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Methods to assess the effect of meat processing on viability of *Toxoplasma gondii*: towards replacement of mouse bioassay by in vitro testing

Marieke Opsteegh [a,*], Cecile Dam-Deisz [a], Paulo de Boer [b], Stephane DeCraeye [c], Andrea Faré [d], Paul Hengeveld [a], Ruud Luiten [d], Gereon Schares [e], Conny van Solt-Smits [f], Bavo Verhaegen [c], Theo Verkleij [b,g], Joke van der Giessen [a], Henk J. Wisselink [f]

[a] *Centre for Infectious Disease Control – Zoonoses and Environmental Microbiology, National Institute for Public Health and the Environment, P.O. Box 1, 3720 BA Bilthoven, The Netherlands*
[b] *TNO (Netherlands Organisation for Applied Scientific Research), Microbiology & Systems Biology, 3704 HE Zeist, The Netherlands*
[c] *Sciensano, Service of Foodborne Pathogens, Rue Juliette Wytsmanstraat 14, 1050 Brussels, Belgium*
[d] *Luiten Vleeswaren, Production Company of Artisanal Meat Products, Edisonstraat 70, 2723 RR Zoetermeer, The Netherlands*
[e] *Friedrich-Loeffler-Institut, Federal Research Institute for Animal Health, Institute of Epidemiology, National Reference Centre for Toxoplasmosis, Südufer 10, 17493 Greifswald-Insel Riems, Germany*
[f] *Wageningen Bioveterinary Research, P.O. Box 65, 8200 AB Lelystad, The Netherlands*
[g] *Wageningen Food & Biobased Research, P.O. Box 17, 6700 AA Wageningen, The Netherlands*

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ABSTRACT

Consumption of meat containing viable tissue cysts is considered one of the main sources of human infection with *Toxoplasma gondii*. In contrast to fresh meat, raw meat products usually undergo processing, including salting and mixing with other additives such as sodium acetate and sodium lactate, which affects the viability of *T. gondii*. However, the experiments described in the literature are not always performed in line with the current processing methods applied in industry. It was our goal to study the effect of salting and additives according to the recipes used by industrial producers. Mouse or cat bioassay is the 'gold standard' to demonstrate the presence of viable *T. gondii*. However, it is costly, time consuming and for ethical reasons not preferred for large-scale studies. Therefore, we first aimed to develop an alternative for mouse bioassay that can be used to determine the effect of processing on the viability of *T. gondii* tissue cysts. The assays studied were (i) a cell culture method to determine the parasite's ability to multiply, and (ii) a propidium monoazide (PMA) dye-based assay to selectively detect DNA from intact parasites. Processing experiments were performed with minced meat incubated for 20 h with low concentrations of NaCl, sodium lactate and sodium acetate. NaCl appeared to be the most effective ingredient with only one or two out of eight mice infected after inoculation with pepsin-digest of portions processed with 1.0, 1.2 and 1.6% NaCl. Results of preliminary experiments with the PMA-based method were inconsistent and did not sufficiently discriminate between live and dead parasites. In contrast, the cell culture method showed promising results, but further optimization is needed before it can replace or reduce the number of mouse bioassays needed. In future, standardised in vitro methods are necessary to allow more extensive testing of product-specific processing methods, thereby providing a better indication of the risk of *T. gondii* infection for consumers.

1. Introduction

Toxoplasma gondii infection is prevalent worldwide (Pappas et al., 2009; Maenz et al., 2014) and was identified as a priority foodborne pathogen in global (Torgerson and Mastroiacovo, 2013; FAO and WHO, 2014; Havelaar et al., 2015), European (Bouwknegt et al., 2018) and Dutch studies (Havelaar et al., 2007; Havelaar et al., 2012; van Lier et al., 2016). Possible sources of human infection are clear since the complete life cycle of *T. gondii* was established in 1970 (Dubey, 2009). Felids are the definitive hosts of *T. gondii*. Upon primary infection, they will

* Corresponding author.
E-mail address: marieke.opsteegh@rivm.nl (M. Opsteegh).

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excrete millions of oocysts within a period of up to approximately 3 weeks. After sporulation, these oocysts are infectious to virtually all warm-blooded animals and humans. Hosts other than felids are intermediate hosts only. They do not excrete the parasite but will develop tissue cysts. These tissue cysts are infectious to intermediate and definitive hosts, enabling *T. gondii* transmission via carnivory. Thus, the two major routes for human infection are ingestion of oocysts and tissue cysts. Oocysts can be present in the environment and cat faeces, on raw unwashed vegetables, and in water or shellfish. Consumption of undercooked or raw meat of infected animals can lead to infection via tissue cysts. The relative contribution of oocysts and tissue cysts to human infections is not well known, but experts assessed that 50% of infections are foodborne (Hald et al., 2016; EFSA Panel on Biological Hazards (BIOHAZ) et al., 2018). Regarding meatborne infections, it is known that the prevalence in livestock increases when the animals have outdoor access (e.g. sheep and organic pigs) and increases with age (Opsteegh et al., 2016). The prevalence of viable *T. gondii* in cattle appears to be low (Opsteegh et al., 2019; Stelzer et al., 2019). However, to estimate the risk of infection from specific meat products, not only the prevalence of infection, but also consumed volumes and preparation habits need to be taken into account. These factors can be combined in a quantitative microbial risk assessment (QMRA), which has been performed for meatborne *T. gondii* infections in the Netherlands. The QMRA results show that heating temperature has a large influence on the predicted risk of infection per portion, and 40% of the total predicted infections were attributed to raw meat products (Opsteegh et al., 2011a). Freezing the meat that is used to prepare these products would avert the risk (Suijkerbuijk et al., 2019), but is not preferred by consumers (Lambooij et al., 2019) and producers. Possibly, the risk from these products is currently overestimated in the QMRA model as, although these products are not heated, they usually still undergo processing that may affect the viability of *T. gondii* tissue cysts. For instance, the effect of salting was implemented in the QMRA model based on a single experiment exposing rodent brains to different salt concentrations (Dubey, 1997), which is not in line with processing in industry and may have influenced the QMRA outputs. In fact, inclusion of additional and more recent data on the effect of salting (e.g. Pott et al., 2013) to fit a new salting effect model, and implementation of this new model in the QMRA has altered the predicted contributions for raw meat products (Deng et al., 2019). Further, as part of the raw products consumed are typical Dutch products, also with the inclusion of more recent data from the literature, the experimental conditions may not reflect the processing used for products in retail in the Netherlands. Therefore we aimed to study the effect of salting and use of additives on the viability of *T. gondii*, according to the recipes used by industrial producers in a public–private partnership.

Currently, mouse or cat bioassay is the 'gold standard' to detect viable *T. gondii.* However it is costly, time consuming and for ethical reasons not preferred for large-scale studies. Therefore, before studying the effect of processing applied for raw meat products on *T. gondii* viability, it was our aim to develop an alternative for the mouse bioassay that can be used to determine the effect of meat processing on the viability of *T. gondii* tissue cysts. As it was perceived that a microscopy-based staining method would not provide enough sensitivity (Opsteegh et al., 2016), we focused on cell culture and a PCR-based method with propidium monoazide (PMA) dye incubation.

Cell culture plays, to some extent, a role as a diagnostic tool for human toxoplasmosis (Contini et al., 1995; Dupon et al., 1995; Miller et al., 2000). However, cell culture for *T. gondii* is, so far, mainly used to study parasite biology (e.g. Buchholz et al., 2013), drug effectiveness (e.g. Couzinet et al., 2000), for antigen production (e.g. Hughes et al., 1986) or the maintenance of strains first isolated by mouse bioassay. In these cases, isolated strains are used and therefore the detection limit or the effect of the sample matrix on the method, are of less concern. It has been demonstrated that the sensitivities of mouse bioassay and cell culture are similar when tachyzoite or bradyzoite suspensions in culture medium are used (Derouin et al., 1987; Couzinet et al., 2000). Cell culture was previously used to detect *T. gondii* in cured meats with limited success (Warnekulasuriya et al., 1998). In those experiments, meat samples were inoculated without prior enzymatic digestion and detection in cell culture was based on microscopical identification of cytopathic effect (CPE) with identifiable parasites. Intrinsic CPE activity of the cured meats affected the suitability of the method. Moreover, since detection was based on microscopy without specific staining, the sensitivity of the method was probably not optimal, as Derouin et al. (1987) demonstrated that identification of *T. gondii* in cell culture by indirect immunofluorescence was more sensitive than examination without staining or using Giemsa-staining. Currently, various quantitative PCR (qPCR) methods are available for sensitive detection and quantification of *T. gondii* DNA. Monitoring the *T. gondii* DNA concentration in cell culture medium using qPCR is easier and possibly more sensitive than microscopy with specific staining. Therefore, qPCR-based detection and quantification was implemented in our experiments, similarly to the method previously described by Zintl et al. (2009) for cell culture-based detection of viable *T. gondii* in ovine placentomes (Zintl et al., 2009), and subsequently used with varying success rates for sheep hearts (Bacci et al., 2016; Vismarra et al., 2017), and Parma ham (Genchi et al., 2017). Cell culture methods have also been used to recover isolates from sea otter brains (Cole et al., 2000), CSF and brain of a pacific harbour seal (Miller et al., 2001), brains from a litter of puppies (Al-Qassab et al., 2009), and brain homogenates or muscle digests from pigeons and cats (Waap et al., 2012). Cell culture has also been applied as a viability test to study inactivation of *T. gondii* oocysts in simple matrices (i.e. buffer solutions or water) (Dumetre et al., 2008; Villegas et al., 2010; Ware et al., 2010), but has not been applied on oocysts in food matrices or environmental samples (Rousseau et al., 2018).

