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Inactivation of *Trichinella* muscle larvae at different time-temperature heating profiles simulating home-cooking

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ABSTRACT

Background: Trichinellosis is caused by consumption of raw or undercooked meat containing infective *Trichinella* muscle larvae (ML). Only few studies on heat-inactivation of *Trichinella* ML are available in literature and more validated data concerning heat inactivation is needed to improve the risk estimation.

Objective and methods: The aim of the present study was to evaluate the two in vitro methods "staining" and "morphological examination" as proxies for *Trichinella* ML heat inactivation in comparison with the mouse bioassay method to get more insight in the relationship between heat, heating time and inactivation of *Trichinella* ML. The second aim was to evaluate whether these methods could replace the bioassay in the light of ongoing animal use reduction in lifescience research.

Tubes containing quantified live *Trichinella* ML were exposed to heat profiles ranging from 40 to 80 °C. Subsequently, inactivation was evaluated using both methylene blue staining and morphological examination, which was validated by bioassay. Results were used to model *Trichinella* inactivation.

Results: Trichinella muscle larvae exposed to 60 °C or higher for 12–12.5 min were not infective to mice. We found that morphological examination was more consistent with the bioassay than methylene blue staining. Modelled inactivation fitted experimental data consistently. Moreover, this study shows that larval *Trichinella* morphology may be used in situations where bioassays are not possible or prohibited.

Conclusions: The relationship between heat and inactivation of larvae obtained from this study could be used in *Trichinella* QMRA models to improve quantification of the risk of *Trichinella* infection.

1. Introduction

Nematode parasites of the genus *Trichinella* are globally distributed zoonotic pathogens. All identified twelve *Trichinella* species and genotypes can infect humans, and four of them (*T. spiralis, T. britovi, T. pseudospiralis, and T. papuae*) can establish infection in pig herds (Pozio and Darwin Murrell, 2006). People acquire infection by consuming raw or undercooked meat that contains infective *Trichinella* muscle larvae (ML), particularly from wild game meat or pork (Pozio et al., 2003; Pozio and Marucci, 2003; Dupouy-Camet, 2000). The symptoms and severity of human trichinellosis relate to the number of infectious larvae in consumed meat and may include fatigue, weakness, fever, diarrhoea and muscle pains. In severe cases, even death can occur (Capo and Despommier, 1996).

Three processing methods to inactivate *Trichinella* prior to human consumption have been recognized by the International Commission on Trichinellosis: cooking, freezing and irradiation (Gamble et al., 2000). Although heat inactivation is considered a very effective control method to prevent human infection, only few studies have been published to evaluate the effect of heat on the inactivation of *Trichinella* ML, two using pork from experimentally infected pigs in combination with rat bioassays (Carlin et al., 1969; Kotula et al., 1983; Randazzo et al., 2011) and a third study evaluating *Trichinella* heat inactivation using an in vitro method (Randazzo et al., 2011).

Recently, the incidence of human trichinellosis from wild boar and from pigs kept under different housing systems was estimated using a

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quantitative microbial risk analysis (QMRA) model (Franssen et al., 2017, 2018). This QMRA contains a heat-inactivation module to calculate *Trichinella* survival based on cooking scenarios and on data provided by Carlin et al. (1969) and Randazzo et al. (2011), although quantification of the temperature effects on *Trichinella* ML in those studies were difficult to combine. The study by Kotula et al. (1983), although a funding study for practical heat-inactivation of *Trichinella* in pork, could not be used to model *Trichinella* inactivation since no larval counts were reported in that publication.

Although the data that were used provided the best information available to formulate a heat-inactivation module for the *Trichinella* QMRA, there is uncertainty in the data caused by differences between the studies by Carlin et al. (1969) and Randazzo et al. (2011), which could have an effect on the estimated number of *Trichinella* larvae surviving heat treatment and modelled number of human trichinellosis cases following consumption of *Trichinella* infected pork.

The aim of the present study was to evaluate two in vitro methods as proxies for *Trichinella* ML heat inactivation in comparison with the mouse bioassay method to get more insight in the relationship between heat, heating time and inactivation of *Trichinella* ML. The second aim was to evaluate whether staining and morphological examination could replace the *Trichinella* bioassay in the light of ongoing animal use reduction in lifescience research.

2. Materials and methods

We designed three experiments to evaluate and validate *Trichinella* ML survival.

In the first experiment (Exp. A1) we mimic oven cooking, and evaluate the intrinsic effect on naked *Trichinella* ML of exposure to time-temperature combinations by microscopy, in combination with the mouse bioassay method (Fig. 1). In the second experiment (Exp. A2), time-temperature combinations mimicking pan-frying were evaluated by microscopy in combination with the mouse bioassay method. In the third experiment (Exp. B) methylene blue staining and morphological examination were evaluated microscopically in more detail, after *Trichinella* ML heat exposure. Prior to the inactivation experiments, the best incubation condition for methylene blue staining was tested with live and dead larvae after heating, which was found at 37 °C for 15 min.

