Parasitology Research (2019) 118:2065–2077
https://doi.org/10.1007/s00436-019-06355-1

PROTOZOOLOGY - ORIGINAL PAPER

Investigation of *Toxoplasma gondii* and association with early pregnancy and abortion rates in New Zealand farmed red deer (*Cervus elaphus*)

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Received: 30 November 2018 / Accepted: 15 May 2019 / Published online: 11 June 2019

Abstract

This study tested for association between *Toxoplasma gondii* and pregnancy and abortion to investigate sub-optimal reproduction in farmed red deer (*Cervus elaphus*). Sera from a sub-sample ($n = 2304$) of pregnant and non-pregnant hinds in early gestation at first pregnancy scan (scan 1) and approximately at the end of second trimester at second pregnancy scan (scan 2) were tested for *T. gondii* antibodies using a validated ELISA. Foetuses and/or uteri from pregnant, non-pregnant, and aborting hinds at scan 1, scan 2, or weaning were tested for *T. gondii* DNA by nested PCR. At scan 1, 31.1% of 861 rising two-year-old (R2) and 28.3% of 357 mixed-aged (MA, ≥ 2 years) hinds were sero-positive. There was no association between scan 1 serology and non-pregnancy at animal (R2, $p = 0.05$ and MA, $p = 0.43$) or herd level (R2, $p = 0.37$). *Toxoplasma gondii* DNA was detected in 3/18 placenta and 4/18 foetal brains from aborting R2 hinds and 15/157 R2 and 3/21 MA uteri from non-pregnant hinds at scan 1. At scan 2, sero-prevalence was higher (odds ratio = 1.6, 95% CI = 1.04–2.48) in aborted (34.3% of 268) than in non-aborted (23.5% of 446) R2 hinds ($p = 0.03$) and 7.9% of abortions between scans were attributable to *T. gondii* exposure. Within-herd sero-prevalence at scan 2 was positively associated with daily abortion rate in R2 herds with aborted hinds ($p < 0.001$) but not in MA herds ($p = 0.07$). *Toxoplasma gondii* DNA was detected in 27/169 uteri, 2/20 cotyledons, and 1/5 foetal brains from aborted hinds at scan 2 and in uteri from 5/33 hinds not rearing a calf to weaning. *Toxoplasma gondii* RFLP genotyping of five loci revealed a unique type I/III genotype pattern, TgRDNZ1, in a foetal brain sample, not been previously reported in deer. These findings provide serological and molecular evidence that *T. gondii* infection is associated with abortion in red deer, possibly in all three trimesters.

Keywords Red deer · *Cervus elaphus* · Ultrasound scanning · Pregnancy · Abortion · *Toxoplasma gondii* · PCR

Abbreviations

DAR	daily abortion rate
DSP	deer slaughter premises
ELISA	enzyme-linked immunosorbent assay
LAT	latex agglutination tests
MA	mixed age (≥ 2 years old)
PCR	polymerase chain reaction
PAF	population attributable fraction
R2	rising 2-year-old (15–24 months)
RFLP	restriction fragment length polymorphism

Introduction

Reproductive performance in rising two-year-old (R2) and mixed-aged (MA, ≥ 2 years old) adult hinds is below optimum in New Zealand farmed red deer (Asher 2003; Asher and

Section Editor: David S. Lindsay

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Pearse 2002; Asher and Wilson 2011). Reproductive efficiency (calves weaned/hinds mated) averaged 75.2% in the last 13 years (Statistics New Zealand 2016) arising from failure to conceive, foetal loss, stillbirth and postnatal losses. A recent study of reproductive efficiency in 85 red deer herds demonstrated that early gestation pregnancy rates of R2 and MA hinds were 85.8 and 93.3%, respectively (Patel et al. 2018).

Causes and risk factors for conception failure and losses to weaning in both R2 and MA hinds have been studied previously (Asher et al. 2005a; Asher et al. 2011; Asher and Cox 2013; Asher et al. 2005b; Audigé et al. 1999b; Audigé et al. 1999c). Abortions in New Zealand red deer have been reported in a few studies (Audigé et al. 1999a; Campbell et al. 2000; Fennessy et al. 1986), with generally low rates, but up to 16% in one herd (Wilson et al. 2012). However, the most substantial data available is presented in a recent study in which the mid-term herd-level mean daily abortion rates were 0.043% and 0.025% in R2 and MA herds, respectively, which equates to an abortion incidence of 3.9% in R2 and 2.2% in MA herds for 90 mid-term gestation days (Patel et al. 2018). Hence abortions can pose a significant economic impact on the red deer farmers and the red deer industry in New Zealand in terms of lost opportunities and lower financial returns.

There are many confirmed and potential infectious and non-infectious causes of abortion in deer. Risk factors for non-infectious causes, as in other species such as cattle and sheep, can include farm management, nutrition, health, and environmental conditions (Bartels et al. 1999; Fthenakis et al. 2012). Pathogens, such as *Toxoplasma gondii*, *Neospora caninum*, *Leptospira* spp., herpes virus and bovine viral diarrhoea virus (BVDv), known for causing abortions in cattle, sheep and other small ruminants have been reported in deer (das Neves et al. 2009; Dubey et al. 2014; Dubey et al. 2013a; Passler et al. 2016; Subharat et al. 2010). *Toxoplasma gondii* has been shown to be a significant cause of abortion in sheep populations in New Zealand (Hartley et al. 1954). In New Zealand farmed red deer, *T. gondii* DNA was detected from 8/9 foetal brains recovered from aborting R2 hinds in a clinical investigation in a herd experiencing a 1% early and 7.2% mid-term abortion rate, with serological evidence of *T. gondii* exposure (Wilson et al. 2012). *Toxoplasma gondii* DNA was detected in non-pregnant uteri, and antibody was detected in blood from non-pregnant, pregnant and aborting hinds from two other R2 herds affected with abortions in the same report. There are reports elsewhere of trans-placental transmission and antibodies for *T. gondii* in deer (Dubey et al. 2014; Dubey et al. 2008). However, an association between *T. gondii* and abortion in farmed deer in New Zealand has not been thoroughly investigated.

Thus, the aims of this study were to test for association between *T. gondii* sero-status and pregnancy outcome in red deer hinds at scanning early in gestation, and mid-term abortion, as determined by repeat pregnancy scanning, and to examine tissue samples from non-pregnant, aborted and aborting hinds and aborting foetuses for *T. gondii* DNA. This study used data and samples form a broader parent study of pregnancy and abortion in farmed red deer in New Zealand (Patel et al. 2018).