Since 2003 viability-PCRs, based on the use of intercalating dyes like ethidium monoazide (EMA) (Nogva et al., 2003) and later PMA (Nocker et al., 2006), have been reported, and currently several bacterial viability PCR kits are commercially available. These dyes permeate selectively into cells that have lost their membrane integrity and intercalate into the DNA. Photoactivation will lead to cross-linkage and irreversibly inhibit DNA amplification in PCR (Fittipaldi et al., 2012). The method has been used for bacterial cells and spores, viruses, fungi, and its use for protozoan parasites was limited to *Cryptosporidium* (Brescia et al., 2009), *Giardia* (Alonso et al., 2014; Ma et al., 2016) and *Acanthamoeba* (Fittipaldi et al., 2011). Recently, a PMA-based method for *T. gondii*, focusing on oocysts, has been described (Rousseau et al., 2019). Although promising, the methods are not always successfully applied, especially in cases of environmental or other complex samples. Incomplete signal reduction for dead cells (mainly PMA) (Ma et al., 2016), and lack of specificity due to membrane permeability (mainly EMA can affect the signal of live cells), are the main issues observed (Fittipaldi et al., 2012). The method has not been used for *T. gondii* tissue cysts or bradyzoites. Zeng et al. (2016) summarises the use of PMA and EMA methods for other foodborne pathogens, but the studies that focused on meat products (e.g. Li and Chen, 2012; Liu and Mustapha, 2014; Zhang et al., 2015), used homogenate rather than digested tissue (Zeng et al., 2016).

In this study, we evaluated two in vitro viability assays (cell culture and a PCR-based method with PMA) using tachyzoites from cell culture, bradyzoites from mouse brains and meat from naturally infected sheep. Because results with the PMA-based viability

assay were inconsistent and did not sufficiently discriminate between live and dead parasites, it was decided to use a cell culture-based method next to bioassay in an experiment to demonstrate the effect of meat processing on viability of *T. gondii*, using naturally infected sheep hearts.

2. Materials and methods

2.1. Study design

After an initial evaluation with tachyzoite-, bradyzoite- or tissue cyst-containing samples to optimise and compare the PMA and cell culture methods, three main experiments were carried out (Table 1). In experiment A the feasibility of a cell culture method was assessed using digests of hearts from naturally infected sheep. Experiment B aimed to evaluate the effect of meat processing protocols including different concentrations of NaCl, sodium lactate and sodium acetate on the viability of *T. gondii* by a cell culture method. In experiment C, the cell culture method and mouse bioassay were used in parallel to determine and compare detection limits and their ability to monitor the effect of processing on the viability of *T. gondii*.

2.2. Parasites and infected sheep hearts

2.2.1. Tachyzoites

T. gondii RH strain (type I) tachyzoites were cultured and harvested from cell culture in rabbit kidney (RK) 13-cells (ATCC® CCL37™, USA) in RPMI 1640 with 3% FBS, 100 U of penicillin/ml and 0.1 mg/ml of streptomycin as described previously (Opsteegh et al., 2011b).

2.2.2. Tissue cysts and bradyzoites

Swiss white outbred female mice (Janvier Labs, France) were inoculated i.p. with 20–30 tissue cysts of the *T. gondii* 76 K (type II) strain at Sciensano (Brussels, Belgium). After a minimum of 5 weeks of incubation, the mice were euthanized using CO_2 and immediately disinfected by submerging them in ethanol. The brains and other tissues (heart, lungs, hind leg muscles) were collected from the individual animals using sterile equipment. The brains were homogenised in the presence of sterile PBS supplemented with penicillin (5317 U/ml) and streptomycin (3600 U/ml) in a Potter homogenator. All collected tissues were transported on ice in 50 ml tubes to the facilities of Wageningen Bioveterinary Research (Lelystad, The Netherlands). To collect cysts, brain tissue was homogenised by passing through a 100 μm cell strainer and suspended in 4 ml of PBS. Tissue cysts were isolated by centrifugation (15 min, 1200*g* at 4 °C) over a 30%-90% Percoll gradient (Sigma Aldrich, The Netherlands). Tissue cysts were harvested from the 30%/90% interface, suspended in PBS and centrifuged at 3000*g* for 15 min at 4 °C (Cornelissen et al., 2014). To release bradyzoites from tissue cysts, a short pepsin digestion (10 min at 37 °C) was used: 1 ml of tissue cyst suspension was mixed with 2 ml of pepsin-HCl solution (Section 2.3), and 1.2 ml of $NaHCO_3$ was used for neutralisation. The parasite concentration was determined by counting under a light microscope using a Bürker-Turk counting chamber.

The use of mice for the culture of tissue cysts was approved by the Ethical Commission of the CODA-CERVA/WIV-ISP, Belgian Institute of Public Health, now Sciensano (20140704-01 and 20170425-01).

2.2.3. Infected sheep hearts

Sheep hearts were obtained from one slaughterhouse in the Netherlands. The hearts were weighted and approximately 5 g were cut, incised, and placed in a meat juice collector (Kabe Labortechnik GmbH, Germany). The meat juice collectors with tissue samples were placed at −80 °C for 3 h and left to thaw overnight at 4 °C. Multispecies ELISA (ID Screen® Toxoplasmosis Indirect Multi-species, IDvet, France) was performed as described in Section 2.4.1 on the collected tissue fluid. Hearts of sheep positive by ELISA were selected for testing by magnetic capture qPCR (MC-qPCR; see Section 2.4.3) to identify hearts containing *T. gondii* DNA for inclusion in the batch of positive tissue. The batch of positive tissue was used to test the effect of meat processing on *T. gondii* viability in experiments B and C. Depending on the number of hearts positive by ELISA, MC-qPCR was performed on individual or pooled samples (approximately 20 samples in each round of MC-qPCR) with a total weight of 50 g. To prepare a negative control batch, ELISA-negative hearts were frozen overnight at −20 °C to ensure that no viable parasites remained present.

2.3. Pepsin-HCl digestion

In the initial evaluation and in experiments A and B, the digestion was based on Dubey (1998), and carried out as follows: meat (50 g) and 75 ml of PBS were homogenised in a blender for 15 s at high speed. The blender was rinsed with 75 ml of PBS, which were added to the meat homogenate. Per meat homogenate (50 g of meat and 150 ml of PBS), 250 ml of pepsin-HCl solution (13.6 ml of 9.5% (v/v) HCl, 1.3 g of pepsin (700 FIP U/g, Merck, The Netherlands), 2.5 g NaCl) were added. The suspension was incubated on a magnetic stirrer for 1 h at 37 °C. The digest was filtered (180 μm sieve) and centrifuged for 10 min at 1200*g*. The supernatant was discarded and the pellet was resuspended in 10 ml of PBS and 6–9 ml of 1.2% $NaHCO_3$ (pH 8.3) until it reached pH >6.5. The suspen-

Table 1
Overview of the study design, with aim and assays and samples applied in each experiment.

