked meat has been shown to be a significant risk factor for infection in different epidemiological studies (Cook, et al., 2000; Jones et al., 2009), including in the UK (Said, et al., 2017). Toxoplasmosis is asymptomatic for most immune competent people; however, it can cause severe disease in immune compromised people, and in pregnant women it may lead to miscarriage, still birth, or congenital defects in the baby (Robert-Gangneux & Darde, 2012). Due to the severe sequelae of infection in these risk groups, which may persist for the lifetime of the host, the disease burden of toxoplasmosis as measured by disability-adjusted or quality-adjusted life years (DALYs or QALYs, respectively) is high (WHO, 2017).

Although *T. gondii* is recognised as a major foodborne pathogen, there is a significant lack of data on the role of retail meat in the transmission of this parasite - something which has been highlighted as a knowledge gap by the European Food Safety Authority (EFSA), and the UK Advisory Committee on the Microbiological Safety of Food (ACMSF, 2012; EFSA, 2007). We previously conducted a survey of retail meat samples in Scotland and demonstrated the presence of *T. gondii* DNA in pork (4.2 %), lamb (6.9 %) and chicken (4.8 %), and a particularly high incidence in venison products (28.4 %) (Plaza, et al., 2020). Game meat, from wild or farmed sources, is growing in popularity and venison in particular is often promoted as a healthier meat choice for consumers. Scotland produces around 3600 tonnes of venison annually, with approximately 100 tonnes from farmed venison and the remainder from the wild cull (Venison Advisory Service). Market demand can often outstrip supply, so the Scottish Venison Sector has launched an initiative through Scotland Food & Drink to significantly expand the farmed venison sector, increasing outputs from 100 tonnes annually to 850 tonnes (Scotland Food and Drink, 2018). Consumption of undercooked venison has been linked to cases of symptomatic toxoplasmosis (Gaulin, et al., 2020; Schumacher et al., 2021), particularly amongst deer hunters

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(Westling, 2021), and consumption of undercooked game meat is considered a risk factor for *Toxoplasma* infection (Cook, et al., 2000; Friesema et al., 2023).

Given that venison is often consumed undercooked, and previous work has demonstrated a prevalence of *T. gondii* DNA of 28.4 % in retail venison in Scotland (Plaza, et al., 2020), the aim of this study was to determine the viability of *T. gondii* in venison products to assess the potential risk to public health and to better inform prevention and control strategies.

2. Materials and methods

2.1. Sample collection and processing

Based on previous research where a sample weight of 50 g was processed for the detection of *T. gondii* (Plaza, et al., 2020), it was calculated that for a 90 % probability of detecting a maximum of 10 positive samples of venison under an assumed prevalence of 32.2 % (95 % CI: 25.2 %–40.1 %), 41 samples of 50 g would need to be processed. Given the inhomogeneous distribution of *T. gondii* tissue cysts in meat, and considering that the aim of the study was not to determine prevalence but to detect viable parasites, two 50 g portions of venison were tested for each product purchased to maximise the chances of detecting parasites. Thus, 23 venison products (8 diced venison, 14 ground venison, 1 steak), giving rise to forty-six 50 g samples, were purchased on a convenience basis from two types of retail outlets (farm shop or supermarket) in four locations in Scotland over a three-week period between September and October 2020. Of the 23 products purchased, 11 were from a farm shop and the remaining 12 products were purchased from supermarkets. All products were purchased fresh and were pre-packaged. Twelve of the products consisted of pure venison only, and 11 contained ground venison supplemented with pork meat. Although the latter products were not pure venison, they were chosen as they represent a popular venison meat product and were shown to have a high *T. gondii* positivity rate in a previous study (Plaza, et al., 2020). Two 50 g portions of meat per product were homogenised individually and digested in acid/pepsin, and 2 ml of the digested pellets were used for DNA extraction, as previously described (Hamilton, et al., 2015). One to 2 mL of sterile saline containing 400 IU/ml penicillin and 400 mg/ml streptomycin were added to the remaining homogenised tissue pellets and samples were stored at 4 °C until required for mouse inoculations (no more than 48 h later) – see Section 2.3. Meat juice was collected from packaging where possible (Hamilton, et al., 2015) and where none was available it was collected following freezing and thawing of the remainder of the sample once 100 g had been deducted for processing.

2.2. Toxoplasma gondii qPCR and meat juice ELISA

DNA was extracted from 2 ml of homogenised tissue pellet per sample using the Wizard® genomic DNA purification protocol (Promega Corporation, U.K), with upscaled volumes to allow for the larger starting material. Each sample was screened for *T. gondii* DNA using the 529 bp repeat element qPCR (Opsteegh, et al., 2010) with slight modifications, as previously described (Hamilton, et al., 2015). Meat juice samples were screened for antibodies to *T. gondii* using a commercial ELISA (ID Screen® Toxoplasmosis Indirect Multi-species, IDvet, Montpellier, France) according to the manufacturer's instructions.

