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Taenia taeniaeformis: Effectiveness of staining oncospheres is related to both temperature of treatment and molecular weight of dyes utilized

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Abstract

Methods to determine viability of taeniid oncospheres following treatments with potential lethality have practical application in efforts to control transmission. Here we investigated several methods, in lieu of infectivity studies, to assess oncosphere viability and determine lethal temperature treatment regimens. In the first experiment, a standard treatment to exshell oncospheres with 0.5% hypochlorite was assessed for influence on oncosphere recovery of *Taenia taeniaeformis* eggs. Recovery of eggs and exshelled oncospheres decreased with increasing time in hypochlorite, which indicated that hypochlorite can damage eggs and oncospheres, translating into potential overestimation of lethality of experimental treatments. Losses in hypochlorite were accentuated when eggs were pretreated at 75 °C, but not lower temperatures, including 65 °C, indicating a sharp threshhold between 65 °C and 75 °C where eggs and oncospheres became hypersensitive to subsequent hypochlorite treatment. To further investigate this change in relation to temperature, non-vital (acridine orange, AO) and vital (propidium iodide, PI; trypan blue, TB) dyes were used to assess staining of oncospheres (exshelled or not) under conditions ranging from room temperature up to 95 °C. The behaviors of dyes as related to internal staining of oncospheres were described using non-linear regression and a sigmoid four-parametric model to determine the inflection point (T_{50}). Each of the dyes differed significantly in T_{50} estimates, e.g. AO (69.22 ± 0.53), PI (73.89 ± 0.52) and TB (79.43 ± 0.45). For these dyes, the T_{50} increased in relation to the increasing molecular weight of the dyes. Collectively, the results suggested that barriers to chemical permeability exist in eggs that breakdown incrementally with increasing temperatures above 65 °C. This staining behavior and the likelihood that the temperatures involved are above a lethal threshhold clarify a basic limitation in the use of vital dyes to assess oncosphere viability. The results may be relevant to other Taenia spp.

Keywords: *Taenia taeniaeformis*; Hypochlorite; Vital dyes; Permeability; Egg membranes

1. Introduction

Eggs of the tape worm *Taenia saginata* infect cattle, leading to development of cysticerci in muscle that can subsequently infect humans. Hence, this parasite has both public health significance as well as economic importance in the cattle industry. Infection in cattle is

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known as bovine cysticercosis or beef measles and cattle become infected by ingesting eggs that contaminate pastures (Sussman and Prchal, 1950; Slonka et al., 1975), water (Slonka et al., 1978) or other feed sources (Sussman and Prchal, 1950; Schultz et al., 1969; Slonka et al., 1975; Slonka et al., 1978; Hancock et al., 1989, Yoder et al., 1994). Processing of several agricultural products generates by-products suitable as inexpensive cattle feeds. Potential contamination with *T. saginata* eggs can limit the economic benefits realized by use of these feeds. The actual location, from field to feedlot, where this contamination occurs remains unresolved. Hence, methods are needed that inactivate the tapeworm eggs in these feeds. Availability of a model system to assess egg viability under experimental treatments would benefit the development of those methods. Due to the cost involved in infectivity experiments, availability of an accurate *in vitro* method would be most useful.

Several criteria have been used to assess taeniid egg viability by methods that exclude infectivity. Taeniid eggs contain an oncosphere surrounded mainly by an oncospheral membrane and an outer embryophore (Nieland, 1968; Chew, 1983). One approach to assess viability *in vitro* is by using enzymatic solutions to digest and remove the embryophore, a process we refer to for simplicity as exshelling, from eggs and then activate oncospheres, which escape from the remaining oncospheral membrane (Silverman, 1954; Stevenson, 1983). Alternatively, sodium hypochlorite (NaOCl) treatment causes exshelling, leaving the oncospheral membrane surrounding the oncosphere visually intact, then oncospheres can be activated with digestive enzymes (Ilsoe et al., 1990). Known problems associated with these approaches include low activation rates (Rajasekariah et al., 1980; Ishiwata et al., 1993) and under-estimation of egg viability (Ilsoe et al., 1990).