Materials and methods

Farms, animals and pregnancy determination

Full detail of farm, farmer consent and animal recruitment is presented in the study by Patel et al. (2018). Briefly, commercial deer farmers throughout New Zealand were invited to take part in the study through deer and mixed practice veterinarians, commercial pregnancy diagnosis scanners or direct contact by the researchers. Based on initial responses, farms were selected considering suitable handling facilities, willingness to participate, fulfilment of the requirements of the project for additional pregnancy scanning and blood, specimen and data collection. This study involved red deer (*Cervus elaphus* subsp. *scoticus* and *hippelaphus*) genotypes from both R2 and MA age groups although it is acknowledged that some deer may have possessed some wapiti (*Cervus elaphus nelson*, *roosevelti*, *manitobensis*) genes. Briefly, 85 New Zealand red deer farms were recruited over two years with 56 farms comprising 49 R2 and 40 MA herds in year 1 and 29 farms comprising 23 R2 and 24 MA herds in year 2. Twenty-eight farms were located in the North Island and 57 in the South Island. Average herd size was 254 (SEM 33) for R2 herds (median 135 (min 15, max 1670)) and 510 (SEM 60) for MA herds (median 303 (min 60, max 2998)). In total, 22,130 R2 and 36,223 MA red deer hinds from 87 and 71 herds, respectively, were included. Hinds were ultrasound scanned for pregnancy early in gestation after a mean interval of 49 days from stag removal (scan 1), and a sub-sample (up to 100 R2 and 200 MA hinds per herd) was scanned again approximately at the end of the second trimester (scan 2) after a mean between-scan interval of 89 days.

Hinds were classified as "pregnant" based on the presence of at least one foetus or part thereof, amniotic membrane, and/or presence of placentomes, or as being "non-pregnant" based on absence of those signs combined with visualisation of a non-pregnant uterus at scan 1. The term "aborting" is ascribed to hinds that had ultrasound evidence of aborting foetuses at scan 1 and scan 2. The term "aborted", used for calculating the daily abortion rate, is ascribed to hinds that were pregnant at scan 1 but not pregnant at scan 2, plus those aborting at scan 2. Daily abortion rate (DAR) ((number aborted at scan 2/number scanned at scan 2)/number of days between scan 1 and scan 2) was calculated to account for differences in interval between scans. This approximated a mid-trimester abortion rate.

Sample collection

All animal manipulations were approved by the Massey University Animal Ethics committee (Protocol number: 12/34) in accordance with the guidelines for ethical conduct available at www.massey.ac.nz/massey/research/research-ethics/animal-ethics/code-ethical-conduct.cfm.

According to a power analysis, with an absolute precision of 5%, 21 hinds each from aborted and non-aborted group at scan 2 or pregnant and non-pregnant hinds at scan 1 were required to detect abortion, assuming a sero-prevalence of 40% (Wilson et al. 2012), with 95% confidence (Cannon and Roe 1982). Serum samples were collected from 21 (or all hinds when the herd size was less) randomly selected pregnant, non-pregnant and all available aborting hinds per herd at scan 1 from late May to mid-August and from up to 21 aborted hinds as available and 21 non-aborted hinds per herd in mid-September to mid-October at scan 2. While this sampling design was cross-sectional, opportunistically, 235 R2 and 131 MA hinds were sampled at both scan 1 and scan 2. Samples were collected by jugular veni-puncture in the Committee's Code of Ethical Conduct and transported chilled to Massey University, and the serum was withdrawn and stored at − 20 °C until testing. In total, 4835 (R2 = 2786 and MA = 2049) and 2932 (R2 = 1780 and MA = 1152) blood samples were collected at scan 1 and scan 2, respectively, to provide the sample pool for selection for serological analyses. The sero-prevalence reported by Wilson et al. (2012) using latex agglutination test (LAT) to detect *T. gondii* antibodies in red deer was used for sample size calculations. However, when evaluated against Western blot, the sensitivity and specificity of LAT were much lower and the true sero-prevalence was lower than the apparent sero-prevalence (Patel et al. 2017). Therefore, the number of hinds required for serological analysis per group (pregnant and non-pregnant hinds at scan 1 and aborted and non-aborted at scan 2) was reduced to 10/group from 21/group based on a sero-prevalence estimate of 33%, as reported by Wilson et al. (2012), with an absolute precision of 5 and 95% confidence.

Non-pregnant and aborting hinds at scan 1, aborted hinds at scan 2, pregnant hinds at scan 1 and hinds not rearing a live calf at weaning, as available, were tracked to deer slaughter premises (DSP) where whole reproductive tracts from the posterior cervix were collected by the senior author or the DSP veterinarian. The authors had no role in killing or live handling of any animals at DSP. At scan 1, uteri from 157 R2 and 21 MA non-pregnant hinds were collected after scan 1. In total, twenty-nine R2 hinds from six herds and three MA hinds from three herds aborting at scan 1 were tracked to a DSP. However, only 23 foetuses were recovered from 35 uteri as ten R2 and two MA hinds had expelled the foetuses between ultrasound pregnancy scan diagnosis on-farm and sample collection at a DSP. Additionally, 19 uteri containing a foetus, from nine R2 and 11 MA cull pregnant hinds from four herds and one uterus containing foetal remnants from one R2 hind, were collected at a DSP. Diaphragm ($n = 40$) and foetal brain ($n = 42$) were taken from collected foetuses. Diaphragm samples were not collected from foetuses recovered from one aborting R2 hind and one pregnant MA hind due to small foetus size (crown rump length < 26 mm). At scan 2, uteri from 138 R2 and 31 MA hinds having aborted were recovered from DSP. Aborting foetuses were recovered from uteri of 11 R2 and five MA aborting hinds. Additionally, farmers from two R2 herds sent a total of four aborted foetuses from R2 hinds recovered directly from paddocks stocked with pregnant hinds. Reproductive tracts from 10 R2 and 23 MA hinds that did not rear a calf to weaning were also collected at DSPs.

Samples were immediately kept in a chiller/freezer and then sent chilled or frozen to Massey University (Palmerston North, New Zealand) where they were processed at the post-mortem facility within 24 to 48 h of arrival. Gross observations from uteri and foetal tissues were recorded at dissection. Uteri, placental, cotyledon and foetal samples were dissected and stored at − 20 °C (fresh specimens) for PCR.