2.3. Mouse bioassay and in vitro isolation of T. gondii

Animal work was conducted in accordance with the Animals (Scientific Procedures) Act 1986 and was approved by the Moredun Research Institute's Animal and Welfare Ethical Review Body (Experiment Number E16/20). Thirty female, outbred CD-1 mice, aged 6–8 weeks, were housed in groups of 6 with access to water *ad libitum*. Venison samples which tested positive for *T. gondii* by qPCR had the remainder of the homogenised pellet inoculated into mice – 1 ml of homogenised tissue inoculated intraperitoneally per mouse. Due to high Ct values in the qPCR (Table 1), indicating low levels of *T. gondii* present in the samples, six mice per sample were inoculated in order to maximise the chance of detecting viable parasites. Mice were monitored twice daily and scored for signs of clinical toxoplasmosis, and humanely euthanised 28 days post-inoculation. At post-mortem, blood was collected for serology, and right lung and brain were collected into 2 ml tubes and stored at −20 °C for DNA extraction, as previously described (Hamilton, et al., 2017). Left lung was collected into a sterile tube containing wash buffer (Hank's Balanced Salt Solution, supplemented with 2 % foetal bovine serum (FBS) and 400 IU/ml penicillin and 400 mg/ml streptomycin), and transported to the laboratory. In the Class II Biological Safety Cabinet, each lung sample was transferred to a sterile Petri dish and finely chopped into approx. 1 mm diameter pieces in 5 ml of wash buffer, before being transferred to a 30 ml centrifuge tube and centrifuged at 400×*g* for 5 min. Supernatant was carefully removed and the pellet was resuspended in 5 ml cell culture media (Iscove Modified Dulbecco Medium, supplemented with 1 % FBS, 400 IU/ml penicillin and 400 mg/ml streptomycin) before being transferred to a T25 cell culture flask containing a confluent monolayer of Vero cells and incubated at 37 °C with 5 % CO_2. After 24 h, media containing chopped tissue was removed and cells washed with sterile PBS (pH 7.2), before adding 5 ml of fresh culture media. Flasks were incubated at 37 °C with 5 % CO_2, and checked daily, for 3 weeks, for parasite growth.

2.4. Genotyping of T. gondii isolates by PCR-RFLP and microsatellite typing

DNA was extracted from tachyzoites in culture using a commercial kit (DNeasy® Blood and Tissue Kit, Qiagen). Isolates were genotyped using 10 PCR-RFLP markers (SAG1, SAG2 (5′ and 3', and alt. SAG2), SAG3, BTUB, GRA6, c22-8, c29–2, L358, PK1 and Apico) as previously described (Su, et al., 2010) and using 15 microsatellite markers, as previously described (Ajzenberg, et al., 2010; Joeres et al., 2023), with minor modifications. For markers M102, AA, and N60, the fluorophore Atto550 was used instead of NED to label amplicons during the multiplex PCR, and MS typing results were numerically adjusted following guidelines recently described (Joeres, et al., 2023). PCR-RFLP typing profiles were determined using the banding pattern of reference strains RH (Type I), Me49 (Type II) and NED (Type III), and each PCR and fragment analysis run for microsatellite typing included reference strains RH (Type I), Me49 (Type II) and NED (Type III).

3. Results

Out of 23 venison products purchased, 5 (21.7 %) were positive for *T. gondii* by qPCR. Of the 5 qPCR-positive samples, 2 (40 %) gave rise to infection in mice, indicating the presence of viable *T. gondii* in meat samples available for human consumption (Table 1). All mice inoculated with venison homogenate in the bioassay, including the mice which subsequently tested positive for *T. gondii*, remained asymptomatic throughout the experiment. Isolates from both positive venison samples were cultured from the lungs of infected mice. Genotyping of tachyzoites in culture by PCR-RFLP revealed both isolates to be ToxoDB genotype #3 (Type II-variant), and microsatellite analysis also revealed them to be Type II, although with slightly different individual profiles (Table 2). Meat juice could only be collected from 13 of the 23 purchased products (despite numerous freeze-thaw attempts), and of these 2 (15.4 %) were positive for *T. gondii* antibodies by ELISA (Table 1). Of the 11 venison products purchased at farm shops, 2 were qPCR- and ELISA-positive, and of the 12 products purchased at supermarkets, 3 were qPCR-positive (meat juice was not available for ELISA). The two products harbouring viable *T. gondii* were from a farm shop.

Table 1
Isolation of viable *T. gondii* from venison.

