TAENIA TAENIAEFORMIS: DIFFERENTIAL STAINING OF ONCHOSPHERES WITH VITAL DYES UNDER CRITICAL TEMPERATURES

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The members of the Committee appointed to examine the dissertation of KALYAN C CHAPALAMADUGU find it satisfactory and recommend that it be accepted.

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ABSTRACT

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Ingestion of *T.saginata* eggs by cattle causes a condition called bovine cysticercosis (BC). Hence, control treatments that inactivate *T. saginata* eggs would be valuable, which requires accurate *in vitro* methods for assessment of egg viability. *Taenia taeniaeformis*, a rodent cestode was used as a model and the affect of 0.5% NaOCl on onchosphere recovery from both untreated and heat treated eggs was investigated. Further, the use of non-vital and vital dyes to assess the egg/onchosphere viability under different heat treatments was also studied.

Onchosphere recovery of *T. taeniaeformis* eggs by exshelling with 0.5% NaOCl for 5, 10 or 20 minutes was assessed that showed quadratic loss in recovered eggs and onchospheres with increased incubation time ($p<0.05$). Also, the effect of heat on the onchosphere recovery was tested by exposing eggs to 55, 65 or 75°C for 10 minutes. Heat treatment at 75°C resulted in remarkable onchosphere loss when exshelled by 0.5% NaOCl ($p<0.05$).

Non-vital dye acridine orange (AO) or vital dye propidium iodide (PI) stained eggs either peripherally or internally, while vital dye trypan blue (TB) showed only peripheral staining in eggs, but showed variable staining in NaOCl exshelled onchospheres. Preliminary

studies at low temperatures indicated differences in the staining behavior of the vital dyes, but not between AO and PI.

Experiments at higher temperature (55-95°C) were performed for better resolution of the staining behavior. AO or PI was used directly with heat treated eggs, while TB was used with onchospheres that were exshelled prior to heat treatment. This is because of the onchosphere loss mentioned earlier. Non-linear regression analysis on the obtained percentage internal staining data with each dye by fitting four-parametric sigmoid model showed that T_{50} for AO was lower that that of PI, which in turn was lower than that of TB.

From these findings, the use of vital dyes appears to be an unacceptable means to assess viability of onchospheres within eggs or onchospheral membranes.

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REVIEW OF LITERATURE

1.1. Biology of *Taenia saginata*

Taenia saginata, commonly called the beef tape worm of humans, is a cestode parasite that belongs to the order cyclophyllidea in the phylum platyhelminthes. The parasite has both public health significance and economic importance in the cattle industry. The adult worm parasitizes the small intestine of the human main host, whereas the larval cysticercus lives in the muscle tissue of the bovine intermediate host. Infection is called Taeniasis in humans and bovine cysticercosis in cattle. Other than cattle, infection is reported occasionally in other hosts such as buffalo, llama and giraffe. The adult tape worm in the gut of human hosts expels terminal segments called gravid proglottids, which contains eggs and passes out independently or along with the feces contaminating the surroundings. Each gravid proglottid contains up to 100,000 eggs (Soulsby, 1965). This large population can cause heavy infections in cattle, when eggs spread out of the proglottids into the surroundings. Sometimes the eggs are released from proglottids before passage into the environment and hence, eggs can be seen in the feces. The taeniid eggs are embryonated before they are released into the environment and the embryos are called onchospheres. After cattle consume the eggs, the early larval stage is released in the intestine of cattle and penetrates into blood or lymph vessels and finally encysts in muscle tissue. Bovine cysticercosis is also referred to as “beef measles” in carcasses because encysted larvae give meat a spotted appearance. Human beings become infected when undercooked or uncooked beef containing cysticercii is consumed.

1.2. Epidemiology

1.2.1. Prevalence of T. saginata (worldwide and in the United States)

T. saginata has a worldwide distribution, but prevalence rates in different regions vary. Based on prevalence, The World Health Organization (1983) roughly grouped areas of the world into three classes: high prevalence (> 10% *T. saginata* carriers in the human population), moderate prevalence (0.1-10% of *T. saginata* carriers) and low prevalence (less than 0.1% *T. saginata* carriers). Prevalence of *T. saginata* is reported to be high in some areas of Africa, while Europe, south-east Asia and countries such as India, Japan and South America have a moderate prevalence. The USA, Canada, and Australia are reported to have prevalence rates of 0.5% (Murrell, 2005).

In the United States, an extensive survey that involved screening stool samples for various intestinal parasites revealed that an average of 10.8 in every 100,000 stool samples examined were positive for *T. saginata* (Ruebush et al., 1978). However, prevalence rate varied by region. The western U.S had a higher rate (144/100,000) compared to the northeast (124/100,000), north central (47/100,000) and the south (6/100,000) (Schultz et al., 1970).

1.2.2. Prevalence of bovine cysticercosis (worldwide and in the United States)

Prevalence of bovine cysticercosis is very high in Africa, Latin America and parts of Asia, while North America and Europe have prevalence rates around 0.03% (Murrell, 2005).

Mann (1983) estimated that around 23 million cattle were infected with bovine cysticercosis on the African continent.

In the United States, the national prevalence rate of bovine cysticercosis has been very low, but epidemics have occurred in one or more feedlots in the states of Arizona (Sussman and Prchal, 1950; Slonka et al., 1975), Virginia (Hammerberg et al., 1978), Texas (Schultz et al., 1969), California (Slonka et al., 1978), Ohio (Fertig and Dorn, 1985) and Idaho (Yoder et al., 1994). The prevalence rates of bovine cysticercosis in these outbreaks, when assessed as a percentage of slaughtered cattle from those respective feedlots, have been in the range of 0.4 to 45%. An infection rate of 67.7% was also reported in one of the pens in an affected feedlot in Texas (Schultz et al., 1969).

1.2.3. Economic losses due to bovine cysticercosis

Bovine cysticercosis is one of the economically important diseases of the cattle industry in the countries where the prevalence rates are high. Losses are mainly due to condemnation of the highly infected carcasses, price curtailments on the lightly infected carcasses and their subsequent freezing to make the meat safe for human consumption. In United States, beef carcasses detected with localized infections will be held at $\leq 9.4^{o}C$ for 10-20 days depending upon the level of infection, before passing on for human consumption (Gracey, 1986). As estimated initially by Pawlowski and Schultz (1972) and updated by Murrell (1991), loss due to condemnation of one whole infected bovine carcass in the developed countries is about US $234, while it costs US $78 in the developing nations. In areas that are highly endemic for bovine

cysticercosis, economic losses are expected to be high. As estimated initially by Mann (1983) and updated by Murrell (1991), the total annual economic loss in Africa due to bovine cysticercosis reached US $1.8 billion. Moreover, as estimated initially by Abdussalam (1975) and updated by Murrell (1991), the total combined annual economic loss due to the porcine and bovine cysticercosis in Latin America is about US $480 million. Even in the United States, outbreaks in the feedlots cause significant losses to the owners (Yoder et al., 1994).