Sample selection for serology

Sample selection for scan 1 serology was determined after the scan 2 serology results were available, from herds categorised as nil, low (R2 > 0–0.03%, MA > 0–0.02%), medium (R2 0.031–0.06%, MA 0.021–0.035%), and high (R2 > 0.06%, MA > 0.035%) DAR groups as previously described by Patel et al. (2018). Twenty-three sera from hinds in 9 R2 and 1 MA herds were tested at the farmer's request after scan 1 and were included in scan 1 serology analysis. Sera from 27 R2 and 16 MA aborting hinds at scan 1 and the equivalent number of sera from pregnant hinds in the same herd were tested for antibodies to *T. gondii*.

Sera from five pregnant and non-pregnant hinds each from 15 R2 herds were tested in year 2. After analysing the results from year 1 scan 1 serology, only sera from those aborted and the equivalent number from non-aborted MA hinds were selected for year 2 serology. Therefore, in total, sera from 861/2786 and 357/2049 sampled hinds at scan 1 were tested from 46 R2 and 18 MA herds.

Sera from aborted hinds at scan 2 from all herds with aborted hinds were tested. Sera from non-aborted hinds (10 per herd) were selected from herds in nil (R2 = 6, MA = 6), low (R2 = 11, MA = 7), medium (R2 = 6, MA = 4) and high (R2 = 7, MA = 5) DAR categories. Therefore, in total, sera from 714/1780 and 372/1152 sampled hinds were tested from 63 R2 and 39 MA herds.

ELISA

An immunoglobulin G (IgG)-based commercial indirect *T. gondii* ELISA test for small ruminants ("Chekit-Toxotest" IDEXX laboratories, Switzerland) was performed as per manufacturer's recommendations, after validating it for use in red deer using Bayesian latent class analyses (Patel et al. 2017). Sera with a S/P(%) of ≥ 15.5 were considered positive, and the term "serological status" refers to positive or negative at that cut-point.

PCR

DNA was extracted from myometrium with attached caruncles, or cotyledon, foetal brain and foetal diaphragm tissue samples, using the DNeasy Tissue Kit (Qiagen, Victoria, Australia) as per the manufacturer's instructions for fresh or frozen tissue samples. Water blanks were included as sample processing controls, after every 8 samples, to confirm the lack of contamination during sample DNA extraction process.

Toxoplasma gondii DNA was detected using a nested PCR protocol for the amplification of the *Pppk-dhps* gene using the primers as described Aspinall et al. (2002) and protocol as described by Roe et al. (2013). A known *T. gondii* isolate (incomplete strain S48, Toxovax®, MSD Animal Health, New Zealand), confirmed by sequencing, was used as a positive control, and water blanks were included as negative controls.

To determine successful amplification of both PCR protocols, 1.5 μl of PCR products were run on 1.5% agarose gel (UltraPure Agarose, Invitrogen, Carlsbad, California, USA) containing ethidium bromide (Invitrogen). All *Toxoplasma* spp.positive amplicons were purified using PureLink PCR purification kit (Invitrogen) and subjected to automatic dye-terminator cycle sequencing with BigDye™ Terminator Version 3.1 Ready Reaction Cycle Sequencing kit and the ABI3730 Genetic Analyser (Applied Biosystems Inc., Foster City, California, USA) using the nested forward and reverse primers for confirmation of the genomic sequence. The sequenced products were aligned using the Geneious Pro 4.8.5 (Biomatters Ltd., Auckland, New Zealand) software and submitted to the National Centre of Biotechnology Information (NCBI) blast nucleotide database for confirmation of correct amplification and species identification.

Where sufficient PCR amplification was produced, *T. gondii* PCR-positive samples were used for genetic typing of seven markers (SAG1, SAG2 (5′ + 3′), SAG3, GRA6, PK1, L358, and Apico) using multilocus PCR restriction fragment length polymorphism (RFLP) analysis using known type I and atypical type II isolates as controls as previously described (Roe et al. 2013; Su et al. 2010).

Statistical analysis

The results from serology in R2 and MA herds were analysed separately. Analyses were performed using SAS software, version 9.4 (SAS Institute Inc., Cary, NC, USA). For analysis purposes, hinds aborting at scan 1 were considered as "non-pregnant", hinds pregnant at scan 1 and scanned not pregnant at scan 2 were considered "aborted since scan 1" and hinds aborting at scan 2 were considered as aborted and therefore included in the DAR calculation and analysis.

At individual animal level, the dependent variable non-pregnancy (non-pregnant (1/pregnant (0)) at scan 1 and aborted (aborted (1)/non-aborted (0)) at scan 2 were binary responses and resembled a binomial distribution. A logistic regression model with "herd" as a random effect was used to model association between non-pregnancy at scan 1 and sero-status (positive (1) or negative (0)) at scan 1. A similar model was used to test association between aborted status at scan 2 and sero-status at scan 1 and scan 2 at individual animal level. A similar logistic regression model with *T. gondii* sero-status (negative (0) or positive (1)) as a binary outcome was used to test for difference in animal level sero-prevalence between age groups, island (north and south) and years (years 1 and 2) at both scans. Odds ratios (OR) with 95% confidence interval and p value from the Chi-squared test were reported for animal level association between non-pregnancy and sero-status at scan 1 and aborted and sero-status at scan 2. A p value < 0.05 was considered to be statistically significant.

The association between presence/absence of haemorrhages (binary variable) in uterus and PCR positivity for *T. gondii* DNA was assessed using logistic regression model. "Herd" was used as a random effect in the model. The odds ratio and p value by chi-squared test are reported.

At the herd level, analysis included those with ≥ 10 sera tested per herd for estimation of within-herd sero-prevalence. The dependent variable at scan 1 was non-pregnancy rate (%) (number not pregnant at scan 1/number scanned at scan 1) whereas DAR was the dependent variable at scan 2. The association between within-herd non-pregnancy rate at scan 1 and within-herd sero-prevalence at scan 1 was assessed using a logistic model. A similar model was used to assess the association between within-herd DAR and within-herd sero-prevalence.

The population attributable fraction (PAF) was calculated as the proportion of aborted hinds in the population tested for *T. gondii* antibodies that was attributable to *T. gondii* exposure (Eq. (1)). The confidence intervals

for PAF were estimated in SAS using the formula provided by Greenland and Rothman (2008).

$$PAF = \frac{P–Po}{Po} \quad (1)$$

where,

PAF is population attributable fraction;
P is proportion of aborted hinds in the population tested for *T. gondii* antibodies;
P_o is proportion of aborted hinds in sero-negative group.