All animal studies were approved by the Ethical Committees of RIVM (Bilthoven, the Netherlands, DEC permit number 2016–0047) and Jilin University affiliated with the Provincial Animal Health Committee (Jilin province, China, Ethical Clearance number IZ-2009-08).

2.1. Heating profiles in tubes and plates

Preliminary experiments were performed to determine heating profiles inside and outside tubes and plates using water without larvae, for biosafety reasons. Heating experiments were carried out using a calibrated digital thermometer (Testo 720 Digital Thermometer, Testo BV, Almere the Netherlands) and programmable digital thermic trackers (Thermo button 22T, range 0–125 °C, Resolution 0.1 °C, sample rate 15 s, Proges Plus, Nantes, France) according to the manufacturer's instructions. Temperatures of both tube or plate content and the water bath were recorded. The lid of an Eppendorf tube was punctured to allow the calibrated thermometer tip to pass into 100 µl water that was used throughout the experiments. The tube was immersed into the water bath until the water reached just beneath the tube lid. Plates were allowed to slightly float in the water bath.

2.1.1. Simulated oven-cooking temperature profile – tube (A1)

In general terms, the time-temperature profile in the core of a larger portion of meat (one or more kilograms) during oven-cooking is characterized by a sigmoidal curve, showing a lag phase from room temperature to around 30 °C, followed by a steep linear increase up to 60–70 °C and a subsequent flattening of the curve towards 80–90 °C, depending on the temperature setting and the type of oven (Skjöldebrand and Hallström, 1980). Typically, the cooking time takes around 90 min in a consumer setting. We simulated a linear increase in temperature of 10 °C per 15 min and a total cooking time of 90 min, which is in accordance with the experiments conducted by Randazzo et al. (2011).

The sensor tip of an accredited digital thermometer (Testo 720 Digital Thermometer, Testo BV, Almere the Netherlands) was passed through the cap of an Eppendorf tube containing 0.1 ml water at room temperature, which was placed in a water bath, in such a manner that the water surface reached the rim supporting the tube caps. Every 5 min, the temperature of the water bath was increased manually by 3, 4 and again 3 °C respectively, to realise a temperature increase of 10 °C per 15 min. After 15 min, the tube attached to the thermometer was removed from the water bath and placed in ice water for 30 s to lower the temperature (below 40 °C). The actual temperature of the water bath and the tube content were recorded every 15 s.

2.1.2. Simulated pan-frying temperature profile tube (A2)

Pan-frying of meat usually involves much smaller portions (100–150 g) and short cooking times (10–20 min) during which the core of the meat is heated quickly to a final temperature of 60–70 °C, which is characterized by a quick incline during the first few minutes, to reach the final temperature asymptotically (Lahou et al., 2015). In Experiment A2, the tube content was exposed for 15 min to a constant temperature of 40, 50, 60, 70 and 80 °C respectively. The temperature of the tube content was recorded every 10 s; the target temperature of the water bath was confirmed using an accredited digital thermometer.

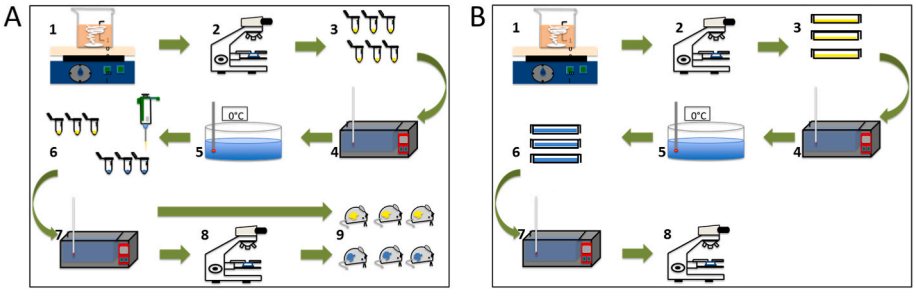

Fig. 1. Process overview of heat treatment experiments.
A: experiments A1 and A2.
1. Artificial digestion to liberate *Trichinella* muscle larvae. 2. Larvae were enumerated by microscopy. 3. Larvae were transferred to Eppendorf tubes in 100 µl aliquots containing 100 larvae each. 4. Larvae were heat-treated in series of six samples. 5. Larvae were cooled on ice to stop the heating process. 6. Methylene blue was added to three vials of each series (Eppendorf vials with blue content) and three other samples were mock treated (vials with yellow content). 7. All vials were placed in water bath at 37 °C for 15 min. 8. Three vials contained Methylene blue were evaluated microscopically. 9. All series were administered to inbred mice per gastric tube.
B: experiment B.
1. Artificial digestion to liberate *Trichinella* muscle larvae. 2. Larvae were enumerated by microscopy. 3. Larvae were transferred to plates in 30 ml aliquots containing 100 larvae each. 4. Larvae were heat-treated. 5. Larvae were cooled on ice to stop the heating process. 6. Methylene blue was added to the plates in 30 ml aliquots. 7. Plates were placed in a water bath at 37 °C for 15 min. 8. Plates containing larvae were evaluated microscopically.