Experiment	Aim	Viability assays	Samples
Initial evaluation	Method optimization	PMA-PCR, Cell culture	Tachyzoites and bradyzoites spiked in cell culture medium or tissue digest
A	Suitability of method for detection from infected tissue	Cell culture	Naturally infected sheep hearts
B	Evaluate effect of meat processing (methods I–V)	Cell culture	Naturally infected sheep hearts spiked with infected mouse tissue (series 1) and tissue cysts (series 2)
C	Evaluate effect of meat processing (methods I–V)	Cell culture, Mouse bioassay	Naturally infected sheep hearts spiked with infected mouse tissue (series 1) and tissue cysts (series 2)
C	Compare detection limit	Cell culture, Mouse bioassay	Tachyzoites spiked in cell culture medium and pepsin digest of negative sheep hearts

PMA, propidium monoazide.

sion was centrifuged for 10 min at 1200*g*, and the pellet was resuspended in 3–5 ml (depending on pellet size) of 0.9% NaCl, PBS or culture medium containing antibiotics, depending on further application. However, it was noticed that the digestion was incomplete (6–10 g of tissue remained on the 180 μm sieve) and digests did not form firm or clearly delineated pellets after 10 min of centrifugation at 1200*g*. Therefore, the homogenisation in the blender, the concentration of HCl and pepsin in the solution, and the centrifugation speed were varied, and the following changes to the digestion protocol were implemented in experiment C: homogenisation was carried out using 10 intervals of 1 s at low speed; pepsin-HCl solution (250 ml) consisted of 8.5 ml of 9.5% (v/v) HCl, 15 g of pepsin (Merck 700 FIP U/g) and 2.5 g of NaCl; centrifugation steps were carried out for 10 min at 1500*g*; the volume of $NaHCO_3$ used for neutralisation was fixed at 15 ml.

2.4. Detection methods

2.4.1. ELISA

A commercially available species-independent indirect ELISA (ID Screen® Toxoplasmosis Indirect Multi-species; IDvet, France) was used for the detection of IgG antibodies against *T. gondii* in sheep and mice. The assay is marketed and validated for sera and meat juice of a variety of animal species including sheep and has previously been used for mice in bioassay experiments (Burrells et al., 2015, 2018). The assay was performed according to the manufacturer's instructions and sera with a sample-positive control ratio (S/P%) ≤40% were considered negative, between 40% and 50% inconclusive, and ≥50% positive.

2.4.2. p30-immunoblot

Mouse sera were additionally tested by immunoblot for IgG antibodies against p30 (TgSAG1), a native affinity-purified *T. gondii* tachyzoite surface antigen (Schares et al., 2017). Two serum dilutions (1:10 and 1:100) were tested as previously described (Burrells et al., 2018).

2.4.3. MC-qPCR

MC-qPCR was performed with previously described minor alterations (Opsteegh et al., 2019) to the original protocol (Opsteegh et al., 2010a). In brief, tissue was cleaned and cut, and an overnight proteinase K lysis was performed. *T. gondii* DNA was selectively extracted from the crude lysate using biotin-labelled capture oligonucleotides (including a TEG spacer) complementary to the 529 bp repeat element (RE) (Homan et al., 2000) and streptavidin-coated magnetic beads (M-270 Streptavidin Dynabeads, Invitrogen, The Netherlands). qPCR for the 529 bp RE was performed on a Roche Lightcycler 480 including a competitive internal amplification control (CIAC) to check for PCR inhibition.

2.5. Viability assays

2.5.1. PMA method

To optimise the PMA method, nine experiments have been carried out (Supplementary Table S1), according to this protocol or with variations indicated in parentheses. The PMA method was applied first to tachyzoites from cell culture (Section 2.2.1), and subsequently also to bradyzoites released from tissue cysts (Section 2.2.2) and suspended in 500 μl of 0.9% NaCl (variation: PBS, RPMI-1640) or 500 μl of in tissue digest (Section 2.3) with parasite numbers ranging from 100 to 10,000. Dead cell controls were prepared by heating the suspensions in 1.5 ml tubes for 10 min (variation: 15 or 20 min) at 70 °C in a heating block. Moreover, DNA controls of 375 fg of DNA isolated from cultured tachyzoites were used. After adding 20 mM PMA (Biotium, USA) to a 50 μM (variation: 100 μM) final concentration, samples were incubated in the dark at room temperature for 5 min, and subsequently exposed for 15 min to a Phast-blue lamp (GENIUL/60 W, Spain). For biosafety reasons, samples were frozen at −20 °C (variation: heat-treated) prior to DNA isolation. DNA was isolated using a DNeasy Blood and tissue kit (Qiagen, The Netherlands) according to the protocol for cultured cells with a final elution in 100 μl of elution buffer. The qPCR for the 529 bp RE was performed as described for the MC-qPCR (Section 2.4.3). For live or killed parasites without PMA incubation and live parasites with PMA incubation, cycle quantification (Cq) values were expected to be similar and a mean reference Cq value was calculated. After PMA incubation the PCR for killed parasites should be inhibited, resulting in higher Cq values or, preferably, failure to detect *T. gondii* DNA.

2.5.2. Cell culture method

For culture of *T. gondii* from tissue digests, 6-well plates with 80–100% confluent RK13 cell monolayers were prepared. RPMI medium was removed from the cells and 1 ml of digest with 1 ml of prewarmed RPMI-1640 was added to each well (500 ml RPMI-1640 with L-glutamine and 25 mM HEPES was supplemented with 10% FBS and 3 ml of penicillin (10,000 U/ml)/streptomycin (10,000 μg/ml) and amphotericin B 25 μg/ml; Lonza, Switzerland). Each digest or digest spiked with different dilutions of tachyzoites or bradyzoites, was added to at least two wells. The digests were incubated on the cells for 2 h at 37 °C and 5% CO_2. The digest was removed from the cells and stored at −20 °C for DNA isolation. The cells were washed with 2 ml of prewarmed PBS. From one well per digest, the cells were scraped, suspended in the PBS and centrifuged for 10 min at 600*g* (two 2 ml tubes per sample). Supernatant was removed and pellets resuspended in 200 μl of PBS and stored at −20 °C until DNA isolation. The other well was maintained for 3–4 weeks at 37 °C and 5% CO_2 in 2 ml of RPMI-1640 with weekly replacement of 50% or 100% of the medium. To monitor growth, culture medium that was taken off the cells was centrifuged at 600*g* for 10 min and the pellet resuspended (200 μl of PBS) and frozen at −20 °C until DNA isolation. After 3–4 weeks, the culture medium was collected and the remaining cells were scraped in 2 ml of PBS and collected for DNA isolation as described above. DNA was isolated, first from initial and final culture medium samples, using DNeasy Blood and Tissue Kit (Qiagen, The Netherlands) using the protocol for cultured cells with a final elution in 100 μl of AE buffer. qPCR for the 529 bp RE was performed as described for the MC-qPCR (Section 2.4.3), and repeated at 1:10 dilution in case of PCR inhibition. Culture samples from intermediate time-points were additionally isolated and tested by qPCR as needed. A reduction in Cq value over time was regarded as an increased concentration of *T. gondii* DNA and therefore parasite growth.

2.5.3. Mouse bioassay

For experiment C, IFNγ KO IFNɣ −/− (C.129S7(B6)-Ifngtm1Ts/J) [GKO] mice (n = 110) were obtained from Friedrich-Loeffler Institut (Germany) (https://www.jax.org/strain/002286). Mice were between 7 and 18 weeks old, and included 52 males and 58 females. After an acclimatisation period of 1 week, mice were used in the experiments. The analgesic buprenorphin hydrochloride (Abbott Laboratories, USA) was added to the drinking water (0.09 mg/kg per mouse per day) from day −1 to day 21 (Lindsay et al., 2005). Two mice per sample were i.p. inoculated with 1 ml of tissue digest on day 0. Mice were monitored and given a health score based on coat condition and demeanour daily from day 1 to day 7, twice daily from day 8 to day 21, and daily from day 22 onwards. Mice were euthanized when predefined humane endpoints were reached. In case mice were euthanized within the first 3 days after inoculation, the remainder of the digest was used to inoculate a replacement mouse. In case mice were euthanized

between 3 and 13 days post inoculation, peritoneal liquid was collected by flushing the peritoneal cavity with 3 ml of PBS. The obtained liquid was stored frozen at −20 °C for DNA isolation. In case mice were euthanized later or at the end of the experiment (6 weeks post inoculation), blood and brain were collected. For euthanasia, mice were anaesthetised using ketamine (70 mg/kg; Alfasan, The Netherlands) and xylazine (7 mg/kg; Dechra, The Netherlands) and bled, followed by cervical dislocation. DNA isolation and *T. gondii* qPCR on peritoneal liquids and brains were performed as previously described (Cornelissen et al., 2014). Sera were tested for IgG antibodies by ELISA and p30 immunoblot as described in Sections 2.4.1 and 2.4.2. A mouse bioassay was considered positive based on a positive reaction in qPCR or serology.

The mouse bioassay was approved by the governmental Central Authority for Scientific Procedures on Animals (CCD) in the Netherlands (AVD401002017896).