Results

Serology association with non-pregnancy at scan 1

Data for sero-prevalence in hinds form herds with and without aborted R2 and MA hinds are presented in Table 1. The sero-prevalence in R2 and MA hinds at scan 1 was 31.1 and 28.3%, respectively, and 29.0% combined. The animal level sero-prevalence at scan 1 did not differ between R2 and MA hinds ($p = 0.80$), between North and South Islands ($p = 0.82$) or sampling years (year 1 vs year 2) ($p = 0.15$). Within age-groups, the animal-level sero-prevalence was not different between islands ($p = 0.66$ in R2 and $p = 0.08$ in MA) or sampling years ($p = 0.08$ in R2 and $p = 0.38$ in MA). The animal level sero-status at scan 1 was not associated with non-pregnancy status at scan 1 in MA hinds (analysed only in year 1, $p = 0.43$) whereas the association was marginally non-significant in R2 hinds (OR = 1.35, $p = 0.05$) (Table 2). Nine of 27 R2 and six of 16 MA hinds identified as aborting at scan 1 were sero-positive.

Table 2 Odds ratios for association between individual hind-level sero-positivity to *Toxoplasma gondii* and non-pregnancy at first pregnancy scan (scan 1) and having aborted by second pregnancy scan (scan 2) and p value determined by logistic regression models

Association, age group	Odds ratio (95% CI)	Chi-squared p value
T. gondii sero-status at scan 1 and non-pregnancy at scan 1		
Rising two-year-old	1.35 (0.99–1.81)	0.05
Mixed age	1.25 (0.72–2.19)	0.43
T. gondii sero-status at scan 2 and aborted by scan 2		
Rising two-year-old R2	1.60 (1.04–2.48)	0.03
Mixed age	0.65 (0.32–1.28)	0.21

At herd level, data from 33 R2 ($n = 821$ hinds) and 10 MA herds ($n = 330$ hinds) were available for analyses at scan 1 (Table 3). No sero-positive hinds were observed in one R2 herd. The mean within-herd sero-prevalence was 32.9% in R2 herds and 25.5% in MA herds. The within-herd scan 1 sero-prevalence was not associated with the non-pregnancy rate in R2 herds ($p = 0.37$) (Table 4). The within-herd mean scan 1 sero-prevalence was not different between sampling years ($p = 0.90$), islands ($p = 0.20$) or between herds with and without aborted hinds as detected at scan 2 ($p = 0.22$).

Serology association with hinds having aborted by scan 2

Sero-prevalence in R2 and MA hinds at scan 2 was 27.6 and 31.7%, respectively, and 30.3% combined (Table 1). The R2

Table 1 Sero-prevalence of *Toxoplasma gondii* in pregnant and non-pregnant hinds at first pregnancy scan (scan 1) and in aborted and non-aborted hinds approximately 90 days later at second pregnancy scan (scan 2) in rising two-year-old (R2) and mixed-age (MA) herds

Scan, age group	Herds with or without aborted hinds at scan 2	No. of herds	% Sero-positive (no. tested)[a] hinds		
			Non-pregnant	Pregnant	All
Scan 1					
R2	With	36	32.7 (294)	26.9 (379)	29.4 (673)
	Without	10	43.0 (79)	33.0 (109)	37.2 (188)
	Overall	46	34.9 (373)	28.3 (488)	31.1 (861)
MA	With	11	35.3 (85)	28.3 (191)	30.4 (276)
	Without	7	26.7 (30)	17.6 (51)	21.0 (81)
	Overall	18	33.0 (115)	26.0 (242)	28.3 (357)
Scan 2					
R2	With	57	34.3 (268)	24.6 (362)	28.7 (630)
	Without	6	na[b]	19.0 (84)	19.0 (84)
	Overall	63	34.3 (268)	23.5 (446)	27.6 (714)
MA	With	32	32.9 (82)	30.3 (225)	33.6 (307)
	Without	7	na[b]	23.1 (65)	23.1 (65)
	Overall	39	32.9 (82)	31.4 (290)	31.7 (372)

[a] Those non-pregnant at scan 2 are those which had aborted since scan 1

[b] na, not applicable

Table 3 Mean, standard error (SE) and range of within-herd *Toxoplasma gondii* sero-prevalence at first pregnancy scan (scan 1) and approximately 90 days later at second pregnancy scan (scan 2) in rising two-year-old (R2) and mixed-age (MA) herds with and without aborted hinds as detected at scan 2

Scan, age group	Herds with or without aborted hinds at scan 2	Herds	Within-herd sero-prevalence (%)		
			Mean	SE	Range
Scan 1					
R2	With	25	30.4	4.4	2.4–80.0
	Without	7	41.9	10.0	11.8–80.0
	Overall	32	32.9	4.1	2.4–80.0
MA	With	8	28.1	6.9	5.6–69.0
	Without	2	15.0	5.0	10.0–20.0
	Overall	10	25.5	5.8	5.6–69.0
Scan 2					
R2	With	21	31.0	4.2	4.5–85.7
	Without	5	25.5	7.2	5.0–40.0
	Overall	26	30.0	3.7	4.5–85.7
MA	With	14	36.5	5.4	7.7–66.7
	Without	5	24.0	5.1	10.0–40.0
	Overall	19	33.2	4.3	7.7–66.7

hinds were less likely to be sero-positive than MA hinds (OR = 0.65, 95% CI = 0.40–0.99, $p = 0.04$) in herds with aborted hinds whereas no difference in sero-prevalence was observed among R2 and MA hinds in herds without aborted hinds ($p = 0.66$). Sero-prevalence at animal level was not different between age groups ($p = 0.13$), sampling years ($p = 0.48$) or islands ($p = 0.86$). Within age-groups, animal level sero-prevalence did not differ between sampling years ($p = 0.22$ in R2 and $p = 0.25$) or islands ($p = 0.89$ in R2 and $p = 0.69$ in MA).

In herds with aborted hinds, the animal level sero-prevalence at scan 2 in aborted hinds was significantly higher than in non-aborted hinds in R2 herds ($p = 0.03$) but not in MA herds ($p = 0.21$) (Table 2). In R2 herds with aborted hinds, sero-positive hinds at scan 2 were 1.6 times (95% CI = 1.04–2.48) as likely to have aborted than sero-negative hinds (Table 2). At animal level, scan 2 sero-prevalence in hinds from herds with aborted hinds was not different that in herds without aborted hinds in R2 ($p = 0.64$) or MA ($p = 0.44$) age groups.