Regarding the use of NaOCl as a rapid exshelling agent, the effect of this treatment on recovery rates of oncospheres is unknown. If detrimental, the method could cause overestimation of lethality from experimental treatments. Further, NaOCl may damage taeniid eggs when exshelling follows exposure to high heat (Laws, 1968). Clarifying the effects that NaOCl treatment has on onchosphere viability assays seems critical before actually implementing its use. For these reasons, we investigated the effects of NaOCl treatment on taeniid egg and oncosphere recovery in relation to time and temperature.

Another approach to assess viability relies on vital dyes. For instance, exclusion of the vital dye trypan blue (TB), has been used with both enzymatically exshelled (Heath and Smyth, 1970; Wang et al., 1997) as well as hypochlorite exshelled oncospheres (Wang et al., 1997; Ciarmela et al., 2005). Vital dyes have the advantage of rapid assessment. However, there is relatively little information on the accuracy of this approach either. A potential pitfall is the impermeability to vital dyes associated with the oncospheral membrane (Silverman, 1954). For that reason, we investigated the accuracy of vital dye staining by evaluating eggs/oncospheres within oncospheral membranes exposed to a range of temperature treatments.

In experiments described here eggs from *Taenia taeniaeformis*, which cycles between rodents and cats, were used due to the unavailability of *T. saginata* eggs. A goal was to establish an experimental system that would allow efficient testing of many treatments, with subsequent validation of promising treatments using *T. saginata* eggs, inclusive of infectivity studies. The focus on *in vitro* studies allowed for thorough testing of methods. Our results unfortunately identified basic limitations for the use of hypochlorite treatment or vital dyes in viability estimates following temperature treatments of *T. taeniaeformis* eggs or exshelled oncospheres. Importantly, the results also revealed basic biological properties that appear to explain the limitations and why these limitations may be difficult to overcome. It is those properties in relation to hypochlorite treatment and vital dye staining that is the subject of this report.

2. Materials and methods

2.1. T. taeniaeformis eggs

Eggs of *T. taeniaeformis* were collected directly from cat feces or the gravid proglottids of a naturally infected cat. Eggs were collected from feces by a sugar floatation technique (Foreyt, 2001) suspended in 500 ml water. The suspension was passed sequentially through 1 mm, 0.495 mm, 180 μm and 120 μm sieves. The filtrate was transferred into 250 ml centrifuge bottles and pelleted at 360 × *g* for 15 min. The pellet was resuspended in 10 ml sucrose solution (specific gravity = 1.27) and centrifuged in 15 ml tubes at 480 × *g* for 5 min. The top 2 ml of the supernatant was collected, diluted in 10 ml of tap water and centrifuged at 480 × *g* for 5 min. The pellet was washed by repeating the addition of water and centrifugation. After the final wash, the supernatant was removed leaving 0.5 ml of the sediment.

Alternatively, eggs were obtained from gravid proglottids of *T. taeniaeformis* by opening proglottids,

using the procedure described by Takemoto et al. (1995), onto a 180 μm sieve with a scalpel. Samples were gently scraped with a glass rod, washed through the sieve with phosphate buffered saline (PBS, pH 7.4) into 50 ml centrifuge tubes and centrifuged at 1600 × *g* for 5 min. The supernatant was removed, leaving 5 ml of the sediment. Total egg numbers were estimated (3 × 20 μl samples) by counting under a compound microscope (Olympus BH-2). Eggs were stored at 4 °C for no more than 4 weeks.

2.2. Exshelling of eggs

NaOCl (Ultra bleach, Food Services of America) was used to eliminate the embryophore, a process we refer to as exshelling, from eggs by a slight modification of a described method (Wang et al., 1997). Exshelling eliminates the embryophore, while apparently leaving the oncospheral membrane intact, although our results (below) indicate that membrane intactness is conditional. Exshelling was performed on the eggs that were collected from feces and eggs isolated from proglottids that were heat treated. Oncosphere recovery was quantified following exposure to NaOCl for different durations of 5, 10 or 20 min. Fifty eggs comprised each replicate in this experiment. When heat treatment was used, NaOCl exshelling was done for 5 min at 21 °C, after the heat treatment. Two hundred eggs were included per replicate in this experiment. NaOCl was added to tubes at 0.5% and the solution was mixed intermittently during incubation. Following exshelling, oncosphere recovery was determined by adding 1.5 ml PBS, centrifugation at 1600 × *g* for 5 min, supernatants were removed and the sediments were washed three times with 1 ml PBS. Final volumes were reduced to 40 μl and the number of oncospheres recovered was determined under a compound microscope.