2.1.3. Simulated pan-frying temperature profile plate (B)

A digital programmable temperature logging button was placed in a plastic 9 × 9 cm counting plate containing 30 ml of water. The plate with temperature logger and closed lid was placed on a support in a water bath, which allowed the plate to slightly float. The plate content was exposed to a constant temperature of 40, 50, 60, 70 and 80 °C respectively. The target temperature of the water bath was confirmed using an accredited digital thermometer. After 15 min, the plate was cooled on wet ice for 2 min. Time and temperature profiles were downloaded from the temperature logging button using the manufacturer's software. Temperature profiles were visualized using Microsoft Excel.

2.2. Parasite exposure to time-temperature profiles

T. spiralis (ISS-534) ML were obtained by the magnetic stirrer digestion method at 37 °C from an experimentally infected mouse according to the OIE Terrestrial Manual (OIE-Manual, 2012).

2.2.1. Simulated oven-cooking temperature profile (A1)

Thirty-six aliquots of 0.1 ml water with 100 *T. spiralis* ML (counted under microscope) were transferred to Eppendorf tubes. All Eppendorf tubes were placed in a thermostat-controlled water bath as described above and exposed to a continuous heat profile described under 2.1.1. At the end of each 15-min interval, one series of six tubes was removed from the water bath and placed in ice water (0–7 °C) for 30 s to lower the temperature beneath 40 °C (step 1–5 in Fig. 1). In this way, larvae were exposed to a cumulative increase in temperature from 30 °C to 90 °C and in time from 15 to 105 min. The temperature of the water baths was controlled using a certified thermostat and validated using a second certified thermometer in the experiments.

2.2.2. Simulated pan-frying temperature profile (A2)

Trichinella larvae were counted and transferred to 36 Eppendorf tubes, resulting in aliquots of 0.1 ml water with 100 ML of *T. spiralis* (isolate ISS-534) and each series of six tubes was placed in the water bath at a different constant temperature for 15 min as described under 2.1.2. Six temperature points ranging from 30 °C to 80 °C were used. After heat treatment, each series of six tubes was removed from the water bath and placed in ice water (0–7 °C) for 30 s (step 1–5 in Fig. 1).

2.2.3. Evaluation of methylene blue staining and morphology (B)

Trichinella larvae in 30 ml of tap water were exposed to the temperature profiles as described in section 2.1.3. To prevent larval loss, experiment B was conducted in single plastic dishes equipped with a numbered grid underneath the bottom (D210 -16 – Square Petri dish with grid, Simport Scientific, Beloeil, Quebec, Canada), without any further manipulation of parasites, since microscopical examination was the endpoint of this experiment. *Trichinella spiralis* (isolate ISS-3, B2) larvae were digested from infected mice according to EU-RM (European-Commission, 2015). One hundred *Trichinella* larvae were added to plastic dishes containing 30 ml of tap water and these were placed with closed lids in a water bath at a constant temperature for 15 min. A temperature range between 30 °C and 85 °C with increments of 5 °C was used. After 15 min in the water bath, the dishes were cooled on ice for 2 min. Subsequently, larvae were stained by adding 10 ml of aqueous methylene blue (MB) solution (1:10,000 w/v) and subsequent incubation at 37 °C for 15 min. The numbers of stained/non-stained and coiled/non-coiled larvae in the dishes were examined microscopically. Temperature and time settings are summarized in experiment B of Table 1.

2.2.4. Methylene blue staining and infection of mice

After removal from the heat treatments described above, 0.1 ml of MB solution (1:10,000 wt/volume in water) was added to three replicates of each series of heat treated *Trichinella* ML and placed into a 37 °C water bath for 15 min. Then, all tubes were removed from the water bath and larvae were examined microscopically for each of these three tubes. MB stained and non-stained larvae were counted, and the shapes of larvae in each tube were recorded as coiled or non-coiled. Larvae were considered coiled when they were in spiral shape; such larvae may exhibit rolling and unrolling motility of the spiral shape (Fig. 3A - C). Dead, non-coiled, larvae showed a comma shape and were non-motile under any circumstance (Fig. 3D - E). In the meantime, three other replicates per series were also placed into the 37 °C water bath for 15 min, but without adding MB solution and without microscopic evaluation. Finally, the contents of the three MB-stained and counted tubes and three non-stained tubes (for evaluation of the impact of the staining step) of each series were administered directly into the stomach of six inbred mice via gastric tubes, each mouse receiving the content of one tube (thus 100 ML) (step 6–9 in Fig. 1).