Herd level analysis was done on 31 R2 ($n = 624$ hinds) and 22 MA ($n = 328$ hinds) herds at scan 2. Overall, 26 (84%) R2 and 19 (86%) MA herds had at least one sero-positive hind (Table 3). The within-herd sero-prevalence at scan 2 was positively associated with DAR in R2 herds ($p < 0.001$) but not in MA herds ($p = 0.07$) (Table 4). The mean within-herd sero-prevalence was not different between herds with and without aborted hinds in R2 ($p = 0.29$) or MA age groups ($p = 0.27$). The within-herd scan 1 sero-prevalence was not associated with DAR at scan 2 ($p = 0.26$) in R2 herds. The mean within-herd sero-prevalence was not different between age groups ($p = 0.84$), sampling years ($p = 0.57$) or islands ($p = 0.80$). Within age-groups, the within-herd mean sero-prevalence did not differ between sampling years ($p = 0.94$ for R2 and $p = 0.28$ for MA) or islands ($p = 0.84$ for R2 and $p = 0.87$ for MA).

In herds with aborted hinds, the PAF for *T. gondii* exposure in R2 hinds tested for sero-status was estimated at 7.9% (95% CI 1.9–13.5). This suggests that on average 7.9% of abortions could be reduced by preventing the *T. gondii* exposure or its effects in R2 hinds.

Data for individual hinds (235 R2, 131 MA) sampled at both scan 1 and scan 2 are presented in Table 5. Overall, sera from 32.8% R2 and 31% of MA hinds were positive. There was no association between sero-status at scan 1 and the aborted status at scan 2 in R2 ($p = 0.21$) or MA ($p = 0.68$) hinds. Paired serology data was available from 26 R2 and six MA aborted hinds

Table 4 Beta coefficient estimate and p value for association between within-herd *T. gondii* sero-prevalence in rising two-year-old and/or mixed-age (MA) herds and proportion of hinds not pregnant at first pregnancy scan (scan 1) and daily abortion rate between scan 1 and second pregnancy scan (scan 2)

Within-herd sero-prevalence association with	Age group	No. of herds	Beta coefficient estimate (SE)	p value
Proportion not pregnant at scan 1	R2	33	0.59 (0.65)	0.37
Daily abortion rate by scan 2	R2	31	2.34 (0.65)	< 0.001
	MA	22	2.00 (1.11)	0.07

Table 5 Association between *Toxoplasma gondii* sero-status of rising two-year-old (R2) and mixed-age (MA) hinds scanned early in pregnancy at first pregnancy scan (scan 1) and their aborted or non-aborted status determined by repeat scanning approximately 90 days later at second pregnancy scan (scan 2)

Age group	No. of hinds	Scan 1 status	Scan 2 status	% Sero-positive at scan 1	Chi-squared *p* value
R2	235	Pregnant	Aborted	8/26 (30.8%)	0.21
		Pregnant	Pregnant	50/209 (23.9%)	
MA	131	Pregnant	Aborted	1/6 (16.7%)	0.68
		Pregnant	Pregnant	30/125 (24.0%)	

from this subset. Of these aborted hinds, sero-conversion was observed in one MA and two R2 hinds. One R2 and one MA sero-positive hind at scan 1 were sero-negative at scan 2. Sixteen R2 and four MA hinds were sero-negative at both scans, and seven R2 hinds were sero-positive at both scans.

PCR and relationship with serology in aborting and pregnant hinds

Data from uteri and foetal tissues collected from aborting hinds at scan 1 and aborted hinds at scan 2 are presented in Table 6.

At scan 1, 11.7% of 230 uteri, 2.8% of 36 cotyledons, 12.8% of 39 foetal brains, 2.8% of 37 foetal diaphragm and 7.7% of 39 placenta samples were positive for *T. gondii* DNA (Table 6). From R2 hinds, *T. gondii* DNA was detected in foetal brains from aborting and pregnant hinds and placenta from aborting hinds and foetal diaphragm samples from one foetus in a pregnant MA hind (Table 6). Both a placenta and uterus sample from one R2 hind were PCR-positive.

Paired serology and tissue PCR data from aborting ($n = 16$), pregnant ($n = 6$) and non-pregnant hinds ($n = 4$) at scan 1 were available from five R2 herds and an aborting hind from one MA herd (Table 6). Of 16 aborting R2 hinds, eight were sero-positive of which two hinds had a sample that was

Table 6 Number positive/number PCR tested for *Toxoplasma gondii* in uteri, cotyledon, placenta and foetal tissue and sero-status early in gestation at first pregnancy scan (scan 1), at second pregnancy scan approximately 90 days later (scan 2) from normal pregnant, non-pregnant, aborting and aborted rising two-year-old (R2) and/or mixed-age (MA) hinds and from hinds that did not rear a calf to weaning

Scan	Age group	Status	Hind sero-status	Uterus	Cotyledon	Foetal brain	Foetal diaphragm	Placenta	Foetal remnants
Scan 1	R2	Pregnant	Positive	1/2	0/2	0/2	0/2	0/2	–
			Negative	1/4	0/4	0/4	0/4	0/4	–
			Not tested	0/3	1/3	1/3	0/3	0/3	–
		Non-pregnant	Positive	0/2	–	–	–	–	–
			Negative	0/2	–	–	–	–	–
			Not tested	15/153	–	–	–	–	–
		Aborting	Positive	1/8	0/4	1/4	0/4	0/4	0/1
			Negative	0/8	0/2	1/2	0/2	0/2	–
			Not tested	4/13	0/12	2/12	0/11	3/12	–
	MA	Pregnant	Not tested	2/11	0/8	0/11	1/10	0/11	–
		Non-pregnant	Not tested	3/21	–	–	–	–	–
		Aborting	Negative	0/1	0/1	0/1	0/1	0/1	–
			Not tested	0/3	0/1	0/1	0/1	0/1	–
Scan 2	R2	Aborted	Positive	5/30	1/1	0/1	0/1	0/1	–
			Negative	10/49	0/4	0/4	0/4	0/4	0/4
			Not tested	7/59	0/5	0/10	1/10	0/6	–
	MA	Aborted	Positive	0/6	–	–	–	–	–
			Negative	4/9	0/1	0/1	0/1	0/1	0/1
			Not tested	1/16	1/4	1/4	0/4	0/4	–
Weaning	R2	No calf at weaning	Not tested	1/10	–	–	–	–	–
	MA		Not tested	4/23	–	–	–	–	–

–, not analysed

T. gondii PCR-positive (one uteri and one foetal brain). Of six pregnant R2 hinds, two were sero-positive of which one was *T. gondii* PCR-positive. The only aborting MA hind was sero-negative and negative on PCR (Table 6).