2.3. Heat treatments

Eggs collected from gravid proglottids were heat treated in several experiments by the following method. Centrifuge tubes (15 ml) containing eggs in PBS (100 μl) were heated in water baths at temperatures ranging from 55 to 95 °C for 10 min depending on the experiment. Untreated control eggs were maintained at 21 °C. After treatment all tubes were cooled for 15 min at room temperature and then the volume of egg suspension in heat treated tubes was adjusted back to 100 μl by adding PBS. Eggs from these treatments were used for staining and hypochlorite treatments. For some experiments using trypan blue (TB), oncospheres exshelled by NaOCl were used. In this case, exshelling in 0.5% NaOCl (5 min) preceded heat treatments and staining.

2.4. Staining with dyes

Fluorescent dyes acridine orange (AO) or propidium iodide (PI) (Molecular Probes, Eugene, USA), were used to stain eggs, while non-fluorescent TB (Invitogen-Gibco, NY, USA), was used to stain oncospheres that were exshelled prior to heat treatment. Acridine orange is membrane permeable green fluorescent dye of molecular weight 301.82 and has excitation and emission wavelengths of 490 and 530 nm, respectively. Acridine orange stains both live and dead cells, and it was used to assess permeability of membranes that surround oncospheres. Propidium iodide is a membrane impermeable red fluorescent dye of molecular weight 668.4 and has excitation and emission wavelengths of 530 and 615 nm, respectively. Propidium iodide is a vital dye that stains dead cells, and it was used to assess viability of oncospheres inside of eggs. Likewise, TB is a vital dye of molecular weight of 891.8 that stains dead cells blue. Hence, it was used to assess viability of oncospheres inside of oncospheral membranes. Two hundred fifty eggs (AO, PI) or 150 exshelled oncospheres (TB) comprised each replicate. Following thermal exposure, eggs were incubated in final concentrations of 6 μg ml^{-1} (AO) or 2 μg ml^{-1} (PI) for 15 min in the dark. Then eggs were washed twice with 2 ml PBS, pelleted at 1600 × *g* for 5 min and the final volume was reduced to 40 μl. The TB staining was performed on exshelled oncospheres by adding 0.4% TB to a final concentration of 0.04%, followed by microscopic examination after 2 min. The first 30 eggs or exshelled oncospheres encountered from each of triplicate sample were examined microscopically at magnification of 100× and 400×. The AO and PI stained eggs were examined under the FITC and Tex red filters respectively, of an epifluorescence microscope (Axioskop 2 plus), while TB stained oncospheres were observed under the compound light microscope.

2.5. Statistical analysis

Completely randomized design (CRD) was used in these experiments. Means of triplicate measurements of recovered oncospheres after NaOCl treatment were analyzed using SAS software (SAS institute, Cary, North-Carolina, USA). One-way analysis of variance

(ANOVA) was conducted, and loss of eggs and exshelled oncospheres under different incubation times of 0.5% NaOCl was analyzed by trend contrast statements. Dunnett's test was used to identify heat treatments that resulted in significant loss of oncospheres when compared to controls.

2.6. Non-linear regression analysis

To analyze the response of oncospheral staining with dyes to heat treatments, non-linear regression analyses were performed using sigma plot 8.0 software (SPSS Inc, Chicago, USA). Each of the stains demonstrated a sigmoid pattern in mean percentage of internal staining across the range of temperatures tested. Hence, a sigmoid four-parametric model (Eq. (1)) (Rutledge, 2004) was fitted to the data observed, and inflection points for each stain were estimated using the following equation:

$$I_T = I_0 + \frac{I_{net}}{1 + e^{-(T - T_{50}/r)}} \quad (1)$$

'I_T' was the computed percentage internal staining at any temperature T (°C), 'I_0' was the minimum percentage internal staining, 'I_{net}' was the net increase in percentage internal staining, 'T_{50}' was the x-coordinate (temperature) of the inflection point, which was the exposure temperature of eggs where internal staining reaches 50% of net increase (I_{net}) and 'r' was the slope coefficient of the internal staining curve.

3. Results

3.1. Oncosphere loss due to NaOCl

Sodium hypochlorite treatments led to the loss of eggs and exshelled oncospheres recovered with increased incubation time (Fig. 1). The mean percentage of eggs and exshelled oncospheres recovered decreased with time of incubation and showed a strong quadratic trend ($p < 0.05$). Although, the highest percentage of exshelled oncospheres was recovered after 10 min, a mean 39% eggs and exshelled oncospheres had already been lost and losses continued with incubation time after 10 min.