1.2.4. Infections in feedlots in United States

Epidemics with high infectivity rates have been reported in feedlots (Schultz et al., 1969; Slonka et al., 1975). It will be important to identify the possible sources of infection in order to design efficient control measures. Adult *T. saginata* is very host specific. Therefore human carriers are the only primary source of infection for cattle. Infection of cattle with *T. saginata* can be accomplished through direct contact or indirect routes. In many of the outbreaks reported in the United States, human carriers of *T. saginata* have been identified in feedlots and were implicated as a possible source of infection through direct contact with animals or by contaminating silage (Schultz et al., 1969), hay (Slonka et al., 1975; Slonka et al., 1978), water (Slonka et al., 1978), or crop fields used for grazing (Sussman and Prchal, 1950). Alternatively, application of raw sewage or sludge contaminated with *T. saginata* egg to pastures was also indicated as a possible route for infection in feedlot cattle (Fertig and Dorn, 1985).

Other studies in the United States indicate the possibility of transmission to cattle by a source of feed external to the feedlot. In such situations, the prevalence rates are generally high and widely distributed, as the same feed may be shipped to several feedlots. An epidemiological

study conducted in the eastern part of Washington State during 1983 and 1984 included data from different slaughter plants and numerous feedlots. This study revealed high prevalence rates for bovine cysticercosis. The highest rate reported from slaughter plants was 6/1000, while it was above 30/1000 in some of the feedlots (Hancock et al., 1989). Another study on an outbreak in one feedlot in south central Idaho reported bovine cysticercosis in 9% of cattle. This outbreak caused an estimated loss of US $150,000 (Yoder et al., 1994). In both of these studies, regionally available potato by-product was suggested as a possible cause of high bovine cysticercosis prevalence.

1.2.5. Diagnosis of bovine cysticercosis in cattle

Post mortem inspection of beef has been the most important method for detection of cysticercii infected carcasses. Predilection sites such as heart, masseter muscles, tongue, shoulder muscles, where the cyst density is reportedly high (Pawlowski and Schultz, 1972), are inspected. Among the various sites, heart usually has the highest cyst density (Kyvsgaard et al., 1990; Maeda et al., 1996). Also, other tissues such as liver, lung, hind limbs, chest muscles, back muscles, intestines and surfaces of the rumen may also harbor the cysts (Mango and Mango, 1972; Kyvsgaard et al., 1990).

1.2.6. Limitations in visual diagnosis

Although routine post mortem inspection of beef carcasses is widely used, it has limitations. The major disadvantage is the low or variable sensitivity of detection that this method offers during meat inspection. Maeda et al. (1996) estimated that the sensitivity of

routine meat inspection in finding cysticercii positive carcasses was 17%. The detection efficiency can be lower in lightly infected cattle with a few cysts (Kyvsgaard et al., 1990). On the other hand, high detection rates around 72% have been reported for the meat inspection procedure (Dewhirst et al., 1967). However, in the same study, cattle from an adjacent pen inspected at a different packing plant were reported to have no infection indicating possible variation due to the person inspecting. Similarly, Rickard and Adolph (1977) also reported high variation in the detection sensitivity of meat inspection (0-72%). The variation was mainly attributed to cyst morphology as both degenerated and viable cysts can be over looked during inspection. In general, the low or variable sensitivity can be due to variations in intensity of infection and relative distribution of cysts among the various tissues along with other factors such as age of infection, sites examined and person conducting the post-mortem. With this level of sensitivity and variability, it is likely that actual prevalence rates of bovine cysticercosis are higher than reported rates (Hancock et al., 1989) and hence many infected carcasses are likely to pass into the food chain (Mann, 1983)

A more recent study reported an immunological assay based on the antigen – enzyme linked immuno sorbent assay (Ag-ELISA), which detects circulating *T. saginata* antigen in serum of the infected cattle (Van Kerckhoven et al., 1998). The Ag-ELISA technique can be 10 times more sensitive than the traditional meat inspection (Dorny et al., 2000). However, this technique is more sensitive (92%) with infections having more than 50 live cysticercii and decreases (12.8%), when the number is less than 50 (Van Kerckhoven et al., 1998). This technique is being used for various sero-epidemiological studies (Dorny et al., 2000).

1.2.7. Control of bovine cysticercosis

Human carriers are the primary source of infection for cattle (Soulsby, 1965). Thus, control of bovine cysticercosis depends on several factors including: (1) identification of human carriers and their treatment with antihelminthics; (2) education of the public on hygiene; (3) ensuring proper sanitary measures and (4) efficient detection of cysticercosis in beef and other integrated efforts at different levels of the society will all help control *T. saginata* (Suroso et al., 2006). However, success will require highly integrated efforts. Also, the cost and limitations in detection of taeniasis and bovine cysticercosis need to be surmounted to achieve the goal. At the feedlot level, it may be possible to reduce chances of bovine cysticercosis outbreaks in the cattle herd by: (1) educating feedlot personnel on parasite transmission routes; (2) identifying and treating human carriers in the feedlot; (3) preventing contamination of feed or water and (4) providing adequate sanitation facilities in cattle premises, surveillance of the cysticercosis infections, cattle origin and avoiding hay from sewage irrigated pastures (Slonka et al., 1978).

1.3. Structure and nature of taeniid eggs

Taeniid eggs will be the focus of studies aimed at finding control measures and these studies require accurate assessment of egg viability *in vitro* or *in vivo*. Hence, before discussing the various methods used to assess viability, a brief review of the structure and nature of the eggs and surrounding membranes is included.

1.3.1. Structure of taeniid eggs

The ultra-structure of taeniid eggs has been studied at the light and electron microscope level. Embryonated eggs of *T. saginata* are around 30-40 μm in diameter and brown in color (Schramlovă and Blažek, 1982). The embryo proper of the taeniid egg is called the onchosphere. It contains six hooks and hence is also called a hexacanth embryo. The onchosphere is surrounded by multiple envelopes. The outermost envelope is the embryophore, which is made of adjacently placed hexagonal blocks that are glued with a cementing substance. These blocks are responsible for the radially striated appearance of the embryophore under a microscope (Schramlovă and Blažek, 1982). From the study on eggs of *T. crassiceps*, these blocks are reported to be synthesized in the embryophore cell (Chew, 1983). Another envelope is the inner onchospheral membrane. This membrane lies adjacent to the onchosphere and is interior to the embryophore. The onchospheral membrane is much thicker than the cellular plasma membrane (Chew, 1983). Scanning electron microscope (SEM) studies on the eggs of *T. taeniaeformis* reveal that the onchospheral membrane is composed of two pairs of laminae separated by vesicles and its formation is connected with the cisternae of endoplasmic reticulum and the embryophore cell. From studies on the different species of *Taenia*, it is understood that the onchospheral membrane is not a simple and single layer like the plasma membrane of a cell, but composed of more than one layer (Nieland, 1968; Schramlovă and Blažek, 1982; Chew, 1983). Embryophore is the thickest of the envelopes surrounding the onchosphere. A study on *T. saginata* and *T. pisiformis* eggs suggest that eggs with relatively thin embryophore are immature, while the ones with thick embryophore are mature (Silverman, 1954b). Hence, this feature of the

egg can give an idea about the maturity of the eggs in *T. saginata* or *T. pisiformis,* where the eggs within a proglottid show wide variations in degree of maturity.