Overall at scan 2, 16% of 169 uteri, 13.3% of 15 cotyledons, 6.7% of 15 foetal brains and 5% of 20 foetal diaphragm samples were positive for *T. gondii* DNA. Of note, the PCR-positive cotyledon sample was from an aborting R2 hind which was sero-positive.

Paired serology and tissue PCR data from aborted hinds ($n = 94$) at scan 2 were available from 15 R2 and five MA herds (Table 6). Overall, 38.3% of 94 were sero-positive. Of the 79 aborting R2 hinds, 30 (38%) were sero-positive of which 5 (6.3%) were *T. gondii* PCR-positive. Of the 15 MA aborting hinds, six were sero-positive but none of them were *T. gondii* PCR-positive.

Uteri from one R2 and four MA hinds present at weaning without rearing a calf were *T. gondii* PCR-positive.

Toxoplasma gondii genotypes

Foetal brain tissue samples from five PCR-positive hinds were selected for genotyping based on the apparent strength of the *Pppk-dhps* gene nested PCR amplicon under the assumption that the DNA was therefore of suitable quality and quantity to amplify in the genotyping PCR. Due to poor DNA quality and subsequent poor DNA amplification, genotyping was only successful for five of the seven representative markers (SAG1, SAG2 (5′ + 3′), SAG3, GRA6, Apico) in one sample (Table 7), with only SAG2 and SAG3 successful in the remaining four samples. In all five samples, the same type I genotype was observed for the SAG2 and SAG3 loci. Additionally, the TgRDNZ1 isolate showed a type II/III for SAG1, type I for GRA6 and type III for Apico loci, suggesting that a unique type I/III genotype pattern has not been previously reported in isolates collected from deer (Table 7).

Pathology

Histopathology was not performed on foetuses as the majority of samples were sent frozen to Massey University, and the preliminary examination (data not shown) showed that the frozen tissues were not suitable for histology. At scan 1, gross examination of scan 1 samples revealed pinpoint, focal and petechial haemorrhages on caruncles and/or entire uterine horn in uteri from 4.5% of 157 non-pregnant R2 hinds from two R2 herds and 13.8% of 29 aborting hinds in one herd. However, none of the uteri with those signs were positive on PCR for *T. gondii* DNA.

At scan 2, gross examination of scan 2 samples revealed pinpoint, focal and petechial haemorrhages on caruncles and/or entire uterine horns in uteri from 49.6% of 137 R2 and 9.6% of 31 MA aborted hinds from 14 R2 and three MA herds, respectively. Twenty-two percent of 68 R2 and two of three MA uteri with haemorrhages were PCR-positive. However, there was no association between PCR positivity and presence of haemorrhages on the uteri samples combined together from aborted R2 and MA hinds ($p = 0.1$).

Toxoplasma gondii DNA-negative uteri from three R2 hinds contained moderate to severely mummified foetuses which were also PCR-negative for *T. gondii* DNA. Signs of mummification, with presence of dark brownish foetal remnants, were observed in two MA hinds, one of which had a uterine sample that was PCR-positive for *T. gondii* DNA. The *T. gondii* DNA-negative uterus with mummified foetal remnants was from a sero-negative MA hind.

Table 7 Summary of multilocus PCR-RFLP typing for *Toxoplasma gondii* isolates from a New Zealand farmed red deer foetal brain sample and reference strains

Strain/isolate	Genetic markers							
	SAG1	(5′ + 3′) SAG2	SAG3	GRA6	L358	PK1	Apico	Reference
RH88 (type I)	I	I	I	I	I	I	I	Dubey et al. (2011)
PTG (type II)	II or III	II	II	II	II	II	II	Dubey et al. (2011)
CTG (type III)	II or III	III	III	III	III	III	III	Dubey et al. (2011)
Atypical type II	II or III	II	II	II	II	II	I	Dubey et al. (2011)
Type 12 (#4)/TgWTDPa1	II or III	II	II	II	II	II	I	Dubey et al. (2014)
TgWTDUS37 (#221)	u-1	II	II	II	I	II	II	Dubey et al. (2013b)
TgWTDAL	u-1	II	II	II	I	III	I	Yu et al. (2013)
ToxoDB#9/TgSikaJl1	u-1	II	III	II	II	II	I	Cong et al. (2016)
Atypical (#216) TgWTDPa4	I	I	III	I	III	I	III	Dubey et al. (2014)
TgRDNZ1	II or III	I	I	I	NT[a]	NT[a]	III	This study

[a] NT, not able to be typed due to lack of DNA amplification

At weaning, 17.4% of 23 uteri from MA hinds were observed with haemorrhages on caruncles and one of those was positive on PCR for *T. gondii* DNA. Pus-like fluid was observed in the uterus from one R2 and two MA *T. gondii* PCR-negative hinds.

Discussion

This is the first study to extensively examine association between evidence of *T. gondii* and both early pregnancy diagnosis and abortion mid-gestation in red deer. This study was prompted by a recent clinical investigation of abortion early and mid-gestation by Wilson et al. (2012) in farmed red deer, showing up to 16% abortion rate. That study alerted to the possibility that abortion, which hitherto was not regarded as significant in red deer, may indeed be contributing to sub-optimum reproductive rates. The results of the current study showed that there was no substantial association between *T. gondii* serological or PCR data and non-pregnancy rate shortly after mating in both age groups. However, this study did detect association with abortion. *T. gondii* sero-prevalence in mid-term aborted hinds was significantly higher than in non-aborted R2 hinds, and the odds of sero-positive hinds having aborted were significantly higher than sero-negative hinds having aborted. Association between *T. gondii* and abortion was further supported by the detection of *T. gondii* DNA in foetal tissue samples and uteri from aborting and aborted hinds sent to DSP at scan 1, scan 2 and after weaning. This finding is consistent with the observation of PCR-positive results in 8/9 aborting foetuses reported by Wilson et al. (2012). Additionally, we have previously identified that within the sampled population, 3.9% of R2 had mid-term abortions (daily abortion rate of 0.043%) compared to 2.2% of MA hinds (daily abortion rate 0.025%) over 90 mid-term gestation days (Patel et al. 2018). Thus, taken together with the differences in sero-prevalence between age groups suggested here, these findings are consistent with a possible infectious or parasitic agent being involved to which the animal develops an enduring immunity. Moreover, these findings support that *T. gondii* is associated with sub-optimal reproductive performance of NZ farmed red deer.