3.2. Oncosphere recovery after heat treatment

Sodium hypochlorite had a marked effect on the recovery of exshelled oncospheres following high heat treatment (Fig. 2). A significant decrease in mean percentage of recovered exshelled oncospheres occurred after treating eggs at 75 °C for 10 min ($p < 0.05$). However, the mean percentage of exshelled oncospheres recovered after treating eggs at 55 or 65 °C for 10 min were not different from the control group (21 °C). The disintegration of oncospheres by NaOCl treatment was microscopically observed with eggs from the 75 °C group (not shown). Results from this and the preceding experiment indicate that 0.5% NaOCl causes artifactual destruction of eggs and oncospheres by NaOCl treatment. The affect was more pronounced with increasing time of exposure and marked elevation in

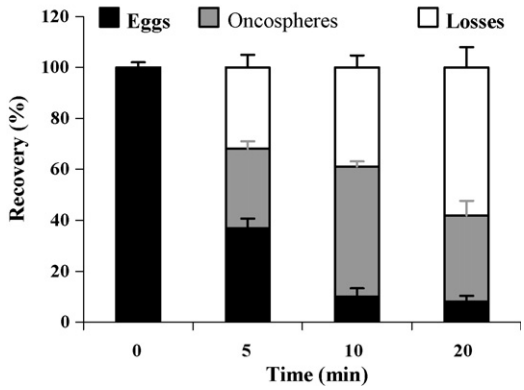

Fig. 1. Influence of incubation time in 0.5% hypochlorite (NaOCl) on *T. taeniaeformis* egg and oncosphere recovery. Eggs were treated with 0.5% hypochlorite for 5, 10 or 20 min. Eggs that received no hypochlorite treatment served as control (0 group). The recovered exshelled oncospheres or eggs or total eggs and exshelled oncospheres lost (losses) were expressed as a mean percentage ± S.E.M of eggs recovered in the time 0 group.

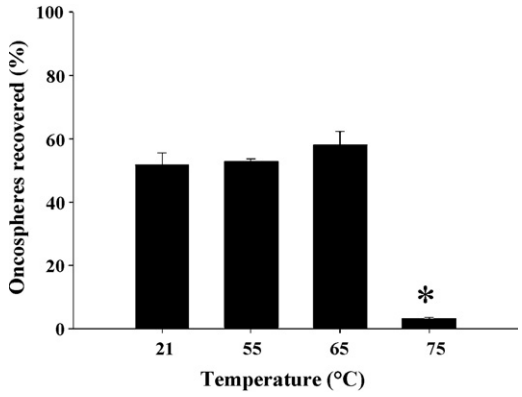

Fig. 2. Effect of thermal treatment of *T. taeniaeformis* eggs on recovery of oncospheres when exshelled in 0.5% hypochlorite (NaOCl). Eggs heat treated for 10 min at temperatures indicated were then exshelled by 0.5% NaOCl for 5 min. The recovered oncospheres were reported as a percentage mean ± S.E.M of the input eggs. Treatment groups indicated by the symbol '*' were significantly different from the control group (21 °C) at $p < 0.05$. Similar results were observed in a replicate experiment.