Venison sample[a]	Product type	Species of deer/rearing condition	Meat Juice ELISA	qPCR	Average qPCR Ct	Mouse Bioassay[c]	Isolate of *T. gondii* cultured	ToxoDB PCR-RFLP genotype
1B	Diced meat	Red/Wild	POS	POS	34.60	2/6	Yes (TgRDUK1)	#3 (Type II-variant)
3A	Diced meat	Red/Wild	POS	POS	32.07	1/6	Yes (TgRDUK2)	#3 (Type II-variant)
13A	Ground meat	Red/Unknown	ND[b]	POS	36.59	0/6	No	
23A	Ground meat	Red/Unknown	ND	POS	37.42	0/6	No	
24A	Ground meat	Red/Unknown	ND	POS	36.31	0/6	No	

[a] Two 50 g portions (A + B) per sample were processed.
[b] ND = Not Done - meat juice could not be collected, despite numerous freeze-thaw attempts.
[c] Number of mice positive (by PCR and/or ELISA)/Number of mice inoculated.

Table 2
Microsatellite (MS) genotyping results for two *T. gondii* strains isolated from retail venison (from red deer), and three reference strains (RH, Me49 and NED).

Isolate	PCR-RFLP Genotype	Microsatellite markers (size in bp)														
		TUB2	W35	TgM-A	B18	B17	M33	IV.1	XI.1	M48	M102	N60	N82	AA	N61	N83
RH	Type I	291	248	209	160	342	169	274	358	209	166	145	119	265	87	306
Me49	Type II	289	242	207	158	336	169	274	356	215	174	142	111	265	91	310
NED	Type III	289	242	205	160	336	165	278	356	209	190	147	111	269	91	312
TgRDUK1	Type II-v	289	242	207	158	336	169	274	356	227	178	140	111	297	85	310
TgRDUK2	Type II-v	289	242	207	158	336	169	274	356	229	174	142	111	263	97	314

4. Discussion

In this study, viable *T. gondii*, capable of causing infection, was isolated from two separate venison products purchased at a retail outlet for human consumption. Given that venison is quite often eaten undercooked, this represents a potential public health risk. Viable *T. gondii* has been isolated from deer tissues previously (Dubey, et al., 2021); however, to our knowledge this is the first study assessing parasite viability in retail meat products. The consumption of raw or undercooked deer meat, or the handling/processing of deer carcases, has previously been linked to cases of symptomatic toxoplasmosis, including ocular toxoplasmosis, in the USA and Canada, with some patients requiring hospitalisation (Conrady, et al., 2022; England et al., 2019; Gaulin et al., 2020; Kohler et al., 2023; McDonald et al., 1990; Ross et al., 2001; Sacks et al., 1983; Schumacher et al., 2021; Vaphiades et al., 2023). It is of note that out of the 43 individual cases documented in the literature, all except one (England, et al., 2019) were reported in immune competent individuals. This further highlights the public health relevance of the results in this study. Furthermore, market research has shown that 30 % of venison in the UK is consumed by people aged 65 years and older (56 Degree Insight, 2020), which represents an age group potentially at risk of immune compromising disorders, thus increasing the risk of developing a more serious *T. gondii* infection.

Of the five *T. gondii* qPCR-positive samples bioassayed in mice, only the diced venison products gave rise to infection. The qPCR Ct values for the ground meat samples were higher than those for the diced meat indicating a lower level of *T. gondii* DNA which may explain this. It may also be explained by the presence of salt (1.5 %) and other additives in these ground meat products, as previous research has demonstrated that low salt exposure during the preparation of some meat products, such as dry-cured hams and sausages, can inactivate *T. gondii* tissue cysts (Fredericks, et al., 2019; Fredericks et al., 2020; Hill et al., 2018). The failure of some qPCR positive meat samples to lead to infection in mice may also be explained by the strain of mouse utilised in this study. Outbred CD-1 mice were chosen as a standard strain used in viability experiments (Gracia, et al., 2022; Kniel et al., 2002); however, given the low levels of parasite DNA present in the samples, as indicated by the high qPCR Ct values, the use of IFN-γ-knockout mice, which are more susceptible to infection, may have resulted in a higher number of mice becoming infected (Opsteegh, et al., 2020; Stollberg et al., 2021). The strain of mouse used to determine viability of parasites in retail meat, as well as alternative methods of detection (Opsteegh, et al., 2020), will be considered carefully in future research.

Genotyping results in this study revealed that both *T. gondii* isolates generated from venison products belonged to *T. gondii* clonal lineage Type II, which is the most predominant genotype in Europe (Fernández-Escobar, et al., 2022), and most commonly associated with clinical cases of toxoplasmosis in this region (Hosseini, et al., 2018). The positive venison products were purchased on the same day from the same retail establishment, so it is likely that they originated from the same animal. However, the microsatellite profile of each isolate was not identical which may suggest that the venison originated from two different animals, or that the same animal was harbouring tissue cysts from two different Type II *T. gondii* strains. Whether the isolates originated from the same animal or not, they still represent two separate products, containing viable parasites, available for consumption potentially by multiple people.