1.3.2. Resistance of taeniid eggs

Taeniid eggs are known for their resistance to a variety of physical and chemical factors. A study conducted in Kenya on the survival of *T. saginata* eggs in open pasture grounds reported a maximum survival time of over one year (413 days) (Duthy and Someren, 1948). On the other hand, studies on the eggs of *T. pisiformis*, *T. hydatigena* and *T. ovis* eggs revealed their resistance to several organic and inorganic compounds (Laws, 1967). For example, compounds such as perchloric acid, silver nitrate, concentrated solutions of sodium chloride, glucose, ammonium sulfate were reported to have little or no influence on the hatching and activation rates of eggs when artificial digestive juices were used (an *in vitro* assessment of egg viability, described in section *1.4.a*). Similar resistance of eggs to diluted (90%) alcohols was also reported. However, the effect of anhydrous alcohols such as methanol, which are capable of dehydrating biological tissues, was high. But, in a similar study most of these alcohols showed no effect on *T. saginata* eggs (Mackie and Parnell, 1967). Other compounds such as aliphatic halogens of paraffins and olefins, amides, quaternary ammonium salts and quinines reduced the hatching and activation rates of eggs (Mackie and Parnell, 1967). A similar study on *T. saginata* eggs demonstrated the resistance of the eggs to chaotropic agents such as urea even under prolonged exposures (30-35 days) (Prokopic and Jelenova, 1980). Impermeability of egg membranes was the suggested reason for the resistance of eggs to the chemicals tested (Nieland, 1968).

However, drying or desiccation of the taeniid eggs is highly lethal to the eggs (Laws, 1968a). The effect of desiccation was comparatively higher in the eggs of *T. hydatigena* and *T. ovis* when compared to *T. pisiformis.* The difference in the permeability of the embryophore membrane was suggested to be responsible for this variation among the different species. Further, removal of the embryophore makes the onchospheres much more susceptible to desiccation (Laws, 1968a).

1.3. *In vitro* assessment of taeniid egg viability

The main criteria for *in vitro* assessment of egg viability have been activating a hatched onchosphere or response to staining with vital dyes (Silverman, 1954a).

1.4.1. Hatching and activation of taeniid eggs

Hatching is removing the embryophore membrane and activation is indicated by a motile onchosphere (Laws, 1967; Heath and Smyth, 1970). Hatching and activation of taeniid eggs has been one of the important methods for assessing egg viability *in vitro*. This method is based on simulating the environment present in the gastro-intestinal tract of the host such as providing digestive enzymatic juices along with maintaining the appropriate pH and temperature. The observed free and active onchospheres serve as a measure of egg viability. Early investigators used enzymes such as pepsin, pancreatin, trypsin and bile or bile salts for accomplishing hatching and activation of taeniid eggs (Silverman, 1954a). Subsequent studies report that this technique had given inconsistent results (Heath and Smyth, 1970). Later, an improved method of hatching and activation using digestive enzymes was described, which showed a mean activation

rate of 82.3% with the eggs of *T. saginata* (Stevenson, 1983). This technique exposed the eggs of *T. saginata* to the artificial gastric juice (pepsin and HCl) for 1.5 hrs, followed by 30 minutes to 3 hrs incubation in artificial intestinal juice (trypsin, bile and sodium bicarbonate). The viable eggs lose the embryophore and the onchospheral membrane after this treatment leaving an activated onchosphere that shows motile hooks and a secretory globule. Bile is one of the most important and necessary requirements in the activation of onchospheres (Stevenson, 1983) and is known to increase the permeability of the onchospheral membrane during the process (Silverman, 1954a). Even in the absence of trypsin, onchospheres can be activated with bile, but can not be freed from the onchospheral membrane. Hence, a combination of trypsin and bile was recommended for accomplishment of active motile onchospheres free of onchospheral membrane (Stevenson, 1983). Alternatively, sodium hypochlorite (NaOCl) has been used as a quick hatching solution and the activation has been accomplished by artificial intestinal fluids containing trypsin, bile and sodium bicarbonate (Ilsoe et al., 1990; Ishiwata et al., 1993). In one study, exshelling and activation of *T. taeniaeformis* eggs using NaOCl and artificial intestinal juices demonstrated a maximum of around 31% activated onchospheres (Ishiwata et al., 1993), when hook movements and visible secretory globule are considered as the criteria of onchosphere activation.

NaOCl has been found to be useful in the hatching of the tapeworm eggs (Laws, 1968b; Brandt & Sewell, 1981a; Lightowlers et al., 1984; Negita and Ito, 1994; Wang et al., 1997). Exshelling of the eggs can be accomplished within 5-8 minutes by using 0.5% NaOCl solution. Treatment with NaOCl removes the embryophore blocks of the eggs releasing the onchosphere enclosed in the onchospheral membrane.

The hatching and activation method has been useful for determining taeniid egg viability in several studies, but has not always been adequate. For instance, this approach under-estimates egg viability by failing to activate the infective eggs (Ilsoe et al., 1990). Silverman (1954b), reported differences in the maturity of *T. saginata* and *T. pisiformis* eggs, even when they are obtained from the same gravid proglottid. Also, the proportion of mature eggs in different gravid proglottids of the same tape worm is different. Further, activation rates of the *T. saginata* and *T. pisiformis* eggs increase with time outside the host at least for a time, which indicates that the mature eggs can only be activated immediately. On the other hand, some of the immature eggs hatched and activated after 2 weeks, while others did not activate even after 2 months indicating maturation of some of the immature eggs outside the host (Silverman, 1954b). This feature of the eggs/proglottids explains in part the shortfall in using activation rates as an accurate indicator of viability in these species of *Taenia.* Even with *T. taeniaeformis* eggs that appear more uniformly matured in terminal proglottids, hatching and activation rates obtained by this method have not been high (Ishiwata et al., 1993). Storage of these eggs, free in saline has been reported to increase their hatchability time (Jones et al., 1960). For instance, Laws (1968b) attributed the failure of *T. hydatigena* egg activation to the changes in the egg membranes following bacterial contamination of the stored samples.