Investigation of association between non-pregnancy and sero-status at scan 1 was a secondary opportunistic objective of this study. Failure to show a significant association between *T. gondii* serology at herd and individual animal levels, or PCR, and pregnancy at scan 1 suggests that *T. gondii* may not be contributing to sub-optimum pregnancy scan rates. However, the non-significance in R2 hinds was very marginal. This implies that the relationship between non-pregnancy and sero-status at scan 1 in R2 hinds may have been biologically relevant at individual animal level but the significance could not be substantiated by statistical analysis, possibly because of sample size. The presence of *T. gondii* antibodies in blood and DNA in uteri of non-pregnant R2 and MA hinds suggests that *T. gondii* may be playing a role in sub-optimum pregnancy diagnosis rates. Hence, ultrasound pregnancy rates early in gestation cannot be used as a proxy for conception, as commonly used previously (Audigé et al. 1999a,b,c). Presence of DNA in uteri from non-pregnant hinds at scan 1 was consistent with reports in two R2 red deer herds by Wilson et al. (2012). Additionally, presence of DNA in foetal tissues from aborting and normal foetuses and antibodies in aborting R2 hinds at scan 1 indicates possible trans-placental transmission in pregnant hinds after infection early in gestation. Hence, despite failure to statistically confirm *T. gondii* association, data from this study does conclusively suggest that early foetal loss occurs in New Zealand farmed red deer and may be contributing to sub-optimum pregnancy rates on farms, and that *T. gondii* may be a contributory cause. Early abortions in experimental studies in other species such as dairy goats in USA (Dubey 1981) and sheep in New Zealand (Wilkins et al. 1987) have been reported. Further study to quantify early foetal loss, particularly in R2 hinds and its causation, is therefore warranted.

It should be noted that the animal-level sero-prevalence may not be directly comparable with previous studies reported in deer since different serological tests were used. For example, the latex agglutination test (LAT) used in the previous NZ studies was shown to have a lower test sensitivity (Se) and specificity (Sp), evaluated using Bayesian latent class analysis (Patel et al. 2017) than the ELISA used here. The ELISA used in this study had a test Se and Sp of 98.8 and 92.8%, compared with 88.7 and 74.3%, respectively, for the LAT, and, therefore, the sero-prevalence data reported here are more robust than those reported using the LAT. Therefore, the prevalence data in farmed deer in New Zealand reported by Reichel et al. (1999) of 52.5% and Wilson et al. (2012) of 42.5% (scan 1) and 69.2% (scan 2) might be an overestimate of the true prevalence, given the lower specificity of that test. In addition, the sample selection of two sera per herd by Reichel et al. (1999) may not be truly representative of a typical herd compared to the sample size per herd in the majority of the herds here.

However, despite assay limitations, a number of reports of *T. gondii* sero-prevalence in deer have been reported from other countries. The animal-level sero-prevalence reported in this study of 29.7% for all hinds at both scans was higher than 6.6% of 348 farmed deer in Ireland (Halová et al. 2013) using LAT. Sero-prevalence of 24.1% of 552 red and 30.4% of 92 roe wild deer reported in Poland (Witkowski et al. 2015) and 39.5% of 81 in free-ranging red deer in Italy (Formenti et al. 2015). Sero-prevalence of 15.6% of 441 wild red deer (Gauss et al. 2006) and 39.2% of 109 wild roe deer (*Capreolus capreolus*) (Gamarra et al. 2008) in Spain, and 21–60% in US in white-tailed deer (*Odocoileus virginianus*) (Ballash et al. 2014; Dubey et al. 2004; Dubey et al. 2008; Humphreys et al. 1995; Lindsay et al. 1991; Vanek et al.

1996) have been reported using a modified agglutination test (MAT). Using the Sabin-Feldman dye test, *T. gondii* antibodies were detected in 15% of 303 wild red deer in the Czech Republic (Hejlicek et al. 1997), 12% of 99 in Norway (Kapperud 1978) and in 12 of 12 pampas deer (*Ozotocerus bezoarticus*) in Uruguay (Puentes and Ungerfeld 2011). Using the direct agglutination test, sero-prevalence of 7.7% of 571 in red deer, 34% of 760 in roe deer and 1% of 866 in reindeer (*Rangifer tarandus*) were reported in Norwegian wild population (Vikoren et al. 2004). Moreover, another Belgian study carried out in 73 roe deer using ELISA and MAT reported a sero-prevalence of 52.2%, using a cut-off of mean plus 3× standard deviation (De Craeye et al. 2011). Thus, it is plausible that this organism is infecting many species of deer worldwide and its effect on reproduction needed further investigation.

The significantly lower sero-prevalence in R2 hinds at scan 2, than MA hinds, and higher abortion rates, likely reflects the immune-naïve status of R2 hinds to *T. gondii* due to limited or delayed exposure. Progressive exposure of older red deer would result in a cumulative increase in sero-prevalence as reported here. Young hinds are therefore more likely susceptible to acute infection and subsequent abortion. Enduring immunity to *T. gondii* as seen in other species, such as sheep (Buxton and Innes 1995; Buxton et al. 1993), would mean the likelihood of abortions due to this organism in MA hinds would be lower than in R2 hinds; however, such phenomenon was not observed in this study as explained by the non-significant association between *T. gondii* sero-status at scan 2 and aborted status at scan 2. Lack of consistency in abortion rate within farms between years observed in the parent study (Patel et al. 2018) is consistent with a pathogen which provide variable exposure rate and timing between years, as expected with *T. gondii*.

The lack of sero-prevalence difference between R2 and MA hinds in herds without aborted hinds suggests that hinds from both age group in those herds may have built immunity towards acute infection and therefore acquire the ability to resist abortion. This is consistent with the proposition that exposure and infection in those situations may have been prior to the risk period of pregnancy and that development of immunity has prevented abortions, as observed in sheep (Hartley 1961; McColgan et al. 1988). At herd level, the positive association between sero-prevalence and abortion rate in R2 herds suggests that the higher sero-prevalence to *T. gondii* is linked to higher abortion rates, assuming the exposure is within the period of risk. Additionally, the majority of the herds included in serology testing at scan 1 or scan 2 were sero-positive. The herds selected for serology were situated in different regions of New Zealand hence suggesting that the organism is ubiquitously present and both epidemiological and immunological patterns support that *T. gondii* is a cause of abortion in New Zealand farmed red deer.