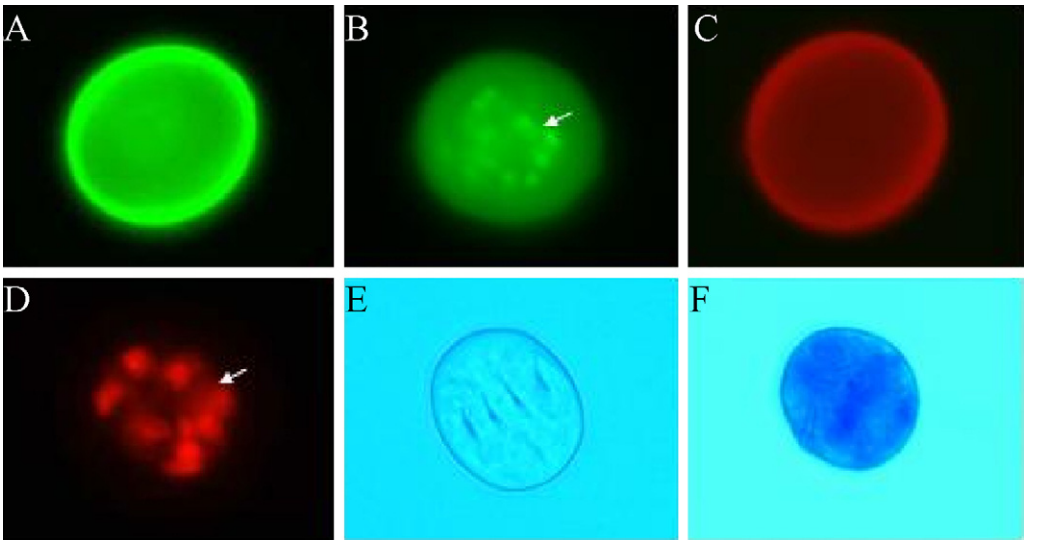

Fig. 3. Staining of *T. taeniaeformis* eggs or oncospheres. *T. taeniaeformis* eggs were stained with acridine orange (A, B) and propidium iodide (C, D) at final concentrations of 6 μg ml^{-1} or 2 μg ml^{-1}, respectively for 15 min at room temperature in the dark and examined using a fluorescent microscope. Stained oncospheres are indicated by arrows. Trypan blue staining (E, F) was performed at final concentration of 0.04% with oncospheres obtained by exshelling in 0.5% hypochlorite for 5 min. Eggs showed peripheral staining with AO (A) or PI (C) and internal staining with AO (B) or PI (D). Hypochlorite exshelled oncospheres either excluded (E) or were stained (F) with TB. Bar scale–10 μm.

NaOCl sensitivity was observed between heat treatments of 65 and 75 °C.

3.3. Staining of taeniid oncospheres with non-vital and vital dyes

Acridine orange stained *T. taeniaeformis* eggs appeared in two principle patterns. Some eggs showed green fluorescence at the embryophore level, which was called peripheral staining (Fig. 3A). Other eggs demonstrated green fluorescence in oncospheres, which was called internal staining (Fig. 3B). Similarly, PI stained eggs either peripherally (Fig. 3C), or internally (Fig. 3D). In some cases, peripheral staining included focal staining, which presumably reflected nuclei of the embryophore cell (not shown). Results obtained with TB were more complicated. Treatment of eggs with TB at room temperature produced peripheral staining only. Even heat treatment at 85 °C produced staining restricted to the periphery. With oncospheres exshelled by 0.5% NaOCl (5 min), internal staining by TB was observed, but variably (Fig. 3E and F).

The mean percentages of eggs demonstrating peripheral and internal staining with AO or PI were similar at 21 °C and did not change markedly with treatment at 55 °C for 10 min (Fig. 4), although 55 °C was expected to approach or exceed a lethal temperature. Exshelled oncospheres that retained the oncospheral membrane routinely showed less than 10% internal staining by TB. These staining experiments indicated that the behavior of the two vital dyes (PI and TB) differed markedly. In contrast, staining behavior of the non-vital dye AO was similar to that of the vital dye PI.

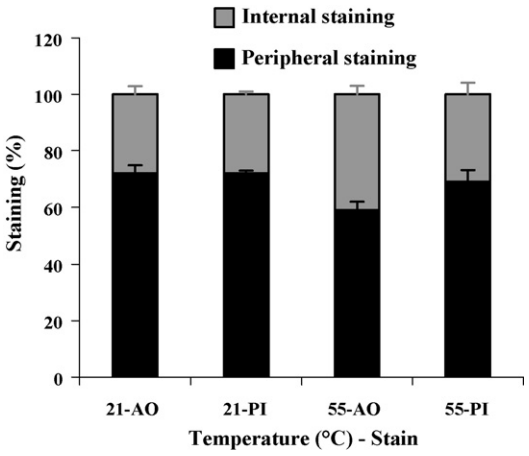

Fig. 4. Acridine orange (AO) or propidium iodide (PI) staining of *T. taeniaeformis* eggs exposed to 21 °C or 55 °C for 10 min. Eggs from each group were stained for 15 min with AO or PI at the final concentration of 6 or 2 μg ml^{-1}, respectively; and examined under fluorescence microscopy. Results show the patterns of staining observed expressed as a mean percentage ± S.E.M of the first 30 eggs examined in each of the three replicates.