Seroprevalence of *T. gondii* in deer varies geographically. A recent study estimated the pooled seroprevalence in wild red deer and roe deer in Europe to be 15 % and 29 %, respectively, with a higher seroprevalence reported in roe deer in Western Europe (Fanelli, et al., 2021). Unfortunately, seroprevalence data for the UK is lacking, with only one historical study in farmed red deer in Scotland reporting 32.5 % (Williamson, et al., 1980). In the present study, meat juice was only available for testing from 13 of the 23 venison products, 11 of which were from wild red deer. Of the 13 samples tested by ELISA, 2 (15.4 %) were positive for *T. gondii* antibodies. Although this result does not directly represent the seroprevalence in deer, it is comparable to the estimated European seroprevalence for wild red deer detailed above. Few studies compare seroprevalence in wild vs farmed deer, but one could hypothesise that farmed deer may have a higher risk of exposure due to the potential closer proximity to farms and domestic cats. The European Food Safety Authority considers *T. gondii* to be a high priority in farmed deer and recommends that prevalence in farmed deer should be investigated using a baseline study so that risk management options (such as cooking and freezing – see below) can be assessed (EFSA, 2013). As deer are herbivores and therefore likely to become infected from ingesting oocysts from the environment, a prevalence survey would also provide

information on environmental contamination, and if genotyping was incorporated it could also provide information on genetic diversity. Such epidemiological information would fill an existing knowledge gap and would be pertinent given the planned expansion of the venison sector in Scotland (Scotland Food and Drink, 2018).

In the last five years, an average of 245 clinical cases of toxoplasmosis were diagnosed annually in England and Wales (UK Health Security Agency, 2022), and an average of 35 clinical cases were diagnosed annually in Scotland (Public Health Scotland, 2023). Currently, the role of foodborne transmission in *T. gondii* infections in the UK is unknown, although the consumption of undercooked meat has been reported as a significant risk factor in England and Wales (Said, et al., 2017). Venison is likely to be viewed as an expensive meat choice for many consumers, or only eaten in "fine dining" restaurants, and therefore not likely consumed as regularly as chicken, beef, or pork, meaning exposure to *T. gondii* could be low. However, an epidemiological study to identify risk factors would need to be conducted to establish this. In a previous study in England and Wales, consumption of game meat was not included in the questionnaire (Said, et al., 2017).

As tissue cysts of *T. gondii* cannot be detected at slaughter, the main control option to reduce foodborne transmission (from any food animal, not only deer) is the thorough cooking or freezing of meat before consumption. Current guidelines vary but EFSA recommends cooking all meat to an internal temperature of 67 °C before consumption (EFSA, 2007), and the UK Food Standards Agency recommends cooking "all red meats until no pinkness remains and the juices run clear" (ACMSF, 2012). Others recommend that wild game meat be cooked to a minimum internal temperature of 71.1 °C (Jones & Dubey, 2012). Freezing meat at −12 °C for at least 2 days has been shown to kill *T. gondii* tissue cysts (Kotula, et al., 1991), although others recommend 3 days at −20 °C (Kijlstra & Jongert, 2008). A combination of freezing and thorough cooking likely presents the best option for reducing transmission (Kuruca, et al., 2023). An alternative control method which could be explored in farmed animals is the use of vaccination to reduce tissue cysts in meat (Sander, et al., 2020). Toxovax®, which is licenced for use in sheep to prevent abortion, has been shown to reduce the burden of tissue cysts in experimentally infected pigs and lambs (Burrells, et al., 2015; Katzer et al., 2014). At present, farmers are unlikely to vaccinate their animals solely for food safety; however, *T. gondii* infection has been associated with abortion in farmed red deer in New Zealand (Patel, et al., 2019), so vaccination could be explored as a "win win" option for deer farmers – improving fertility and also making the meat safer. This would require further investigation but may be relevant given the planned expansion of the farmed deer sector in Scotland.

In conclusion, this study has demonstrated the presence of viable *T. gondii* in venison for human consumption highlighting a potential public health problem, and certainly something deer hunters/game keepers or anyone working with deer carcases should be aware of. Although this study focused on venison, due to the previously reported higher incidence of *T. gondii* in these products (Plaza, et al., 2020), the determination of parasite viability in all meat products is recommended to better understand the role of retail meat in foodborne toxoplasmosis.

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CRediT authorship contribution statement

J. Thomson: Conceptualization, Investigation, Methodology, Writing – review & editing. **M. Joeres:** Investigation, Methodology, Writing – review & editing. **G. Schares:** Writing – review & editing. **E.A. Innes:** Writing – review & editing. **C.M. Hamilton:** Conceptualization, Funding acquisition, Investigation, Methodology, Writing – original draft.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

No data was used for the research described in the article.

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