The association between *T. gondii* sero-positivity and abortion at scan 2 was further supported by the presence of DNA in uteri from aborted hinds and aborting foetal tissue. Detection of *T. gondii* DNA in foetal tissues is consistent with reports of detection of *T. gondii* in foetuses during early to mid-pregnancy reported in American white-tailed deer (Dubey et al. 2014; Dubey et al. 2008). The detection of *T. gondii* DNA in foetal tissues from the present study and detection of antibodies in foetal heart blood from an aborted foetus submitted to a national laboratory in New Zealand (Orr and Thompson 1993) suggests that the organism can infect the foetus and cause subsequent abortion in NZ farmed red deer. Furthermore, the observation of mummified foetuses, uteri with signs of mummification and foetal remnants provides evidence that abortions are occurring, after scan 1, and that foetuses are getting completely or partially resorbed before expected calving.

The majority of abortions reported here were in early to mid-gestation. Abortions due to *T. gondii* in mid-gestation have been reported in sheep (Blewett et al. 1982; Buxton and Henderson 1999; Buxton et al. 1993). Moreover, reports from these studies together with detection of *T. gondii* in foetuses at mid-pregnancy in white-tailed deer (Dubey et al. 2008) suggest that *T. gondii* can cause abortion in early to mid-gestation, when the immune status of the foetus might not have developed fully as reported in sheep (Buxton and Finlayson 1986; Salami et al. 1985).

Histopathology and immunochemistry on samples positive on PCR would have been helpful in establishing a causal relationship between *T. gondii* and abortion. However, as the majority of samples from DSPs were sent frozen, it was not possible to undertake histopathology; hence, since sections were not prepared, and immunochemistry was not performed. Partial genotyping of a limited number of *T. gondii*-positive foetal brain tissue samples in this study suggested the presence of a unique type I/type III genotype. Unfortunately, due to the highly autolysed nature of these tissues, the quality of the DNA appears to have been compromised. This resulted in poor PCR amplification of the typing amplicons larger than 500 base pairs and inhibiting further successful typing. Ideally, foetal tissues could have been bio-assayed or cultured as is frequently performed in other countries to improve genotyping success (Dubey et al. 2017); however, this option was not available for this study. Despite these limitations, this albeit limited genotyping information adds to our understanding of *T. gondii* isolates circulating among animals in New Zealand. Previously, atypical type II *T. gondii* isolates in tissues from native birds and Hectors dolphins (*Cephalorhynchus hectori*) which had died from disseminated toxoplasmosis had been successfully genotyped (Howe et al. 2014; Roe et al. 2013). Moreover, a type I isolate from an aborted lamb was used to develop

a *T. gondii* licenced vaccine for sheep (Wilkins et al. 1988). Elsewhere, genetic characterisation has been successful for *T. gondii* isolates from sika deer (*Cervus nippon*) in China (Cong et al. 2016), elk (*Cervus canadensis*) and white-tailed deer (*Odocoileus virginianus*) in the United States (Dubey et al. 2014; Dubey et al. 2013b; Gerhold et al. 2017; Yu et al. 2013). However, to date, none of these isolates, or those found in the ToxoDB database (http://ToxoDB.org), match the genotype identified in the New Zealand red deer.

To the authors' knowledge, this is the first report to estimate the proportion of aborted hinds that could be attributable to *T. gondii* exposure. However, more studies are needed to evaluate the cost and benefits of possible intervention. The PAF is a useful measure for informing planning for and evaluating an intervention, such as vaccination, to reduce abortions due to *T. gondii* exposure. This measure has been used previously to inform interventions for abortions due to exposure to other infectious pathogens in cattle (Davison et al. 1999; Sanhueza et al. 2013). The PAF reported in this study was for R2 hinds at scan 2 at which a significant association was observed between sero-status and aborted status in R2 hinds. Additionally, the observations of early foetal losses coupled with the presence of *T. gondii* DNA in aborting maternal and foetal tissues and antibodies in serum at scan 1 suggest that the true PAF may be higher than that observed here.

The findings from this study at both scans suggest that *T. gondii* can infect pregnant hinds early- and mid-gestation and may cause abortions. Therefore, the sub-optimal reproductive performance in farmed red deer in New Zealand is likely partly due to *T. gondii* infections. However, further studies will be needed to definitively establish the causal relationship between *T. gondii* and abortion by challenging the pregnant R2 hinds with *T. gondii* and monitoring the fate of pregnancies.

Conclusion

There was marginal statistical evidence for reduction in pregnancy rates associated with *T. gondii*, but the observation of early abortions and detection of *T. gondii* DNA from aborting foetal and maternal tissues, corroborated by antibody in maternal blood suggests that at least some pregnancy losses may be attributed to *T. gondii* infections. The serological, molecular and pathology findings at scan 2 from this study provide robust evidence that *T. gondii* is associated with early and mid-gestation abortions in R2 hinds and potentially in MA hinds that are first exposed to the organism at the time of pregnancy. The abortion rate attributable to *T. gondii* exposure in R2 hinds was 7.9%. These findings confirm that *T. gondii* plays a role in sub-optimum reproductive performance in NZ farmed red deer.

Acknowledgements This study was supported by the Deer Reproductive Efficiency Group based in Southland. It was funded by AgResearch, Agmardt, DEEResearch, MSD Animal Health, Southland Deer Farmers Association, Massey University and individual farmers, particularly Landcorp Farming Ltd. The in-kind contribution of all participating farmers is gratefully acknowledged, as is the assistance of a large number of veterinary practices and scanners for scanning and blood sample collection and DSP staff and veterinarians for tissue sample collection. We thank Dr. Fernanda Castillo-Alcala (School of Veterinary Science, Massey University, New Zealand) for her assistance in histology. We are also thankful to the technical team at the School of Veterinary Science and visiting veterinary science students for their assistance in laboratory work and the staff at the post-mortem facility to allow dissection of maternal and foetal tissues in the post-mortem premises.

Compliance with ethical standards

Conflict of interest The authors declare that there is no conflict of interest.

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