2.6. Meat processing

Sheep hearts were ground to 6 mm and subsequently to 3 mm using a meat grinder (Bizerba FW 32 T; Bizerba Nederland B.V., The Netherlands). Separate batches of negative and positive meat were prepared and, after grinding, divided into portions of 50 g (experiment B) or 30 g (experiment C). Different combinations of NaCl (AkzoNobel, The Netherlands), sodium lactate (Corbion, The Netherlands) and sodium acetate (Van Hees, The Netherlands) were added to final concentrations in the meat sample according to Table 2. Sodium lactate was added using a 58.8% (w/v) stock solution. Treated samples were stored for 20 h at 7 °C before proceeding with pepsin digestion and inoculation in mice or on cell culture.

2.7. Data analysis

Generalised linear modelling was performed to evaluate the effect of reference Cq value, parasite type and matrix on the increase in Cq value for PMA-treated killed parasites. Logistic regression analysis was performed to determine the effect of tachyzoite concentration, method (cell culture and mouse bioassay), and presence of digest on the detection of viable *T. gondii*. To compare the effectiveness of the different processing methods in experiment C, Pearson's χ^2-test was performed to evaluate overall difference and a *z*-test to compare column proportions. All statistical analyses were performed in SPSS 24.0.0 (IBM).

3. Results

3.1. PMA method

Incubation with PMA should lead to lower, and preferably undetectable, levels of *T. gondii* DNA from inactivated parasites. Initial PMA experiments comparing tachyzoite suspensions in PBS, RPMI-1640 and 0.9% NaCl indicated that the increase in Cq values for killed parasites was most consistent in 0.9% NaCl (experiments 1 and 2, Supplementary Table S1). Moreover, increasing the final concentration of PMA from 50 μM to 100 μM did not appear to result in a further reduction in Cq values for inactivated samples (experiment 3, Supplementary Table S1), therefore final concentrations of 50 μM PMA and suspension in 0.9% NaCl were used in further experiments. An increase in Cq value was observed when PMA incubation was used on killed tachyzoite suspensions, bradyzoites, or *T. gondii* DNA. The increase was independent of the starting DNA concentration, as indicated by a lack of significant effect of the reference Cq value (Table 3). The increase in Cq value was associated with type and matrix, with a larger increase for DNA samples compared with tachyzoites; and a smaller increase for suspension in RPMI and PBS compared with 0.9% NaCl (Table 3). In 86.8% of samples, detectable *T. gondii* DNA was still present after PMA treatment (Table 4). The results were not interpretable for every sample (26.4% not interpretable) and, especially in combination with pepsin-digested tissue, the proportion of non-interpretable results was high (70%) (Table 4).

Table 2
Final concentrations of sodium lactate, sodium acetate and sodium chloride in samples for processing methods I–V.

Processing method	% Na lactate (dry matter)	% Na acetate	% NaCl
Method I	1.19	0.26	1.20
Method II	0.61	0.29	0.15
Method III	1.08	0.32	1.60
Method IV	1.40	0	0
Method V	1.00	0	1.00

3.2. Cell culture method

After initial experiments with dilution series of tachyzoites from cell culture (data not shown), different numbers of bradyzoites (5000, 1000, 500 and 100) were inoculated on RK13 cells with or without digest. Experiments with digest were performed in triplicate. The maintenance of RK13 cells after inoculation with bradyzoites showed a clear decrease in Cq values after 2 weeks (ΔCq between 6.9 and 10.7) and 3 weeks (ΔCq between 6.3 and 10.2) incubation (Fig. 1). For 12 out of 72 (16.7%, 95% confidence interval (CI) 8.1–25.3%) samples with digest and none of the 24 samples without digest, PCR inhibition was observed. Samples with inhibition were excluded from the analysis.

3.3. Experiment A: detection of viable *T. gondii* in naturally infected sheep using a cell culture method

For this experiment 20 sheep hearts were obtained from a slaughterhouse. Indirect ELISA on tissue fluid indicated that 10 out of 20 were serologically positive. All were tested by MC-qPCR on 50 g of heart tissue and three seropositives were also positive by MC-qPCR (Table 5). Pepsin-HCl digestions were performed on 50 g of the remaining heart tissue and digests inoculated on RK13 cells. Contamination during DNA isolation was observed with the 2 h inocula in three out of six blank controls (Cq values: 34.3, 33.7, 31.7), and therefore positive results at 2 h are unreliable (data not shown). The tachyzoite controls (both in cell culture medium and in tissue digest) were positive, but did not show growth as is illustrated by a lack of decrease in Cq value (Table 5). Passage of the same uncounted tachyzoite stock on the RK13 cells in a T75 flask did result in further growth, confirming that the tachyzoites were viable. Maintenance of the RK13 cells and qPCR on the culture medium did not provide clear results for the sheep and PCR inhibition was observed for many samples at different time points (Table 5). Sample A17 showed a decrease in Cq value from 2 weeks to 3 weeks, and was also positive by MC-qPCR. Additional qPCR testing of the digest and repeated testing of the culture medium (adding the 1 week samples and 10^{-1} dilutions) were performed for A3, A8, A11, A17, and the tachyzoite controls. A3 and A11 were negative in digest and cell culture. A8 gave a single uncertain positive result in cell culture after 2 h at 10^{-1} dilution (Cq value: 36.8), and was considered negative. The increase in *T. gondii* DNA concentration for A17 was confirmed when testing the 10^{-1} dilutions (Table 6).

Table 3
Parameter estimates from a generalised linear model with an increase in cycle quantification (Cq) value for propidium monoazide-treated killed *Toxoplasma gondii* as a dependent variable and reference Cq value as a covariable, and sample type (DNA, bradyzoites, tachyzoites) and matrix (pepsin digest, RPMI medium, PBS, 0.9%NaCl) as cofactors.

Parameter		β-coefficient	S.E.	95% Confidence Interval	X^2	df	*P*
(Intercept)		6.477	2.628	1.326–11.628	6.073	1	0.014
reference Cq value		0.005	0.098	−0.188–0.197	0.002	1	0.962
type	DNA	2.492	0.849	0.828–4.157	8.612	1	0.003
bradyzoites	1.102	1.273	−1.393-3.598	0.749	1	0.387	
tachyzoites	Reference	.	.	.	.	.	
matrix	digest	−2.807	1.721	−6.180–0.565	2.662	1	0.103
RPMI	−3.122	1.365	−5.797–−0.446	5.228	1	0.022	
PBS	−2.077	1.016	−4.068–−0.086	4.178	1	0.041	
0.9%NaCl	Reference	.	.	.	.	.	
(Scale)		4.966[a]	1.139	3.167–7.785			

[a] Maximum likelihood estimate.

Table 4
Testing *Toxoplasma gondii* viability using a propidium monoazide (PMA)-based assay. Increase in cycle quantification (Cq) values for killed parasites after PMA incubation compared with reference Cq value (mean of Cq values for sample with live parasites with and without PMA incubation, and killed parasites without PMA incubation), % of samples in which *T. gondii* DNA was not detectable any more after PMA treatment, and % of samples with non-interpretable results. Detailed results are provided in Supplementary Table S1.

Variable		Increase Cq value mean (min–max)[a]	Not detectable (%)[a,b]	Not interpretable (%)[b]
Type	Tachyzoites	5.8 (1.0–14.9) (*n* = 23)	3/23 (13.0%$_1$)	8/32 (25%$_1$)
	Bradyzoites	7.0 (2.8–9.6) (*n* = 4)	1/4 (25.0%$_1$)	5/9 (55.6%$_1$)
	DNA	8.4 (5.7–11.5) (*n* = 11)	1/11 (9.1%$_1$)	1/12 (8.3%$_1$)
Matrix	0.9% NaCl	7.5 (6.4–8.5) (*n* = 27)	5/27 (81.5%$_1$)	7/34 (20.6%$_1$)
	Digest	4.4 (2.8–5.9) (*n* = 2)	0/2 (0%$_1$)	7/10 (70%$_2$)
	RPMI	4.3 (2.2–6.3) (*n* = 3)	0/3 (0%$_1$)	0/3 (0%$_{1,2}$)
	PBS	5.4 (1.9–8.8) (*n* = 6)	0/6 (0%$_1$)	0/6 (0%$_1$)
Total		6.7 (1.0–14.9) (*n* = 38)	5/38 (13.2%)	14/53 (26.4%)

[a] For five samples in which no *T. gondii* DNA could be detected after PMA treatment, no Cq value was obtained. However, an increase in Cq value is calculated by assuming a Cq value of 40.
[b] Pearson's χ^2-test indicates differences between the proportion of non-interpretable samples by matrix ($P = 0.004$). Each subscript number denotes a subset of categories whose column proportions do not differ significantly from each other.