3.4. Response curves of staining under increasing temperature treatments

Higher temperature treatments were used to better resolve the staining behavior of dyes under anticipated lethal conditions for *T. taeniaeformis* eggs (AO, PI) or exshelled oncospheres surrounded by oncospheral membranes (TB). The data in Fig. 5 A–C show that a high percentage of eggs or exshelled oncospheres were stained internally only after high temperature treatments, but the temperature at which the highest percentage of staining occurred differed for each dye.

Nonlinear regression analysis (Eq. (1)) of the data was performed to compare each dye based on the inflection point (T_{50}) for percentage staining. This parameter reflects the point at which internal staining was 50% of the net increase in percentage internal staining (I_{net}). The model identified inflection points for AO and PI curves, but not for the TB curve when 85 °C was included as the maximum treatment (Fig. 6, Table 1). The result with TB reflected the high rate of change in internal staining between 75 and 85 °C treatments, with no plateau detected. Therefore in a separate experiment, mean internal staining (%) with TB was assessed on hypochlorite exshelled oncospheres that were treated at 21 °C, 75 °C or 95 °C for 10 min. The response at 21 °C and 75 °C were similar to the previous data. Internal staining for the 95 °C treatment was 99% (± 1). This observation was incorporated into the previous data (Fig. 5C) and non-linear regression (Eq. (1)) was performed that defined the inflection point.

The four-parameters defined by the regression model, corresponding r^2 values and p values for each curve that indicated the best fit are shown in Table 1. Differences among the stains were determined based on the mean T_{50} value. The T_{50} for each of the three stains differed significantly in reference to a 95% confidence level. The T_{50} for AO was lower than that for PI, which in turn was lower than that for TB. Therefore PI and TB behaved differently even though, they are both vital dyes.

Finally, we considered the possibility that staining behavior was related to the molecular weight of dyes used for staining (as discussed later). This possibility was supported by an increasing T_{50} associated with increasing molecular weight for the dyes (Table 1).

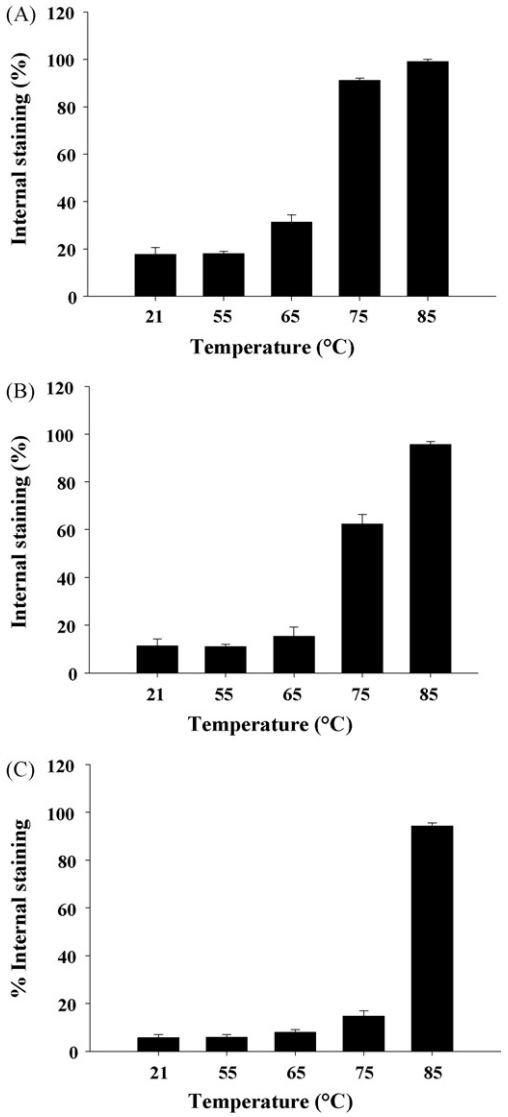

Fig. 5. Responses to high temperatures in internal staining of oncospheres by dyes. *T. taeniaeformis* eggs (A, B) or hypochlorite exshelled oncospheres (C) were heat treated for 10 min at temperatures indicated and then stained with: A, acridine orange (AO, 6 μg ml^{-1}); B propidium iodide (PI, 2 μg ml^{-1}); or C, trypan blue (TB, 0.04%). Oncospheres were obtained by exshelling in 0.5% sodium hypochlorite for 5 min prior to heat treatment. Results in all cases show the proportion of internally stained oncospheres expressed as mean percentage ± S.E.M of the first 30 eggs or oncospheres examined in each of three replicates in every group. Replicate experiments showed similar results (not shown).