1.4.2. Use of vital dyes for the assessment of taeniid egg viability

Another criterion for assessment of taeniid egg viability has been the staining of onchospheres with vital dyes (Silverman, 1954a). Vital dyes are known for their capability to differentiate between dead and live cells in studies on cell cultures.

In assessing taeniid egg viability *in vitro*, vital dyes have been used directly with intact eggs and onchospheres. Past research showed that neutral red and trypan blue are useful dyes for assessing taeniid egg viability. These dyes have been used with onchospheres that are hatched and activated using digestive enzymes. Neutral red has been used for visualization of activated onchospheres, as it stains only activated onchospheres that are free of onchospheral membrane, but not the inactive onchospheres enveloped by the onchospheral membrane (Heath and Smyth, 1970; Ishiwata et al., 1993). Trypan blue has been used for measuring egg viability with hypochlorite hatched but not activated onchospheres (Wang et al., 1997; Ciarmela et al., 2005) and onchospheres that are hatched and activated with digestive enzymes (Heath and Smyth, 1970; Wang et al., 1997).Viability with trypan blue is assessed based on dye exclusion from onchospheres that are considered viable. Apart from these dyes, biochemical stains such as D-(3-[4, 4-dimethylthiazol-2-yl]-2, 5-diphenyltetrazolium bromide or MTT has been used with the intact eggs (Madeline et al., 2001) for determining the egg viability. A colorless MTT dye will be reduced to blue formazan by the dehydrogenase enzyme present in the mitochondria of the living cells but not dead cells of onchosphere. Incubation for 5 hrs in the medium containing stain as well as the enzyme substrate complex is needed for this procedure.

When using stains for assessing egg viability, one should be aware of the nature of the egg membranes. As discussed earlier, the egg membranes are known for their resistance to penetration of various organic and inorganic chemicals (Mackie and Parnell, 1967; Nieland, 1968). Also, Silverman (1954a) reported that the onchospheral membrane of a hatched but not activated onchosphere was impermeable to vital stains and the permeability increases with the

activation of the onchosphere. If this is true, then use of the vital dyes with these onchospheres may not give an accurate measure of the egg viability. Even, the use of vital stains with the activated onchospheres may not always give a satisfactory measure of egg viability, as viable eggs may not always be activated (Ilsoe et al., 1990) and hence the permeability may not change. This might become a concern while using vital dyes as egg viability indicators.

1.5. *In vivo* assay

Measuring infectivity of eggs by using an animal model has been an alternative approach, used by researchers working on tape worms. From studies on the life cycle of various species of *Taenia*, it is known that the most suitable animal model for finding the egg infectivity of a particular species of *Taenia* would be their corresponding intermediate hosts in which the cysticercus larval forms encyst. For instance, the intermediate host of *T. taeniaeformis* can be a mouse or a rat. Hence, studies with *T. taeniaeformis* use either mouse or rat as the animal model. Similarly *T. saginata*/cattle, *T. pisiformis*/rabbit, *T. ovis*/sheep and *T. solium*/pig are valuable models in studies on the respective tape worms.

1.5.1. T. taeniaeformis/Mouse model

The ease of handling, cost-effectiveness and the similarity in the host parasite relationship between mouse and *T. taeniaeformis* parasite with that of the other tape worms/host make this model useful in the studies related to the immunity and development of efficient vaccines against taeniid antigens (Lightowlers et al., 1984, Bogh et al., 1988), and determining control measures for eradicating tape worms of human importance (Olsen and Nansen, 1990).

The eggs are fed orally to the experimental mice and after giving sufficient time (two or more weeks) for the embryonated eggs to develop into the cysticercii larval stages, the mice are killed and their carcasses (mostly liver) screened for the cysticercii larva. Based on the number of cysts found in the carcass, the percent infectivity of the eggs is assessed. The cysts sometimes can be classified as fertile and sterile based on the gross morphology of cysts (Conchedda and Ferretti, 1984). After nearly 2 months post infection, the fertile larvae in the susceptible mice can be around 5-6 mm in size with a thickening on the external surface of the cyst, which identifies the future scolex. On the other hand, sterile larvae (small or sometimes 2-3 mm) are the larval forms that fail to develop successfully in the host or those in different stages of destruction or resorption. They are generally small with absent or underdeveloped scolex thickening (Bortoletti et al., 1985). Multiple studies showed that the percent infectivity of *T. taeniaeformis* eggs in the mouse intermediate host has been highly variable.

Before using this model, one should be aware that the behavior of this model depends on factors related to *T. taeniaeformis* and the mouse intermediate host. First, the larval form of the parasite is not as host specific as that of the adult worm. It has been reported that some strains of *T. taeniaeformis* can be highly infective to rats while their infectivity in mouse intermediate host is negligible and vice-versa (Brandt & Sewell, 1981b). Although mice and rats are the most common intermediate hosts, *T. taeniaeformis* also infects other rodents such as vole. More interesting is that there are isolates of *T. taeniaeformis* reported, which are infective to only voles but not to mice or rats (Nonaka et al., 1994). Hence, before starting a study, parasite strain to be used should be mouse adapted, when mice are used as intermediate host.

Apart from the strain of parasite, other factors that influence the percent infectivity of *T. taeniaeformis* eggs are egg viability and the dosage. The effect of egg dose depends upon whether the mouse strain is susceptible or resistant to infection. In the case of a susceptible strain, the response to egg dose is positively correlated (Mitchell et al., 1980). On the other hand, if the intermediate host is resistant, a similar response is not seen. But, sometimes, an increase in the number of sterile larvae may be seen (Conchedda and Ferretti, 1984). In some instances, resistant and susceptible mice strains fed with low/intermediate egg number may not show the difference, but at very high numbers, the difference can be clearly seen (Mitchell et al., 1980). The authors suggested that the high egg dose may elicit a strong immune response that minimizes the infection in mice.

Further, viability of the egg batch is also important, especially when the mice are infected with high egg doses. Mitchell et al. (1980) suggested that high doses containing a low percentage of live eggs can elicit a strong and quick immune response affecting the infectivity of few viable eggs.

Another aspect of this system to be considered is the strain of mice infected. Various strains of inbred, hybrid, nude and severe combined immune-deficient (SCID) mice have been used. Some studies observed development of larvae in the liver (Bortoletti and Ferretti, 1985) and associated host cellular responses (Letonja et al., 1984) at the microscopic level, while others studied larval development at the macroscopic level (Orihara, 1962; Mitchell et al., 1977; Conchedda and Ferretti, 1984) in detail. From these studies, it is evident that various strains of mice differ in their susceptibility to the infection with *T. taeniaeformis* eggs. This is also the case with rat strains used in infectivity studies (Williams et al., 1981).