2.2.5. Evaluation of bioassay

Six weeks post inoculation, all mice were weighed, diaphragms were checked microscopically for *Trichinella* ML and the whole carcass, including the checked diaphragm was digested individually according to the OIE Terrestrial Manual (OIE-Manual, 2012). Subsequently, ten drops of 20 μl (thus 200 μl in total) of *Trichinella* ML suspension isolated from each mouse were checked for *Trichinella* ML under the microscope. If *Trichinella* ML were found in diaphragm and/or one of the ten drops, then that mouse was recorded as infected and the number of larvae in all ten drops was counted. Subsequently, the total number of larvae was calculated from the larval count in 200 μl in relation to the total volume of digest fluid. The number of larvae per gram (LPG) was calculated by dividing the total larval count by the mouse bodyweight in grams. If no *larvae* were found in diaphragm or any of the drops, then the mouse was recorded as not infected.

2.3. Statistical analysis

For simulated oven-cooking (experiment A1), tubes are slowly heated in a water bath. We can describe the temperature of the tube T_{tube} by a linear model with the temperature of the water bath T_{bath} as continuous predictor plus an intercept (β_0) and error (ε):

$$T_{tube} = \beta_0 + \beta_1 T_{bath} + \in, \text{where } \in \tilde{} N(0, \sigma^2)$$

Table 1
Exposure of *Trichinella spiralis* larvae to different time-temperature profiles simulating consumer home-cooking.

Exp.	Temperature range	Increment	Temp. profile	Morphology examination	MB staining	Bio-assay
A1	30–90 °C	10 °C	10 °C increase per 15 min	Yes	Yes	Yes
A2	30–80 °C	10 °C	Constant for 15 min	Yes	Yes	Yes
B	35–80 °C	5 °C	Constant for 15 min	Yes	Yes	No

A1: *Trichinella* larvae in Eppendorf vials were exposed to gradually increasing temperatures during a 90 min period simulating time-temperature exposure during oven cooking. Every 15 min, 6 Eppendorf vials containing larvae were taken out of the water bath for further analysis.
A2: *Trichinella* larvae in Eppendorf vials were exposed to different constant temperatures for 15 min each, simulating time-temperature exposure during pan-frying.
B: *Trichinella* larvae in Petri dishes were exposed to different constant temperatures for 15 min each, simulating time-temperature exposure during pan-frying. After 15 incubation for 15 min Eppendorf vials containing larvae were taken out of the water bath for further analysis.
Morphology examination: larvae were examined and counted both before and after heating. MB staining: Methylene Blue staining during 15 min at 37 °C.

For simulated pan-frying (experiments A2 and B) it takes some time for the water bath to reach the constant temperature which was set. The actual temperature starts at an intercept of about room temperature, and rises to a plateau which is very close to the desired temperature. We postulate the following relation between actual temperature and set temperature:

$$T(t) = T_{set} - (T_{set} - T_0)e^{-\frac{t}{\kappa}}$$

Here, $T(t)$ (observed temperature) and t (time) are measured, T_{set} is the temperature setting of the water bath. The coefficients to be determined are: T_0, the initial temperature of the water bath, and κ, a heat diffusion parameter.

The average temperature over the 15 min preceding time t is

$$T(t) = T_{set} - \frac{(T_{set} - T_0)}{15}\int_{t-15}^{t} e^{-\frac{\tau}{\kappa}}d\tau = T_{set} + \kappa\frac{(T_{set} - T_0)}{15}\left[e^{-\frac{\tau}{\kappa}}\right]_{t-15}^{t} = T_{set} + \kappa\frac{(T_{set} - T_0)}{15}\left(1 - e^{\frac{15}{\kappa}}\right)e^{-\frac{t}{\kappa}}$$

2.3.1. Dose-response relation and evaluation of morphology and MB

In order to assess the consistency of counted larvae, either via morphology or MB, with infection status of the mice, a dose-response model is needed to relate the larvae that are fed to the mice (the dose) to the probability of infection. We use the dose-response model proposed in Teunis et al. (2012), which results in a sigmoid curve depicting probability of infection as function of larval dose. There are two estimates for the doses administered: counts according to methylene blue, and according to morphology, and a-priori we do not know which of these two most accurately reflects the actual number of living larvae. Hence we fit the dose-response model for two doses, one obtained by methylene blue and one obtained by morphology. We fit the models to the infection outcomes of the bioassay, and obtain estimates of the infectivity. Fitting was performed in Stan (StanDevelopmentTeam, 2018), and all statistical computations were performed in R (RCoreTeam, 2019).

As a next step, we may use both fitted models to predict infection in each mouse. The model outcome showing the best concordance to the actual observed infection status corresponds to the most accurate counting method.