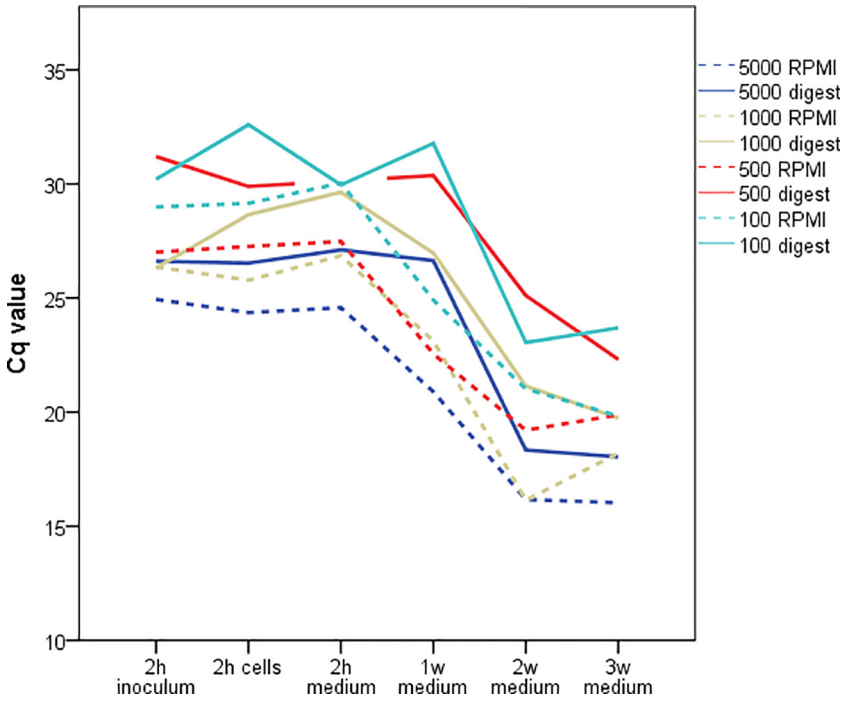

Fig. 1. *Toxoplasma gondii* DNA in cell culture after inoculation with bradyzoites in culture medium (RPMI, dashed lines) or pepsin digest of sheep heart samples (solid lines). Cycle quantification (Cq) values in quantitative PCR for *T. gondii* for inocula, cells or culture medium (2 h, 1 week (1w), 2 weeks (2w) and 3 weeks (3w)) after 100, 500, 1000 or 5000 viable bradyzoites spiked in tissue digest or cell culture medium were inoculated and maintained by cell culture on RK13 cells. Dilutions in tissue digest were performed in triplicate and mean Cq values are shown.

Table 5
Detection of *Toxoplasma gondii* in 20 naturally infected sheep (A1-A20) in experiment A. Sera of sheep were assessed by ELISA, and heart musculature by magnetic capture quantitative PCR (MC-qPCR) and cell culture. Results are for sheep hearts and tachyzoite controls. Five sheep hearts (A2, A4, A10, A12 and A16) were negative in all tests and have been omitted from the table.

Experiment	ELISA (S/P %)	MC-qPCR (Cq value)	Cell culture 2 weeks	Cell culture 3 weeks
A1	pos (99)	neg	neg	neg
A3	pos (147)	32.5	neg	neg
A5	neg (11)	neg	inh.	neg
A6	doubt (48)	neg	neg	neg
A7	pos (73)	neg	neg	neg
A8	pos (151)	34.1	neg	inh.
A9	pos (94)	neg	neg	neg
A11	neg (21)	inh.	35.1	neg
A13	neg (31)	neg	inh.	neg
A14	pos (94)	neg	neg	neg
A15	pos (167)	neg	neg	neg
A17	pos (161)	31.8	26.8	24.5
A18	pos (174)	neg	neg	inh.
A19	doubt (47)	neg	inh.	neg
A20	pos (105)	neg	neg	neg
Tachyzoites[a] in digest A5	NA[b]	NA	17.5	19.1
Tachyzoites[a] in medium	NA	NA	17.0	16.3

Results: pos, positive; neg, negative; doubt, doubtful; inh., PCR inhibition was observed; S/P%, OD sample-to-positive ratio; Cq, cycle quantification.
[a] Uncounted tachyzoite spike consisted of 500 μl of medium harvested from tachyzoite culture on RK13 cells. The same stock was passaged successfully.
[b] NA, not applicable.

Table 6
Additional testing by quantitative PCR for experiment A samples with positive results in cell culture at 2 or 3 weeks (Table 5). Cycle quantification values are presented. Samples A3, A8 and A11 were negative in additional testing and have been omitted from the table.

Sample	Dilution	Digested sheep heart	2 h	1 week	2 weeks	3 weeks
A17	10^0	inh./31.1	inh.	neg	30.0	24.5
	10^{-1}	neg/neg	34.8	36.0	30.6	27.6
Tachyzoites[a] in digest A5	10^{-1}	ND[b]	22.3	17.0	21.0	22.9
Tachyzoites in medium	10^{-1}	NA[c]	19.5	16.6	20.6	19.9

Results: neg, negative; inh., PCR inhibition was observed.
[a] Uncounted tachyzoite spike consisted of 500 μl of medium harvested from tachyzoite culture on RK13 cells. The same stock was passaged successfully.
[b] ND, not determined.
[c] NA, not applicable.

3.4. Experiment B: effectiveness of meat processing based on a cell culture method

For this experiment 49 sheep hearts were obtained from the slaughterhouse. Meat juice of one sheep heart gave an inconclusive result in ELISA (S/P% 42.7%), all others were considered seropositive (S/P% ranging from 51.2% to 208.5%). As there were too many seropositives for individual testing, 40 tissue samples were selected and tested in pools of two samples (2 × 25 g) for MC-qPCR. Ten out of these 20 pools tested positive (Cq values ranging from 27.9 to 36.5). The remaining tissue of the 10 sheep hearts included in the five pools with the lowest Cq values (27.9–31.1) were used to prepare a positive batch for meat processing. To prepare a negative batch, four sheep hearts with the lowest S/P% in ELISA and negative MC-qPCR in pooled testing were selected and frozen overnight at −20 °C prior to use in the viability assays. Six sheep hearts from three MC-qPCR positive pools were individually tested without processing to further evaluate the ability to detect natural infection with *T. gondii* by cell culture. As only one out of three MC-qPCR positives in experiment A was also positive by cell culture and consistent positive results are essential to evaluate the effect of processing, the positive batch used for processing and for unprocessed controls was additionally spiked in experiment B. First, the batch was spiked with tissue (heart, hind legs and lungs homogenised once using a meat mincer with a 5 mm hole diameter disc (Braun)) of five experimentally infected mice before mincing (series 1 and 2). To provide even more certainty about the presence of *T. gondii* tissue cysts, the positive batch samples were additionally spiked with tissue cysts (one tissue cyst/g) isolated from mouse brains on the second day (series 2). Pepsin digestions were performed on 50 g portions in two series on two consecutive days with three individual sheep samples, the five processing methods, a negative untreated control and three positive untreated controls on each day. Pepsin-HCl digestion was performed after 20 h incubation of the samples at 7 °C. Bradyzoite (10^3/well) and tachyzoite (10^3/well) controls in medium or in combination with digest of the untreated positive and negative controls were additionally inoculated on cell culture.

After 3 weeks maintaining the cell cultures, the tachyzoite controls (with and without digest) showed growth of *T. gondii*, indicated by a reduction in Cq values over time (Fig. 2). None of the processed samples showed growth of *T. gondii* on cell culture, however also only one out of six unprocessed positive controls showed growth, therefore these negative results for processed samples do not necessarily indicate that processing was effective. None of the individually tested sheep hearts was found positive. The bradyzoite controls (with and without digest) did not show growth (data not shown).