The larvae in resistant mice are damaged and destroyed during the early phase of infection in the host, which is not the case in susceptible strains of mice. From studies conducted using resistant C57 and susceptible C_3H mice, it was reported that by around 15 days post infection, the larvae in the livers of resistant C57 mice are either destroyed completely (Bortoletti et al., 1985) or if present, they are mostly sterile (Conchedda and Ferretti, 1984), while normal larvae are recovered from susceptible C_3H mice, when infected orally with appx. 30 - 40 eggs (Conchedda and Ferretti, 1984; Bortoletti et al., 1985). Infections in relatively resistant strains of mice such as CF1 may be intermediate as some of the larvae may be destroyed, while some develop normally (Bortoletti et al., 1985). Early larval infections occur in all the strains of mice, but the sustainability of the infection varies with strain of mouse used (Bortoletti et al., 1985). This variation among the different inbred strains of mice is attributed to the difference in their genetic composition that influences the onset of the cestode larvae lethal immune response (Mitchell et al., 1977; Mitchell et al., 1980). The inbred strains having C5 and/or C_4 component deficiency in their complement were susceptible to the infection (Mitchell et al., 1977).

Another factor to be considered especially when resistant mouse strains are used as a host is the time span between the oral inoculation of *T. taeniaeformis* eggs and the necropsy time of the mouse intermediate host, which influences the percent infectivity observed at necropsy. Only sterile larvae are usually recovered from resistant mice, and their recovery also goes down with increase in time post infection (Mitchell et al., 1980; Conchedda and Ferretti, 1984). This observed effect is probably due to the complete resorption of some larvae by the host immune system during the course of the experiment.

The sex of the mouse also influences the infectivity of *T. taeniaeformis* eggs. Males are reported to be slightly more susceptible to infection than females (Mitchell et al., 1977; Mitchell et al., 1980).

Studies on the immune response to larval *T. taeniaeformis* infections led investigators to use immune deficient SCID and nude mice as an intermediate mouse host. SCID lacks both functional T and B lymphocytes, while only T lymphocytes are absent in nude mice. Infectivity of *T. taeniaeformis* eggs in SCID mice such as CB-17 *scid/scid* ranged from 17% to as high as 50% (Ishiwata et al., 1992; Lagapa et al., 2002). Also, by infecting SCID mice orally with hatched onchospheres of *T. taeniaeformis*, 100% infectivity has been reported (Lagapa et al., 2002). Resistance to larval cestode infections in mice has been attributed mainly to T-cell responses (Ishiwata et al., 1992). Both primary infection and secondary infections can be established in SCID mice (Ishiwata et al., 1992). This situation is different with immune competent mice, in which secondary infection given within 24 hrs after primary infection is eliminated. (Conchedda and Ferretti, 1984). This reveals that surviving cysticercii larvae in the SCID mice may not experience the host immune response even after 4 weeks post primary infection. Nude mice such as female BALB/c.nu/nu and CBA/H.nu/nu were also reported to be susceptible to *T. taeniaeformis* infection (Mitchell et al., 1977). Infectivity of around 50% has been reported with these mouse strains.

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Chapter II

TAENIA TAENIAEFORMIS: DIFFERENTIAL STAINING OF ONCHOSPHERES WITH VITAL DYES UNDER CRITICAL TEMPERATURES

Abstract

Onchosphere recovery of *T. taeniaeformis* eggs incubated for 5, 10 and 20 minutes in 0.5% NaOCl was assessed. Also, effect of heat on the recovery of onchospheres was studied by exposing eggs treated for 10 minutes at 55, 65 or 75°C to 0.5% NaOCl for 6 minutes. Additionally, the efficiency of the non-vital dye acridine orange (AO) and the vital dyes propidium iodide (PI) and trypan blue (TB) in assessing taeniid egg/onchosphere viability was studied, which were exposed to 85 or 95°C in addition to the above mentioned time-temperature combinations. A quadratic decrease in the onchosphere recovery was observed related to an increase in exposure time to 0.5% NaOCl ($p<0.05$). A considerable number of onchospheres were lost even before all the eggs were exshelled. Remarkable loss of onchospheres occurred by hypochlorite exshelling the eggs treated at 75°C ($P<0.05$). Preliminary studies showed a difference in the behavior of the vital dye TB, but not between AO and PI. Subsequent staining studies were conducted that better resolve the differences in the behavior of the stains. Non-linear regression analysis of observed percentage internal staining data with each stain was done by fitting a sigmoid four-parametric model using sigma plot software 8.0 and the different stains were compared based on the x-coordinate of the inflection point (T_{50}). At 95% confidence level, all three stains differed in that they had different T_{50} temperatures. The T_{50} for AO was relatively lower (69.22 ± 0.53) than that for PI (73.9 ± 0.52), which in turn was lower than that for TB (79.4 ± 0.45).

Key words*: T. 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 known as bovine cysticercosis (BC) 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. Methods that prevent this mechanism of BC transmission are needed. One general approach is to devise treatment methods for feeds that inactivate the tape worm eggs. Development of these methods requires a model system to assess egg viability. Due to the cost factor involved in infectivity experiments, development of an accurate *in vitro* method would be useful. Due to low availability of *T. saginata* eggs, we chose to investigate *Taenia taeniaeformis*, which cycles between rodents and cats and determine feasibility of this goal.

Several criteria have been used to assess taeniid egg viability by methods that exclude infectivity. Taeniid eggs contain an onchosphere surrounded mainly by an onchospheral membrane and an outer embryophore (Nieland, 1968; Chew, 1983). One approach used to assess viability *in vitro* is by using digestive enzymatic solutions to exshell and activate onchospheres (Silverman, 1954; Stevenson, 1983). Alternatively, exshelling is accomplished by sodium hypochlorite (NaOCl) treatment followed by onchosphere activation with digestive enzymes

(Ilsoe et al., 1990). 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).

Another approach incorporated 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 onchospheres (Wang et al., 1997; Ciarmela et al., 2005) to assess viability of various taeniid species.

Vital dyes have the advantage of rapid assessment. However, there is relatively little information on the accuracy of this approach. The potential pitfall is the impermeability associated with the onchospheral membrane to vital dyes (Silverman, 1954). Because, vital dyes are commonly used for measuring egg viability, we investigated the accuracy of this approach by evaluating eggs/onchospheres exposed to a range of temperature treatments.

Although NaOCl is used as a quick exshelling agent, there is relatively little information on the recovery rates of onchospheres following NaOCl exshelling. Further, past research observed that NaOCl could damage taeniid eggs, if exshelling followed exposure to high heat (Laws, 1968). Our preliminary studies suggested that NaOCl treatment was detrimental to recovery of onchospheres, which would translate into inaccurate measurements in viability assays. Hence, we investigated the effects of NaOCl on onchosphere recovery and use of non-vital and vital dyes to assess egg/onchosphere viability following temperature treatments anticipated to damage onchospheres.