2.3.2. Inactivation model

A useful inactivation model should include both time (t) and temperature (T). We include these in a generalised linear model, with binomial family, and logit link. Note that the logit function resembles a sigmoid curve bounded between zero and one, which makes it particularly suitable for modelling inactivation. Furthermore, actual counts are used in the model fitting, not just the fractions of survival, ensuring proper weighing of the data. We pose the following relation for the probability parameter, which is the probability of survival,

$$logit^{-1}(p) = \beta_0 + \beta_1 t + \beta_2 T + \beta_3(t \times T) + \beta_4\log(t) + \beta_5\log(T) + \beta_6(\log(t) \times \log(T))$$

The logarithmic terms were included since we cannot exclude such a dependence from the onset. A backward elimination based on Akaike Information Criterion (AIC) was performed for variable selection.

3. Results

For simulated oven-cooking, the temperature increase of the Eppendorf tube content was characterised by a linear equation: $T_{tube} = 0.9816 \times T_{bath} - 0.1374$, ($R^2 = 0.9996$) (Fig. 2A). The linear model for relating the temperature of the water bath and the temperature of the tube yielded a non-significant intercept, and a highly significant ($p < 10^{-16}$) slope of 0.98. Given the almost perfect straight line relationship, it was decided not to adjust the temperature of the tube to the temperature of the water bath.

3.1. Simulated oven-cooking (A1)

The average weight of mice at six weeks after inoculation was 19.9 ± 1.6 g, therefore the animals can be considered as a homogeneous group.

Bioassay results showed that muscle larvae were found in mice that received *Trichinella* ML which had been heat treated at temperatures up to 50 °C, although three mice in these groups remained uninfected (one mouse in the 40 °C group and two mice in 50 °C group) (Table 2). No muscle larvae were found in mice that received heat-treated *Trichinella* ML from 60 °C onwards.

The infection status results obtained from diaphragm examination fully agreed with the results from the digested mouse carcasses. As shown in Table 2, larvae per gram (LPGs) in infected mice were lower in the experiment where larvae underwent cumulative heat treatment compared to larvae that were heat treated at a constant temperature. One animal in group A and one in group D in Exp. A1 died shortly after inoculation and they were excluded from further analysis (Table 2).

3.2. Simulated pan-frying temperature profile (A2)

The values of T_0 and κwere 21.2 and 0.49 respectively for this time-temperature profile. Fig. 2B shows that a good fit is achieved using these fitted parameters.

Bioassay results showed that muscle larvae were found in mice that received *Trichinella* ML that had been heat treated up to 50 °C (Table 2).

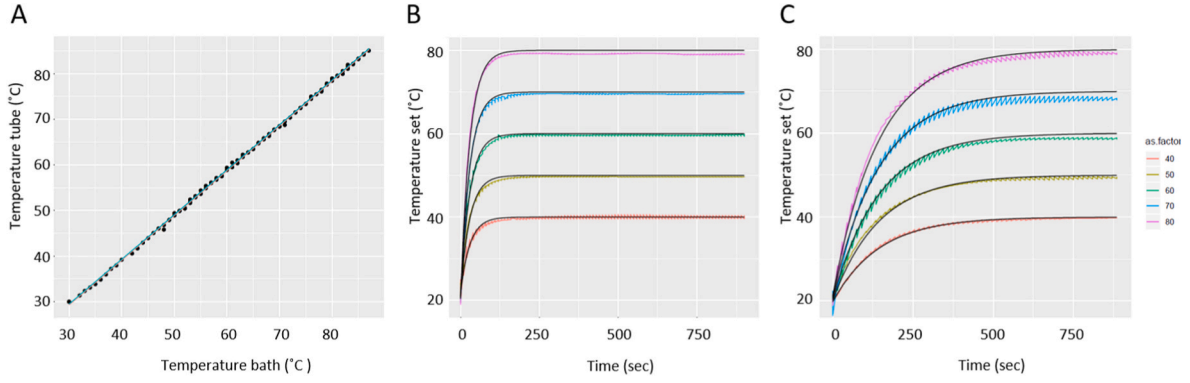

Fig. 2. Heating profiles used for inactivation experiments. **A.** The linear model for relating the temperature of the water bath and the temperature of the tube yielded a non-significant intercept, and a highly significant ($p < 10^{-16}$) slope of 0.98. B. Temperature profile for Eppendorf tube content over time (Exp. A2). C. Temperature profile for closed plate content over time (Exp. B).

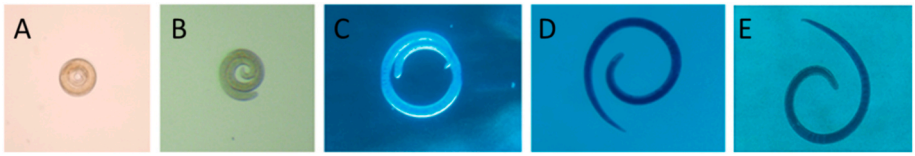

Fig. 3. Morphological features and methylene blue staining of *Trichinella* ML after heat-treatment (Exp. B).
A: Non-heated, coiled, live *Trichinella* ML. **B:** partially uncoiled and slightly stained *Trichinella* ML. **C:** almost non-coiled and stained *Trichinella* ML. **D:** fully non-coiled and fully stained *Trichinella* ML. **E** fully non-coiled and only partially stained.