3.5. Experiment C: comparison of cell culture and mouse bioassay using a tachyzoite dilution series

To compare sensitivity of the cell culture and mouse bioassay method, a 10-fold dilution series of tachyzoites was prepared in a negative heart digest and in RPMI-1640. Consistent positive results were obtained with inocula containing 10 or more tachyzoites in RPMI for the mouse bioassay, and 100 or more tachyzoites in RPMI for the cell culture method (Table 7). One mouse inoculated with 10

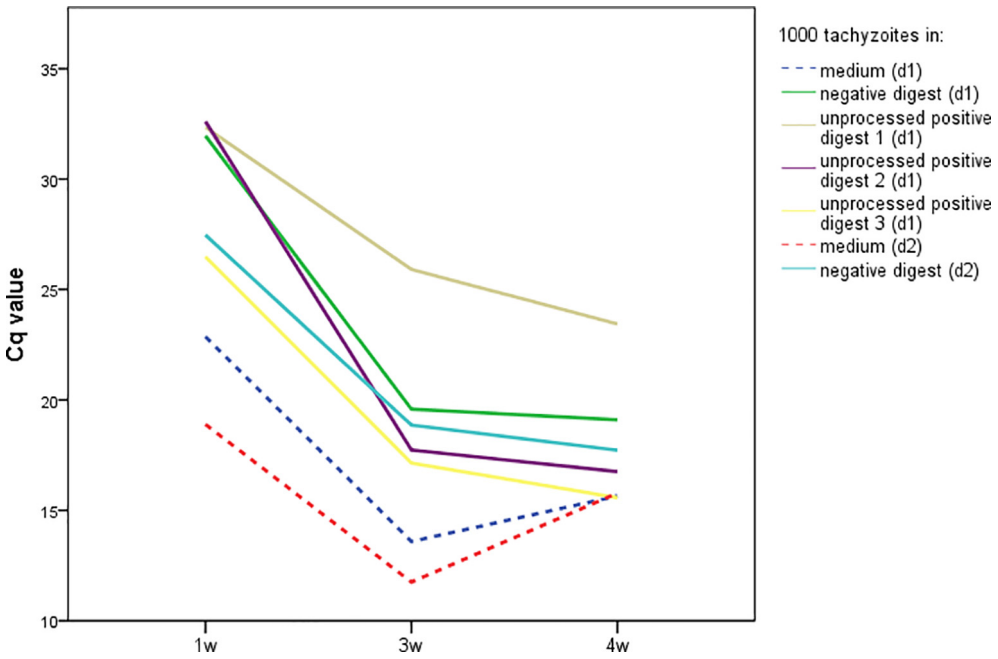

Fig. 2. *Toxoplasma gondii* DNA in cell culture after inoculation with 10^3 tachyzoites in culture medium (dashed lines) or pepsin digest of sheep heart samples (solid lines) (experiment B). Cycle quantification (Cq) values in quantitative PCR for tachyzoite controls (10^3 per well) inoculated with digest of three unprocessed positive controls (P1, P2 and P3), digest of negative control (N) or in RPMI-1640 medium on a 6-well plate with RK13-cells on first (d1) and second (d2) days of experiment B. DNA was isolated from culture medium harvested after 1 week (1w), 3 weeks (3w) and 4 weeks (4w) of maintenance.

Table 7
Comparison of mouse bioassay and cell culture for detection of viable *Toxoplasma gondii* (experiment C). Different tachyzoite concentrations in cell culture medium or pepsin-digested sheep heart were assessed. Viability was assumed if a positive PCR or serological reaction was obtained for bioassay mice, or when the concentration of *T. gondii* DNA in cell culture medium increased.

	Cell culture medium		Pepsin-digested sheep heart	
Tachyzoites	Mouse bioassay	Cell culture	Mouse bioassay	Cell culture
0	0/2	0/1	0/2	0/1
10	6/6[a]	1/2	4/4[a]	1/3
10^2	6/6[a]	2/2	6/6	0/3
10^3	4/4	2/2	4/4	3/3
10^4	4/4	2/2	4/4	2/2

[a] In each of these groups one mouse was found dead at 9 days p.i. and not tested, but time of death and clinical picture were consistent with *T. gondii* infection, therefore these mice are considered positive.

tachyzoites in RPMI survived until the end of the experiment and was positive by qPCR on brain (Cq value 33.7), but not by ELISA or p30 immunoblot. All other mice inoculated with tachyzoites were euthanized or died between five and 9 days post inoculation; all mice that were euthanized were positive by qPCR on peritoneal fluid (Cq values between 12.0 and 17.3). Multivariable logistic regression analysis on the data in Table 7 demonstrated the probability that a test is positive increases with higher tachyzoite concentrations (odds-ratio (OR) 49.8 for log-transformed tachyzoite concentration, 95% CI 3.71–669, $P = 0.003$), and the probability is lower in cases where pepsin digests are assessed by cell culture (OR 0.011, 95% CI 0.00–0.84, $P = 0.042$) (data in Table 7). The use of a mouse bioassay to examine spiked pepsin digests (OR 10.3, 95% CI 0.22–479, $P = 0.234$) or spiked medium (OR 12.9, 95% CI 0.32–524, $P = 0.175$) did not significantly increase the probability of a positive test result compared with examining spiked medium by cell culture (reference category).

3.6. Experiment C: effect of processing

For the final experiment 53 sheep hearts were obtained from the slaughterhouse. Out of 53 sheep hearts, 16 were negative, six doubtful, and 28 positive by ELISA. The 14 sheep hearts with the highest S/P% in ELISA were tested individually by MC-PCR, and an additional 12 sheep hearts were tested in pools (two hearts per pool matched by S/P%). Only one sample tested positive by MC-qPCR (Cq value 29.2). This was an individual sheep (S/P% 241%) and only 39 g of tissue remained. Therefore, the portions for processing were reduced from 50 g to 30 g, and the hearts of 11 serologically positive but MC-qPCR negative sheep were added to make up a total of at least 660 g. Tissues of 10 experimentally infected mice were added to the positive batch (16.9 g of muscle tissue and 5.8 g of hearts and lungs). Similarly to experiment B, processing was performed on two consecutive days with an additional spike of 25 tissue cysts per portion on the second day (series 2). Processing methods I–IV were carried out in duplicate each day; method V was only included in duplicate on the first day. Four positive untreated controls, a negative control and a negative control spiked with tissue cysts were subjected to pepsin-HCl digestion and inoculated on cell culture or in mice. To confirm tissue cyst viability, controls with 5, 10, 20 or 40 tissue cysts in RPMI without digestion were used in the mouse bioassay.

Fungal contamination was encountered in the cell culture method, and no clear results were obtained for the processing

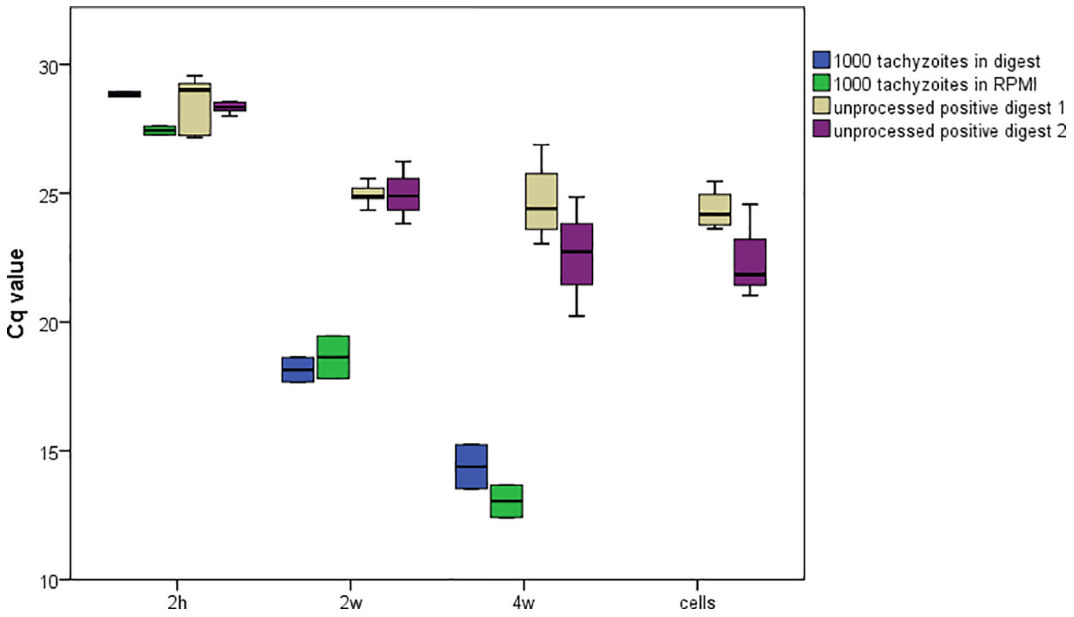

Fig. 3. *Toxoplasma gondii* DNA in cell culture after inoculation with unprocessed positive batch samples (series 1), or 10^3 tachyzoites in culture medium or tissue digests (experiment C). Boxplots for five replicates of two positive unprocessed controls and two replicates for 10^3 tachyzoite controls in culture medium (RPMI) and negative heart digest (digest). DNA was extracted from cell culture medium after 2 h, 2 and 4 weeks (2w, 4w) of maintenance on RK13 cells and cells scraped from the wells after 4 weeks (cells).

methods (data not shown). The positive controls that were left untreated from the first day of processing did show growth, but not as clearly as the tachyzoite controls (Fig. 3). Results of the positive unprocessed samples of the second day were less consistent (data not shown).