2. Materials and methods

2.1. T. taeniaeformis eggs

Eggs of *Taenia* species were either collected directly from the cat feces or the gravid proglottids collected from feces of a naturally infected cat. Fecal samples which were positive by sugar floatation technique (Foreyt, 2001) using 3 gms of fecal sample were weighed and 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 x *g* for 15 minutes. The pellet was resuspended in 10 ml sucrose solution (specific gravity = 1.27) and centrifuged in 15 ml tubes at 1500 rpm for 5 minutes. The top 2 ml of the supernatant was collected into another 15 ml tube and centrifuged twice at 480 x *g* for 5 minutes after adding 10 ml of water. After the final wash, the supernatant was removed leaving 0.5 ml of the sediment.

Gravid proglottids of *T. taeniaeformis* were identified from the feces of a naturally infected cat and were stored in PBS (pH 7.2) at 4^{o}C. The proglottids were opened by a slight modification of the procedure described by Takemoto et al. (1995). Proglottids were cut open on a 180 μm sieve with a scalpel and gently scraped with a glass rod, washed through the sieve with PBS into 50 ml centrifuge tubes and centrifuged at 1600 x *g* for 5 min. The supernatant was removed leaving 5 ml of the sediment. In both the cases, egg numbers were estimated (3 X 20 μl samples) and 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 exshell eggs by a slight modification of a described method (Wang et al., 1997). Exshelling eliminates the embryophore,

but retains the onchospheral membrane. Exshelling was performed on the normal eggs that were collected from feces and eggs isolated from proglottids that were heat treated. Onchosphere recovery was quantified following exposure to NaOCl for different durations of 5, 10 or 20 minutes. Fifty eggs comprised each replicate in this experiment. When heat treatment was used, NaOCl exshelling was done for 5 minutes 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 onchosphere recovery was determined by adding 1.5 ml PBS, centrifugation at 1600 x *g* for 5 minutes. 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 onchospheres recovered was determined under a compound light microscope (Olympus BH-2).

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 minutes depending on the experiment. Temperatures of water bath are constantly monitored during the treatment period using a thermometer. Untreated control eggs were maintained at 21°C. After treatment all tubes were cooled for 15 minutes 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), onchospheres exshelled by NaOCl were used. In this case, exshelling in 0.5% NaOCl (5 min) preceded heat treatments and staining.

2.4. Staining with AO, PI or TB

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 onchospheres 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. As AO stains both live and dead cells, it was used to assess permeability of membranes that comprise the egg. 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. Because, PI is a vital dye that stains dead cells, it was used to assess viability of onchospheres 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 onchospheres inside of onchospheral membranes. Two hundred fifty eggs (AO, PI) or 150 onchospheres (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 minutes in the dark. Then eggs were washed twice with 2 ml PBS, pelleted at 1600 x *g* for 5 minutes and the final volume was reduced to 40 μl. The TB staining was performed on onchospheres by adding 0.4% TB to a final concentration of 0.04%, followed by microscopic examination after 2 minutes. The first 30 eggs or onchospheres encountered from each of triplicate samples were examined microscopically at magnification of 100x and 400x. The AO and PI stained eggs were examined under the FITC and Tex red filters respectively, of an epifluorescent microscope (Axioskop 2 plus), while TB stained onchospheres were observed under a compound light microscope (Olympus BH-2).

2. 5. Statistical analysis

Completely randomized design (CRD) was used in these experiments. Means of triplicate measurements of recovered onchospheres 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/onchospheres 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 onchospheres when compared to controls.

2.6. Non-linear regression analysis

To analyze the response of onchospheral staining with dyes to heat treatments a non-linear regression analysis using sigma plot 8.0 software (SPSS Inc, Chicago, USA), was performed. Due to the sigmoid pattern of mean percentage of internal staining demonstrated by each of the stains across the range of temperatures tested, a sigmoid four- parametric model (eq.1) was fitted to the data observed on various stains individually and inflection points for each stain were estimated using the following equation:

$$I_T = I_0 + \frac{I_{net}}{1 + e^{-\left(\frac{T - T_{50}}{r}\right)}} \qquad \text{.....} \quad \textbf{[Eq. 1]}$$

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

3. Results

3.1. Onchosphere loss due to NaOCl

Sodium hypochlorite treatments lead to the loss of eggs and onchospheres recovered with increased incubation time (Fig. 1). The mean percentage in the loss of eggs and onchospheres recovered increased with time of incubation and showed a strong quadratic trend ($p<0.05$). Although, the highest percentage of onchospheres was recovered after 10 minutes, a mean 39% loss in recovery of eggs and onchospheres had already occurred and continued to increase with incubation time after 10 minutes.

3.2. Onchosphere recovery after heat treatment

Sodium hypochlorite had a marked effect on the recovery of onchospheres following high heat treatment (Fig. 2). A significant decrease in mean percentage of recovered onchospheres was observed by treating eggs at 75°C for 10 minutes ($p<0.05$). However, mean percentage of onchospheres recovered after treating eggs at 55 or 65°C for 10 minutes were not different from the control group (21°C). The disintegration of onchospheres 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 onchospheres by NaOCl treatment. The affect was more pronounced with increasing time of exposure and marked elevation in NaOCl sensitivity was observed between heat treatments of 65 and 75°C.

3.3. Staining of taeniid onchospheres 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 3, A). Other eggs demonstrated green fluorescence in onchospheres, which was called internal staining (Fig 3, B). Similarly, PI stained eggs either peripherally (Fig 3, C), or internally (Fig 3, D). 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 onchospheres exshelled by 0.5% NaOCl (5 min), internal staining by TB was observed, but variably (Fig 3, E 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 minutes (Fig. 4). Exshelled onchospheres that retained the onchospheral membrane routinely showed less than 10% internal staining by TB. From these preliminary staining experiments, 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.

3.4. Response curves of staining under increasing temperature treatments

Higher temperature treatments were used to better resolve the staining behavior of these dyes under the anticipated lethal conditions for *T. taeniaeformis* eggs (AO, PI) or exshelled onchospheres (TB). The data in the figures 5 A-C, show that a high percentage of eggs or onchospheres were stained only after high temperature treatments and the behavior of stains varied.

Nonlinear regression analysis (eq.1) on obtained data with each stain was performed to compare them based on the inflection point (T_{50}). This parameter reflects the point at which internal staining is 50% of the net increase in percentage internal staining (I_{net}). The model identified inflection points for AO and PI curves, but not for 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 onchospheres that were treated at 21, 75 or 95°C for 10 minutes. The response at 21 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, the corresponding r^2 values for each curve that indicated the best fit are shown in Table 1 with related p values. Differences among the stains were determined based on the mean T_{50} value. At a 95% confidence level, all the three stains differed in that they had different T_{50}. 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 (as discussed later). This possibility is supported by an increasing T_{50} associated with increasing molecular weight for dyes used (Table 1).