Table 2
Mouse bioassay results after exposure to different time-temperature profiles.

Experiment	Group	T_i	T_f	Larval count at T_i	Non-stained larvae at T_f	Coiled	Infected/Total mice	LPG
A1	A	30	40	100–107	82–91	Yes	4/5*	10–1025
A1	B	30	50	92–107	31–84	Yes	3/6	171–1440
A1	C	30	60	98–104	96–104	No	0/6	0
A1	D	30	70	104–107	0	No	0/5*	0
A1	E	30	80	99–103	0	No	0/6	0
A1	F	30	90	95–110	0	No	0/6	0
A2	A	30	30	111–130	95–98	Yes	6/6	4200–15,000
A2	B	40	40	112–124	95–111	Yes	6/6	247–1292
A2	C	50	50	113–122	96–113	Yes	6/6	173–902
A2	D	60	60	114–152	100–134	No	0/6	0
A2	E	70	70	113–140	0	No	0/6	0
A2	F	80	80	104–120	0	No	0/6	0

A1: *Trichinella* larvae in Eppendorf vials were exposed to gradually increasing temperatures during a 90 min period simulating time-temperature exposure during oven cooking. Every 15 min, 6 Eppendorf vials containing larvae were taken out of the water bath. After cooling on ice, larvae from three vials were counted and examined morphologically, and larvae from all vials were administered to mice per gastric tube.
A2: *Trichinella* larvae in Eppendorf vials were exposed to different constant temperatures for 15 min each, simulating time-temperature exposure during pan-frying. After incubation for 15 min, Eppendorf vials containing larvae were taken out of the water bath. After cooling on ice, larvae from three vials were counted and examined morphologically, and larvae from all vials were administered to mice per gastric tube.
T_i: initial temperature at t = 0; **T_f**: final temperature at t_{final}; **LPG:** larvae per gram. *One animal died after inoculation in both group A and D of Experiment A1.

No muscle larvae were found in mice that received heat-treated *Trichinella* ML from 60 °C onwards.

The MB staining results showed that the percentage of live, non-stained *Trichinella* ML remained at around 80% when temperature was below 60 °C and it dropped to 0% above 60 °C. Morphological examination showed that larvae maintained their coiled shape below 50 °C and presented as non-coiled shape above 60 °C (Fig. 4, Fig. 6).

3.3. Evaluation of methylene blue staining and morphology (B)

The values of T_0 and κ were 20.4 and 2.4 for this time-temperature profile. Fig. 2C shows that a good fit is achieved using these fitted parameters.

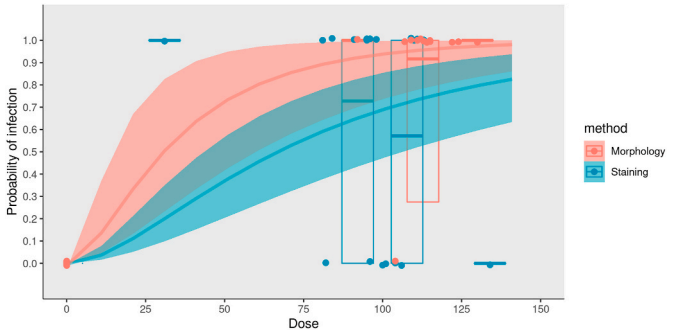

Fig. 4. The dose-response relation for infection in mice after administering a dose of *Trichinella* ML, with the dose taken from the MB staining results (blue shaded area) or the morphological examination results (red shaded). Original mice infection endpoints are added slightly jittered to the plot. Further, we show boxplots of the infection status, aggregated over dose-ranges of 25 ML (LPG), to show the relation between experiment and predicted curve more clearly. The middle horizontal line in the bars represents box plot median values, which closely match the predicted sigmoid surface plot for morphological evaluation (in red), but not for staining (in blue).

In experiment B temperature increments were smaller and morphological features were not always consistent with staining results, for instance fully stained but partially coiled larvae were seen (Fig. 3C), and fully non-coiled shaped (dead) and partially stained larvae (Fig. 3E). Therefore, since coiledness is easier to assess (Fig. 3A and B), non-coiled larvae were defined as total number of larvae before heat treatment minus the number of coiled larvae at a given temperature in the analysis.

In the bioassay, none of the mice became infected with non-coiled *Trichinella* larvae that were heat-treated at or above 60 °C for 15 min, both in experiments A1 and A2, although larvae did not stain with MB after treatment at 60 °C.

3.4. Statistical analysis

3.4.1. Dose-response and evaluation of morphology and MB

Best fitting values for survival probability p_m were 0.10with (95% credible interval 0.05–0.20) for morphology, and 0.04 (95% CI 0.03–0.07) for staining. The dose-response curves are presented in Fig. 4; clearly, morphological examination yielded much greater consistency with observed mice survival (Fig. 5).