All mice became infected when inoculated with the digest of the unprocessed positive controls or the tissue cyst control. The number of infected mice was reduced when the tissue had been processed with additives prior to digestion and inoculation, however none of the methods was shown to be fully effective (Table 8). Cq values for peritoneal fluid varied from 12.3 to 17.7 and for the brains from 23.6 to 36.4 (Supplementary Table S2). The mice euthanized 6 weeks post inoculation were tested for IgG antibodies by IDvet ELISA, but none of these mice tested seropositive. When tested in p30-immunoblot, one mouse tested positive at a dilution of 1:100 (method III in series 2), two further mice at 1:10 dilution (method I and II in series 1), one of which was also positive by qPCR on the brain (method I) (Supplementary Table S2). Statistical analysis on the overall results indicates differences between groups (χ^2-test $P < 0.0005$), but lacks power to find statistical differences between the processing methods.

4. Discussion

Raw meat products were predicted to attribute most to meat-borne *T. gondii* infections in the Netherlands by QMRA modelling (Opsteegh et al., 2011a). However, the effect of salting is based on experiments described in the literature and may not be applicable to meat processing in industry. Therefore, it was our aim to study the effect of meat processing on *T. gondii* viability. In these

Table 8
Effect of processing methods I–V (experiment C, Table 2) on infectivity of *Toxoplasma gondii* in positive sheep heart spiked with infected mouse tissue (series 1 and 2) and tissue cysts (one per g) (series 2). *T. gondii* viability was evaluated by mouse bioassay and the number of positive mice (pos/n) is based on positive quantitative PCR (qPCR) results for peritoneal liquid (for mice euthanized within 13 days p.i.) and/or brain, as well as of serological findings in a *T. gondii* immunoblot (p30 IB). Each subscript number in column pos/n denotes a subset of categories whose column proportions do not differ significantly from each other. Detailed results are provided in Supplementary Table S2.

Processing	pos/n	Series 1				Series 2			
Na-lactate/Na-acetate/NaCl		Euthanasia <41 days p.i.	qPCR peritoneal	qPCR brain	p30 IB	Euthanasia <41 days p.i.	qPCR peritoneal	qPCR brain	p30 IB
I: 1.19/0.26/1.20	$2/8_{1,2}$	0	ND	2/4	1/4	0	ND	0/4	0/4
II: 0.61/0.29/0.15	$5/7_{1,2}$[a]	0	ND	1/3	1/3	2	2/2	3/4	0/2
III: 1.08/0.32/1.60	$2/8_{1,2}$	0	ND	0/4	0/4	0	ND	1/4	1/4
IV: 1.40/0/0	$6/7_{1,2}$[a]	3	3/3	3/4	0/1	3	3/3	3/3	ND
V: 1.00/0/1.00	$1/4_{1,2}$	0	ND	1/4	0/4	ND	ND	ND	ND
Unprocessed	$8/8_1$	4	4/4	2/2	ND	4	4/4	2/2	ND
Tissue cysts	$8/8_1$	4	4/4	ND	ND	4[b]	3/3	2/2	ND
Negative	$0/4_2$	0	ND	0/2	0/2	0	ND	0/2	0/1[c]

[a] In each of these groups one mouse was found dead and peritoneal fluid and brain were not tested. Since the time of death or clinical picture was not consistent with toxoplasmosis these mice were excluded.
[b] One mouse was found dead at 9 days p.i., not tested, but time of death and clinical picture were consistent with *T. gondii* infection and the mouse was considered positive.
[c] One mouse was omitted from p30 immunoblot testing by mistake.

experiments the infectivity or viability of the parasite is the outcome measure of interest. Currently, bioassay using either cats or mice is the standard method to demonstrate *T. gondii* viability but, for ethical reasons, this is not preferred for large scale testing as has, for example, become evident from the recent closure of these facilities at the United States Department of Agriculture (USA) (USDA Press release No. 0042.19). Therefore, our second aim was to work towards an in vitro method to replace the mouse bioassay in this type of experiment. We focused on a PCR-based assay using PMA incubation to selectively detect DNA from viable parasites, and a cell culture assay, similar to what has been described by Zintl et al. (2009). The findings presented provide a useful base for future research, but further optimization of methods and additional testing of processing methods is needed.

Our study design consisted of an initial evaluation of in vitro viability assays and selection of the most promising method for more thorough evaluation and comparison to the mouse bioassay. The experiments with viable and killed *T. gondii* tachyzoites and bradyzoites indicated that the PMA method did not provide consistent results. Moreover, although available DNA was suppressed in samples spiked with killed parasites (demonstrated by an increase in the Cq value), these samples still gave a positive result by qPCR, thus not providing clear discrimination between viable and killed parasites. Therefore, the PMA method was not included in experiments A–C. Possibly, additional repetitions of the PMA experiments, evaluation of other influencing factors such as PCR target and amplicon length (Fittipaldi et al., 2012), or the use of PMAxx™ (Randazzo et al., 2016), could have resulted in more consistent data and better discrimination between viable and killed parasites. However, thorough evaluation of a PMA-based method for discrimination of viable and inactivated oocysts also failed to completely suppress PCR signals for killed oocysts (Rousseau et al., 2019). Meat digests have a high turbidity and a background of host cell material, and possibly present a complex matrix for an effective PMA-based viability assay.

The cell culture method performed well with tachyzoites and bradyzoites in culture medium, and inoculation of tissue digest spiked with bradyzoites resulted in decreasing Cq values in *T. gondii* qPCR, indicating parasite multiplication. Cq values decreased by 10 Cq values maximum (Fig. 1), indicating approximately a thousand-fold increase in *T. gondii* DNA concentration. Maintenance did not result in free tachyzoites visible by light microscopy, confirming that monitoring *T. gondii* DNA content by qPCR helps increase the sensitivity of the in vitro method. As the decrease in Cq values with naturally infected sheep heart (A17) and unprocessed controls (experiment C) was not as clear as those for tachyzoite controls, clear criteria should be established about when to consider the increase in *T. gondii* concentration as an indication of growth or viability. To enhance growth, different cell lines (e.g. HeLa, VERO) (Hughes et al., 1986) (Jabari et al., 2018) or the addition of growth promotors (Nagamune et al., 2008; Goroll, T., Daugschies, A., Entzeroth, R., Bangoura, B., 2017. Development of an alternative assay to study the infectivity of *T. gondii* (P31), ApiCOWplexa, Madrid, p. 138.) might be useful. RK13 cells were used for the cell culture assay, as they are easy to maintain and are successfully used in our hands to proliferate RH-strain tachyzoites for antigen production (Opsteegh et al., 2011b). RH tachyzoites are well adapted to our cell culture in RK13 cells, and the same growth cannot be expected of *T. gondii* bradyzoites isolated from naturally infected sheep, which are most likely infected with type II strains (Opsteegh et al., 2010a).

In the cell culture method, issues with PCR inhibition and fungal growth were encountered, and both of these issues appear to be related to the presence of tissue digest. It is important to tackle these issues, as failure to obtain a test result may result in a lack of statistical power. There is still some digest present after 4 weeks of maintenance, because it is not possible to remove all tissue digest after 2 h of incubation (the RK13 cell monolayer is affected by the digest and will easily detach from the flask) and only 50% of medium is replaced during maintenance. It will be important to find a better balance between removal of digest and loss of RK13 cells, for example by adding fresh RK13 cells after inoculation and additional wash steps once monolayers have recovered. To prevent fungal and bacterial growth, and since it is often not possible to work under sterile conditions with animal tissues derived from a slaughterhouse, antibiotics and antimycotics are used. Despite the addition of amphotericin B, problems with fungal growth were encountered as described by Genchi et al. (2017). Since there are not many different antimycotics available, rinsing in ethanol and (overnight) incubation of the meat in PBS with antibiotics and amphotericin B prior to cutting and grinding may help prevent fungal contamination.