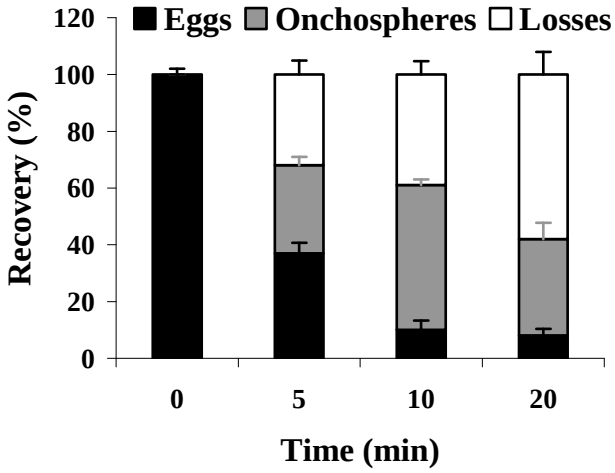

Figure 1. Influence of incubation time in 0.5% hypochlorite (NaOCl) on *T. taeniaeformis* egg and onchosphere recovery. Eggs were treated with 0.5% hypochlorite for 5, 10 or 20 minutes. Eggs that received no hypochlorite treatment served as control (0 group). The recovered onchospheres or eggs or total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

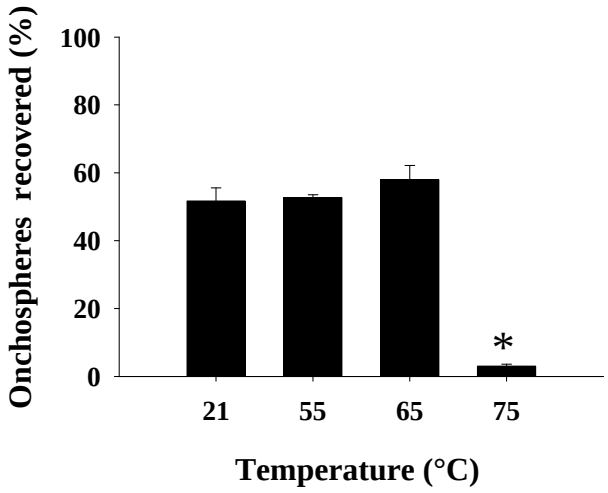

Figure 2. Effect of thermal treatment of *T. taeniaeformis* eggs on recovery of onchospheres when exshelled in 0.5% hypochlorite (NaOCl). Eggs heat treated for 10 minutes at temperatures indicated were exshelled by 0.5% NaOCl for 5 minutes. The recovered onchospheres were reported as a percentage mean ± 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 when repeated the experiment (not shown).

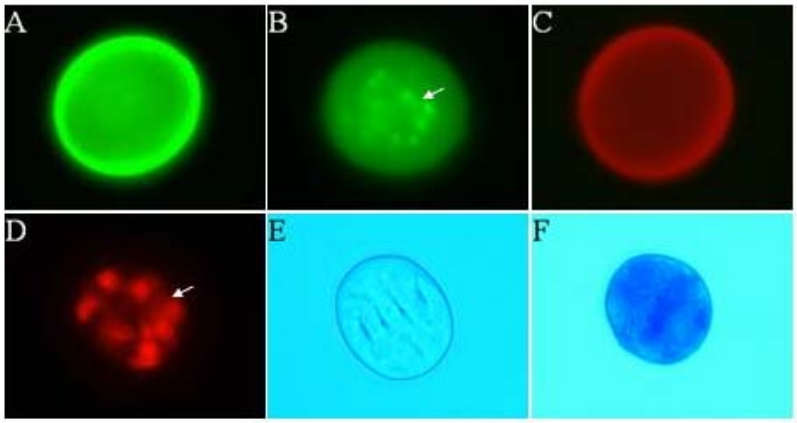

Figure 3. Staining of *T. taeniaeformis* eggs or onchospheres. *T. taeniaeformis* eggs were stained with acridine orange (A,B) and propidium iodide (C,D) at final concentrations of 6 μg ml^{-1} or 2 μg ml^{-1}, respectively for 15 minutes at room temperature in the dark and examined under fluorescent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

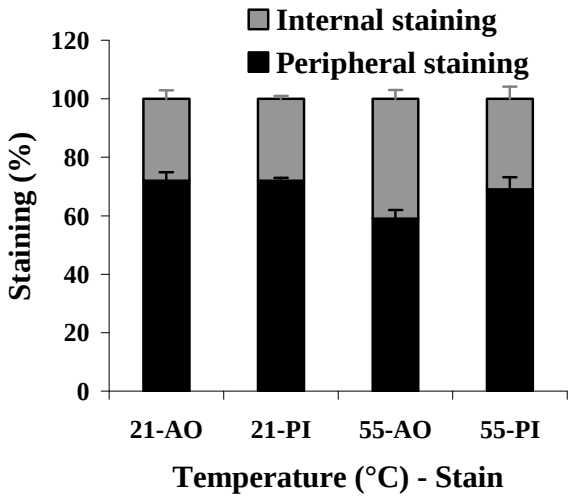

Figure 4. Acridine orange (AO) or propidium iodide (PI) staining of *T. taeniaeformis* eggs exposed to 21 or 55°C for 10 minutes. Eggs from each group were stained for 15 minutes with AO or PI at the final concentration of 6 μg ml^{-1} or 2 μg ml^{-1}, respectively; and examined under fluorescence microscopy. 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.

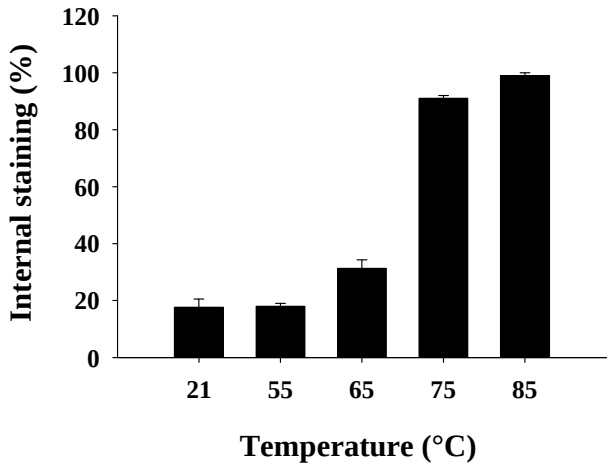

Figure 5A. Acridine orange (AO) staining of heat treated *T. taeniaeformis* eggs. Eggs heat treated for 10 minutes at temperatures indicated were stained with AO at final concentration of 6 μg ml^{-1}. Results show the proportion of internally stained eggs expressed as mean percentage ± S.E.M of the first 30 eggs examined in each of three replicates in every group. Experiment when repeated showed similar results (not shown).