3.3.2. Inactivation model

The best fitting inactivation model was

$$logit^{-1}(p) = -1.38 \times 10^3 + 8.56t + 1.09 \times 10^1 T - 4.06 \times 10^{-2}(t \times T) + 8.31 \times 10^2 \log(t) + 2.94 \times 10^2 \log(T) - 2.53 \times 10^2(\log(t) \times \log(T))$$

All coefficients were significant at the 0.05 level, except the log of the temperature which had a p-value of 0.08. Fig. 6 shows experimental data and predicted inactivation overlaid, depicting both the time and temperature dependence.

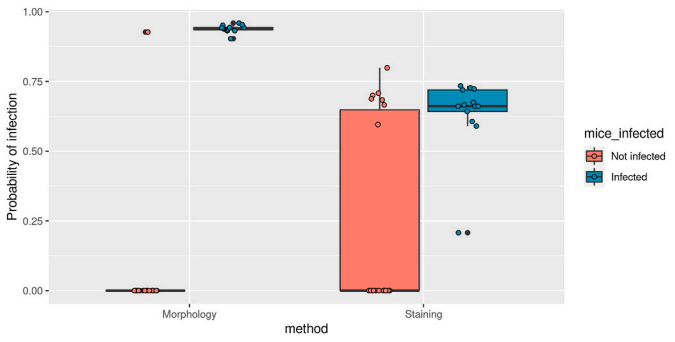

Fig. 5. Boxplots of the predicted probability of infection (based on doses according to either the MB staining or morphological examination method), split in the data for the infected and uninfected mice. Clearly, the dose obtained using the morphological examination method is more discriminating in distinguishing infection.

4. Discussion

The aim of the present study was to evaluate two in vitro methods as proxies for *Trichinella* ML heat inactivation in comparison with the mouse bioassay method to get more insight in the relationship between heat, heating time and inactivation of *Trichinella* ML. The second aim was to evaluate whether staining and morphological examination could replace the bioassay in the light of ongoing animal use reduction in lifescience research.

We used two scenarios to investigate the heat inactivation of *Trichinella* ML in our study. In the first scenario, we gradually increased the temperature and exposed larvae to each chosen temperature for 15 min in a range between 30 °C and 90 °C, thereby exposing the larvae to cumulative time-temperature combinations (comparable with oven-cooking). In the second scenario, we exposed *Trichinella* ML to a range of constant temperatures for 15 min each (comparable to pan-frying).

The qualitative results of the present study (complete larval inactivation with 15 min at 60 °C, but not at 50 °C) are in close agreement with the data of Kotula et al. (1983) (inactivation at 55 °C after 7 min with 99% confidence) and Carlin et al. (1969) (total inactivation at 60 °C internal meat temperature for oven-cooking), but not with those of Randazzo et al. (2011) (50% inactivation at 60 °C for 15 min). Carlin et al. (1969) described temperature inactivation of *Trichinella* ML in 5–7 lb (2.3–3.6 kg) chunks of oven-cooked pork, originating from experimentally infected domestic pigs. Fifteen grams of least well-done centre parts of oven-cooked pork was fed to rats on two consecutive days in a rat bioassay to evaluate heat inactivation of *Trichinella* ML. The researchers did not determine *Trichinella* larval burden in the pork directly, but instead larval counts in the diaphragms were used to indicate successful infection of the pigs. This study lacks quantification of larvae in the pork before cooking and before administering oven-cooked meat to rats.

Randazzo et al. (2011) conducted in vitro experiments to evaluate intrinsic temperature sensitivity of *Trichinella* ML in water, both for freezing and cooking. *Trichinella* ML with- and without capsule were exposed to a range of temperatures and larval survival was determined by microscopic evaluation of motility in combination with a previously developed methylene blue (MB) vitality stain (Randazzo and Costamagna, 2010), but without evaluation of infectivity using a bioassay (Randazzo et al., 2011). This study reported roughly 56% survival of *Trichinella* ML after exposure to a cumulative temperature range from 30 to 60 °C for in total 60 min (10 °C increase per 15 min).

In our study, we used naked Trichinella larvae, since no (protective) effect of the capsule surrounding *Trichinella* larvae could be observed in the study of Randazzo et al. (2011), in comparison with naked larvae.

In the present study, the temperature exposure profile as used by Randazzo et al. (2011) was used, to provide optimal comparison conditions. When compared to bioassay results, the morphological examination of larvae showed more consistency than MB staining results. In contrast, the actual survival and infective capacity of *Trichinella* ML in mice was lower than suggested by the MB staining method.