4. Discussion

Heat treatment of cattle feeds is one option that may have practical value in efforts to counter contamination by *T. saginata* eggs. Experiments described here focused on the use of methods other than infection to assess viability of taeniid eggs after heat treatment, utilizing *T. taeniaeformis* as a model organism. Specifically, we investigated the effect of NaOCl on exshelling oncospheres in relation to egg and onco-

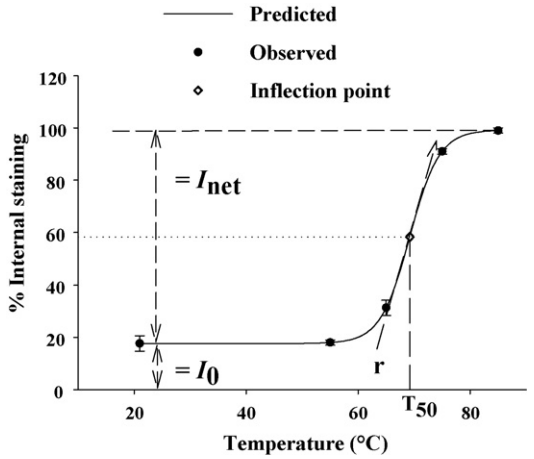

Fig. 6. Sigmoid four-parametric non-linear regression model. Internal staining data observed with acridine orange (Fig. 5A) was used as an example, to show the four parameters estimated by the model. For the internal staining curve of each stain, 'I_{net}' is the net increase in percentage internal staining due to heat treatment, r indicates slope coefficient of the curve, 'T_{50}' indicates x coordinate temperature (°C) of the inflection point, where the internal staining is 50% of I_{net} and 'I_0' indicates minimum percentage internal staining. Observed data points are means ± S.E.M of three replicates in each treatment. Data for all stains is summarized in Table 1.

sphere recovery. We also tested the use of standard dyes for assessing viability of oncospheres following heat treatments. In each case significant short comings were identified for use of these methods in assessing lethality of experimental treatments on *T. taeniaeformis* eggs.

An important observation in this study was that different dyes, including vital dyes, displayed distinct behavior in staining of eggs and exshelled oncospheres under a range of temperatures. Although PI and TB differ in chemical properties, both vital dyes are expected to stain dead oncospheres following a lethal treatment unless other factors complicate this situation. In our experiments, PI and TB differed in ability to stain exshelled oncospheres under both low and high temperatures as indicated by the lower T_{50} for PI compared to TB. Staining of oncospheres in wholly intact eggs by TB was poor regardless of heat treatment. Use of hypochlorite exshelled oncospheres demonstrated enhanced staining only with 85 °C and 95 °C treatments. If hypochlorite is assumed to have caused leaky oncospheral membranes under conditions used in this study, the estimated T_{50} for TB should have been lower than PI. Further, the high percentage of internal staining (>90%) at temperatures of 85 °C excludes the possibility of a bleaching effect by residual hypochlorite on TB that could affect the staining estimates. Having excluded these possibilities, the discrepancy in staining by these vital dyes under expected lethal temperature conditions indicated the influence of other egg related factors.

A further discrepancy involved the similar staining behavior of AO, a non-vital dye, and PI, both under low and intermediate temperatures. Nevertheless a significant difference was observed with T_{50} in response to high temperatures between AO and PI.

One factor that may account for these observations is the impermeability of the oncospheral membrane. Earlier research reported the impermeability of the oncospheral membrane to vital stains such as neutral red (Silverman, 1954; Heath and Smyth, 1970). Even though neutral red is a membrane permeable dye, the potential problem of impermeability was indicated by its ability to stain only activated and live oncospheres that have lost the oncospheral membrane (activation in enzymatic solutions). Although, AO is not a vital dye (as it stains both live and dead cells), the low percentage of internal staining in control eggs in this study confirmed the general impermeability of egg membranes.