Sensitivity of the cell culture assay and the mouse bioassay appeared comparable with tachyzoite dilution series prepared in RPMI (Table 7), but the experiment may have lacked power to find significant differences. The results did, however, clearly indicate that the cell culture method was hampered by tissue digest. For the datapoints included in Table 7, no fungal growth or PCR inhibition was observed, therefore a cytopathogenic effect of the digest on the tachyzoites, the RK13 cells, or both is assumed. Therefore, not only replication of *T. gondii* but also resistance to meat digest needs to be considered when a cell line is selected. Moreover, optimization of the pepsin digestion or a switch to trypsin digestion (Zintl et al., 2009; Villena et al., 2012) may be needed to improve the cell culture method. Tissue digestion is critical and difficult to assess, as it needs to be sufficient to homogenise tissue and release bradyzoites, but should not inactivate the bradyzoites. The most commonly used pepsin-HCl digestion protocol was described by Dubey (1998), and most literature refers to this protocol without providing further details. However, blender speeds may vary, and may need to be adjusted depending on the tissue, and the concentration of the HCl stock is not provided in the original reference (7.0 ml of HCl per 500 ml of acid pepsin solution). Moreover, pepsin is available with varying biological activities declared in different units, which may lead to confusion. In our case, incomplete digestion was observed (i.e. too much tissue remained on the sieve). Based on personal communication and comparison with other protocols (e.g. Jacobs et al., 1960; Verhelst et al., 2015; Schares et al., 2017; Hill et al., 2018) the blending protocol and pepsin and HCl concentrations were varied. With the incomplete digestion in experiments A and B, digestion may have contributed to the low recovery from naturally infected samples. In the final experiment C (including mouse bioassay) we used lower HCl and increased pepsin concentrations, and results for the unprocessed positive controls were more consistent, showing growth for both replicates in the first series. In future, for further evaluation of digestion protocols, the digestion of tissue, the survival of bradyzoites and the cytopathogenic effect on the cells need to be considered.

In this study viability assays were used to study the effects of processing on *T. gondii* in meat. To study these effects it is important to perform the experiments on tissues of infected animals with intracellular tissue cysts. Artificial spiking might influence not only the effect of the processing methods but also the performance of the viability assays. It was our aim to use naturally infected animals, and sheep were selected as the seroprevalence in sheep is known to be high (Opsteegh et al., 2010b), there is good concordance between the detection of antibodies and presence of *T. gondii* by MC-qPCR (Opsteegh et al., 2010a) and mouse bioassay (Opsteegh et al., 2016), and the heart has been identified as predilection site (Opsteegh et al., 2016). Unfortunately, in the described experiments, the concordance between serology and

MC-qPCR was low compared with a previous study (Opsteegh et al., 2010a) and additional spiking was needed. Probably, the reduced sample size (50 g or 25 g versus 100 g up to the whole heart in the study published in 2010) has affected the concordance, as the concentration of tissue cysts can be low and the distribution is not homogeneous. A minimum of 25 g was used for MC-qPCR, leaving less tissue to be included in the positive batch. It might be better to omit the MC-qPCR and instead select positive animals based on ELISA, and perform DNA isolation and qPCR on the digests for confirmation. For future experiments, experimental infection might be a more reliable source of positive tissue, however the use of experimentally infected animals does not guarantee a consistent positive result for unprocessed tissue or an even distribution of *T. gondii* over the different portions (Sommer et al., 1965; Abdulmawjood et al., 2014).

In the presented experiments, meat processing was limited to meat homogenisation, mixing in NaCl, sodium lactate and sodium acetate, and storage at 7 °C for 20 h. For storage conditions, the highest acceptable temperature and shortest time from production to consumer for industrial meat processing companies were chosen. Meat was cut to 3 mm particles according to industrial processing and has been shown not to affect *T. gondii* viability (Sommer et al., 1965). Most raw meat products contain other additions such as sauces or spices. These are probably not very influential on *T. gondii* viability, as pH variation in the range of 5–7 (Pott et al., 2013) as well as condiments such as black pepper and garlic (Navarro et al., 1992) have been shown to have little effect. Therefore, processing was limited to mincing and addition of NaCl, sodium lactate and sodium acetate, thereby making the results applicable to a range of raw meat products available in the Netherlands.

With regard to the effect of processing, the mouse bioassay results demonstrate that none of the methods was 100% effective. With a maximum of eight mice per processing method, the power to find statistical differences is limited and indeed, the statistical analysis on the overall number of positive mice does not indicate differences between the processing methods. However, taking clinical, qPCR or serological reactions into account (Supplementary Table S2), methods I, III and V showed a larger effect with none of the mice reaching the humane endpoints for euthanasia, compared with methods II and IV. Methods II and IV had very low salt concentrations (0.15% for II and 0% for IV) but relatively high sodium acetate (0.29% in II) or sodium lactate (1.4% in IV), indicating that NaCl was probably the most influential ingredient. Adding 1.4% sodium lactate has previously been reported to be effective (Hill et al., 2004, 2006). In those experiments, infected pork loins were injected with a solution containing 1.4% sodium lactate in combination with sodium tripolyphosphate and sodium diacetate, and tested by cat bioassay. Although those other additives were not effective when used without the sodium lactate, it cannot be ruled out that they enhanced the effect of sodium lactate. Possibly, the method (injection versus mixing in additives into minced meat) is also influential. Our results indicate that mixing meat with 1.4% sodium lactate is not very effective.

For the processing method I (1.2% NaCl), *T. gondii* DNA was detected by qPCR in the brains of two out of eight mice, one of which was also positive by p30-immunoblot. For method III (1.6% NaCl), two out of eight mice were positive, one by qPCR on brain and another one by p30-immunoblot. Method V (1.0% NaCl) was only included in series 1, and one out of four mice was positive by qPCR on brain. Only one of the PCR-positive mice tested positive by p30-immunoblot and none of the mice was positive by ID.Vet ELISA. By contrast, in only one out of the three p30 immunoblot positive mice was *T. gondii* DNA detected in the brain. A lack of concordance between PCR results and serology has been previously reported for bioassay in mice (Burrells et al., 2015, 2018; Opsteegh et al., 2019) as well as wild mice (Galal et al., 2019). It remains to be studied whether failure to detect *T. gondii* parasites or DNA in serologically positive mice indicates a lack of sensitivity of the direct detection method (i.e. a sample is tested and tissue cysts can be missed), or an antibody response can also be induced without the presence of viable and multiplying parasites. A highly specific PCR was used and there was no indication of contamination at any point in this experiment, therefore failure to detect antibodies in PCR-positive mice suggests that including PCR-based detection increases sensitivity of the mouse bioassay.

The effect of processing, and salting in particular, has been studied previously. Three studies used matrices and processing methods (Navarro et al., 1992; Pott et al., 2013; Abdulmawjood et al., 2014) similar to our study. In our study, the effect of processing (one out of eight mice positive after 20 h with 1.6% NaCl, 1.08% sodium lactate and 0.315% sodium acetate) appears to be stronger than in the study by Pott et al. (2013) in which infective tissue cysts were still detected when homogenised muscle tissue of experimentally infected mice was incubated for 8 days in RPMI supplemented with 2.0% NaCl (Pott et al., 2013). Also, Navarro et al. (1992) reported more survival of infective tissue cysts in sausages prepared with muscle tissue of experimentally infected pigs; after 24 h of incubation, six (1.25% NaCl), three (2.0% NaCl) and two (2.5% NaCl) out of six mice became infected (Navarro et al., 1992). Abdulmawjood et al. (2014) prepared raw meat products (teewurst, mettwurst and salami) according to conventional and organic industrial processing. For all three products, one or two mice became infected, demonstrating that inactivation was incomplete (Abdulmawjood et al., 2014), which corresponds with our results. In summary, direct comparison of the presented results with the literature is difficult due to variations in salting concentration and duration, but it appears that the effect was stronger than presented previously by Navarro et al. (1992) and Pott et al. (2013).

In conclusion, in our hands a PMA-based viability assay was inconsistent in discriminating between viable and killed *T. gondii* in tissue digests. Results with the cell culture method were more promising with detection of natural infection in a sheep and positive results for part of the unprocessed controls. Moreover, cell culture appeared to perform similarly to mouse bioassay when tachyzoites were spiked in culture medium. However, further optimization of the cell culture method is needed and tissue digestion has been identified as a critical point, since the presence of digest affected sensitivity, and issues with PCR inhibition and fungal contamination were encountered. Due to a lack of consistent positive results for unprocessed portions and fungal growth, conclusions with regards to processing could not be based on the cell culture results. Mouse bioassay results indicate that mainly NaCl is influential, and adding NaCl, sodium lactate and sodium acetate in the low concentrations used in these experiments reduced the number of infected mice, but does not guarantee that all *T. gondii* that could potentially be present in raw meat products are rendered non-viable. Additional experiments are needed to find effective concentrations for combinations of these additives. After further optimization, hopefully in vitro methods can replace or reduce the number of mouse bioassays needed. A reliable in vitro method would allow more extensive evaluation of current and future processing methods, thereby providing a better indication of the risk of *T. gondii* infection via consumption of various meat products.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.ijpara.2020.04.001.

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