We used the bioassay results in a previously developed mouse dose-response model (Teunis et al., 2012) to describe the proportional larval inactivation after exposure to heat. In that study, a single-hit probability (p_m, defined as survival of one *Trichinella* larva after ingestion) for *Trichinella* infections in humans has been determined at $p_m = 0.01$. Exposure of exactly one male and one female larva is expected to lead to infection with probability $p = 0.01 \times 0.01 = 10^{-4}$ (Teunis et al., 2012). In the present study, $p_m = 0.1$ was determined for *T. spiralis* infections in mice (probability of infection $0.1 \times 0.1 = 10^{-2}$). The difference could potentially be explained by the difference between host species (humans and mice) and strain-differences as we considered *T. spiralis*, whereas Teunis et al. (2012) considered many species aggregated in the single hit probability model. From comparing single-hit probabilities, it is plausible to conclude that mice are more susceptible to *Trichinella* spp.

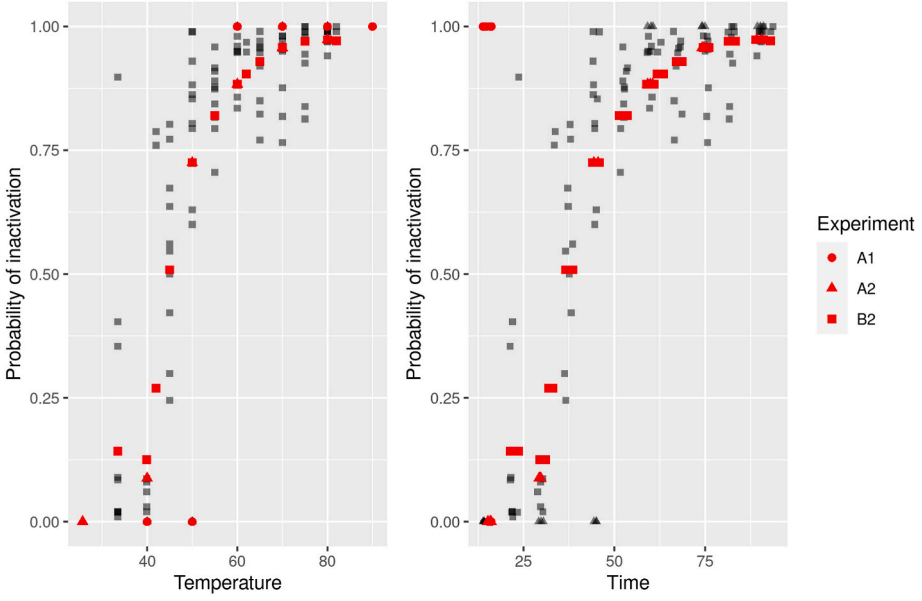

Fig. 6. Observed and prediction of *Trichinella* muscle larvae inactivation as function of temperature and time. **A.** Probability of inactivation as a function of temperature. **B.** Probability of inactivation as function of time. Predicted *Trichinella* ML inactivation over temperature and time are shown in red. The original data points are shown in black. Symbols indicate experiment, and size indicates number of ML spiked.
Although the points at 100% inactivation at short heating times seem outliers, they are actually predicted perfectly. When studied closer, these points correspond to temperatures at 60 °C or higher at 15 min, which sufficed in our experiments to inactivate all larvae.

infections than humans are. Host species difference has been observed before: mice are less susceptible to *Trichinella* infections than pigs, and both mice and pigs are more susceptible to *T. spiralis* than to other *Trichinella* species (Kapel, 2000).

In our bioassay experiment A1, one mouse in the 40 °C group and two mice in 50 °C group did not acquire infection. This is consistent with the proportional larval inactivation model used in the current study. Lahou et al. (2015) showed with simulated home pan frying experiments that the core temperature in 100 g portions of minced, intact and sliced pork reached 60 °C within 4–6 min in seven out of nine trials (Lahou et al., 2015). Cooking these portions of pork for a total time of 15 min would expose *Trichinella* ML to 60 °C during ten minutes, inactivating 96% of *Trichinella* larvae in our model. At this inactivation rate, the surviving larvae may all have the same sex, which does not lead to infection, since both males and females are needed to generate new borne larvae and hence infection.

In addition, our experiments show that there is no minimum required time at a certain temperature to inactivate all *Trichinella* ML. Rather, there is a fraction of the initial larvae that survives, depending on the entire time-temperature profile. Using our proportional larval inactivation model, we can extrapolate to relevant time-temperature combinations, which can be used to calculate exposure time at a given temperature and inactivation rate.

5. Conclusions

Morphological evaluation correlates with heat inactivation as demonstrated by mouse bioassay, but methylene blue staining underestimates *T. spiralis* ML heat inactivation in comparison with mouse bioassay. From these results, inactivation parameters can now be used to fine-tune the existing heat inactivation model that is part of the Trichinella QMRA. Moreover, this study shows that evaluation of larval *Trichinella* morphology as proxy for inactivation may be used in situations where bioassays are not possible or prohibited.

Declaration of competing interest

The authors declare that they have no competing financial interests.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.exppara.2021.108099.

CRediT author statement

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Data availability statement

The datasets generated during and/or analysed during the current study are available from the corresponding author on reasonable request.

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