The behavior of dyes used here leads us to suggest that the internal staining of the oncosphere in response to temperatures between 65 °C and 95 °C reflects the diameter of the membrane pores created by heat denaturation, rather than viability of oncospheres. A likely membrane to be involved is the oncospheral

Table 1
Summarized data obtained by fitting non-linear sigmoid four-parametric regression model to the observed internal staining (mean % ± S.E.) curves of acridine orange (Fig. 5A), propidium iodide (Fig. 5B) and trypan blue (Fig. 5C)

Stain (MW)	Regression parameters				r^2-Value[a]
	I_{net}[a]	r[a]	T_{50}[a]	I_0[a]	
AO (300.82)	81.56 ± 2.50	2.64 ± 0.28	69.22 ± 0.53 a	17.65 ± 1.39	0.99
PI (668.40)	86.63 ± 4.72	2.99 ± 0.79	73.89 ± 0.52 b	11.10 ± 2.04	0.98
TB (891.82)	92.58 ± 1.66	1.90 ± 0.17	79.43 ± 0.45 c	6.46 ± 0.82	0.99

MW: Molecular weight; AO: Acridine orange; PI: Propidium iodide and TB: Trypan blue. (a, b, c): T_{50} identified by different superscripts are different at $p < 0.05$

[a] All values in the respective columns are significantly different from 0 at $p < 0.05$.

membrane, but involvement of other egg membranes cannot be excluded. This suggested involvement of membrane pores stems from the association between increasing T_{50} for AO < PI < TB and the molecular mass of these dyes in which AO < PI < TB. This relationship may indicate an increasing mean pore size of the egg membranes with increasing temperature. The temperature conditions (65–95 °C) used are all expected to be lethal to oncospheres. Hence, these dyes appear to provide an unacceptable means to assess viability of oncospheres within eggs or oncospheral membranes at these temperatures. Furthermore, the similarity in mean percentage internal staining of AO and PI regardless of temperature up to 55 °C, indicates equal limitations for use of these non-vital or vital dyes, respectively, to make viability estimates. These conclusions may not apply to methods that lead to a breach in the oncospheral membrane (e.g. enzymatic treatments). However, the variability of these methods undermines reliable assessment of oncosphere viability.

Sodium hypochlorite has also been used for exshelling of taeniid eggs in conjunction with viability studies (Wang et al., 1997, Ciarmela et al., 2005). Our results identified two short-comings of 0.5% NaOCl for this purpose. One is that use of hypochlorite caused losses in recovery of eggs and exshelled oncospheres that increased with time of incubation. Loss in oncospheres was experienced even before all eggs had been exshelled. This effect represents a basic problem for use of hypochlorite when attempting to assess viability for a population of eggs. A likely explanation for these losses is a time-dependent damage by hypochlorite oxidation on oncospheres.

A second short-coming is related to the first, but was especially pronounced when hypochlorite was used to exshell eggs following temperature treatments. A dramatic loss was observed with temperatures between 65 and 75 °C. That loss was associated with disintegration of the oncospheres, which was directly observed by microscopy. Earlier research reported similar detrimental effects of 1% NaOCl on *T. pisiformis* eggs after treatment at 65 °C for 2 h (Laws, 1968). Because enhanced staining was observed with AO and PI at 75 °C compared to 65 °C, the hypochlorite effect might also reflect a change in oncospheral membrane permeability. OCl^- has a mass of 50.46, which is smaller than AO or PI and could account for the extreme effect observed at 75 °C. This consideration may also indicate that egg membranes have a permeability threshold of less than 50 molecular mass.

A primary interest was to determine if the *in vitro* methods investigated could be reliably used to assess viability of taeniid eggs, and if not, why not. Our results indicate that use of vital dyes to stain oncospheres enclosed in eggs or onchospheral membranes under relatively low and higher temperatures poses a problem for the accurate assessment of oncosphere viability. This short-coming is unfortunate given the simplicity of the methods. The apparent problem of premeability suggests a general obstacle for use of vital dyes to assess lethality of treatments on taeniid egg viability. Further, inclusion of 0.5% NaOCl as an exshelling agent may additively compromise the accuracy of viability estimates due to degeneration of a portion of eggs or oncospheres. The sharp increase in destruction of eggs and oncospheres by NaOCl following high heat treatment poses an even greater limitation on the use of NaOCl in this context. From these conclusions, serious reservations exist for use of methods that incorporate 0.5% NaOCl or vital dyes to assess *T. taeniaeformis* egg viability. It will be important to determine how well these results apply to other *Taenia* spp.

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