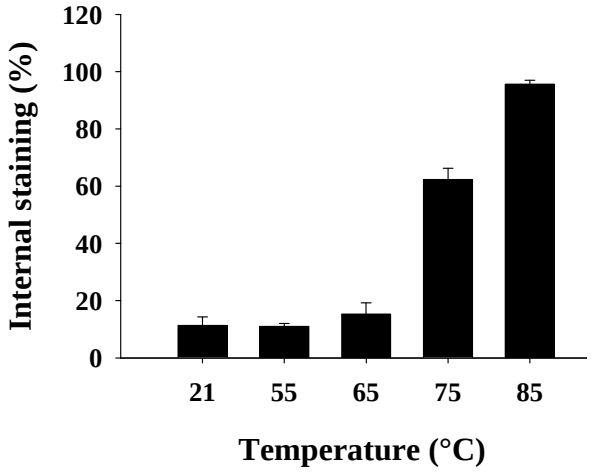

Figure 5B. Propidium iodide (PI) staining of heat treated *T. taeniaeformis* eggs. Eggs heat treated for 10 minutes at temperatures indicated were stained with PI at final concentration of 2 μg ml^{-1}. Results show the proportion of internally stained eggs expressed as mean percentage ± S.E.M of the first 30 eggs examined in each of three replicates in every treatment. Experiment when repeated showed similar results (not shown)

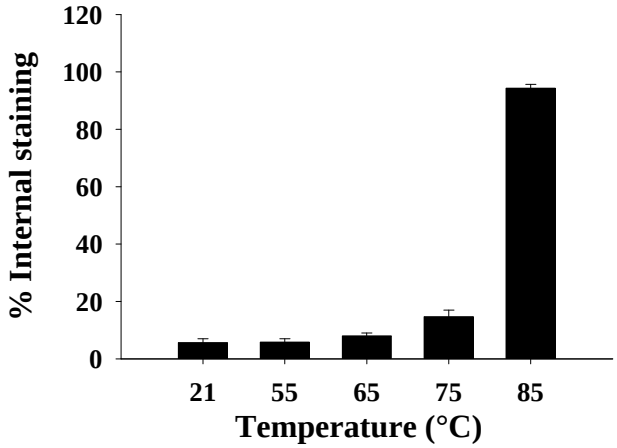

Figure 5C. Trypan blue staining of heat treated *T. taeniaeformis* onchospheres. Onchospheres obtained by exshelling in 0.5% sodium hypochlorite for 5 minutes were treated for 10 minutes at temperatures indicated and stained with TB at final concentration of 0.04% at room temperature. Results show the proportion of internally stained onchospheres expressed as mean percentage ± S.E.M of the first 30 onchospheres examined in each of three replicates in every group. Experiment when repeated showed similar results (not shown).

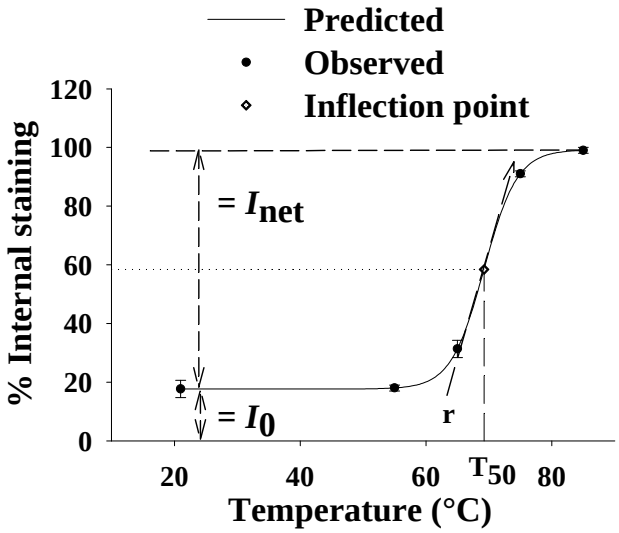

Figure 6. Sigmoid four-parametric non-linear regression model. Internal staining data observed with acridine orange (Fig 5A) was used as an example, to show the four parameters estimated by the model. For the internal staining curve of each stain, 'I_{net}' is the net increase in percentage internal staining due to heat treatment, r indicates slope coefficient of the curve, 'T_{50}' indicates x coordinate (temperature in $^{\circ}$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.

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

Stain (MW)	Regression Parameters				r^2-value*
	I_{net}*	r*	T_{50}*	I_0*	
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.

*All parameters in the respective columns are significantly different from 0 at $p<0.05$.

[a,b,c]T_{50} identified by different superscripts are different at 95% confidence level.

All parameter values were associated with standard error (S.E.).

4. Discussion

Experiments described here investigated the use of methods other than infection to assess viability of *T. taeniaeformis* eggs after heat treatment. Specifically, we investigated the effect of NaOCl for exshelling onchospheres on egg and onchosphere recovery and the performance of viability stains. In each case significant short comings were identified for use of these methods.

An important observation in this study was that different vital stains displayed distinct behavior in eggs and onchospheres under a range of temperatures. Although PI and TB differ in chemical properties, both vital dyes are expected to stain dead onchospheres following a lethal treatment unless other factors complicate this situation. In our experiments PI and TB differed in ability to stain onchospheres under both low and high temperatures as indicated by the lower T_{50} for PI compared to TB. Staining of onchospheres in eggs by TB stain was poor regardless of heat treatment. Use of hypochlorite exshelled onchospheres demonstrated enhanced staining only with 85 and 95°C treatments. Even if hypochlorite was assumed to cause leaky onchospheres 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 in T_{50} in response to high temperatures between AO and PI.

One factor that may account for these observations is the impermeability of onchospheral membrane. Earlier research reported the impermeability of the onchospheral membrane to vital stains such as neutral red (Silverman, 1954a; Heath and Smyth, 1970), Even though, neutral red is a membrane permeable dye, its ability to stain only the activated and live onchospheres that have lost the onchospheral membrane (activation in enzymatic solutions), indicated the potential problem of impermeability associated with the onchospheral membrane. 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 impermeability of egg membranes.

The behavior of dyes used here lead us to suggest that the internal staining of the onchosphere in response to temperatures between 65 and 95°C reflects the diameter of the membrane pores created by heat denaturation, rather than viability of the onchosphere. A likely membrane to be involved is the onchospheral membrane, but involvement of other egg membranes can not 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 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 onchospheres. Hence use of vital dyes appears to be an unacceptable means to assess viability of onchospheres within eggs or onchospheral membranes. This conclusion may not apply to methods that lead to a breach in egg and onchospheral membranes (e.g. enzymatic treatments). However, the variability of these methods undermines reliable assessment of onchosphere viability, also.

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 identify two short

comings of 0.5% NaOCl for this purpose. One is that use of hypochlorite caused an increasing loss in recovery of eggs and onchospheres with increasing time of incubation. Loss in onchospheres was experienced even before all eggs had been exshelled to onchospheres. 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 the degenerating effect of hypochlorite oxidation on onchospheres.

A second short coming relates to the first but was especially pronounced when hypochlorite was used to exshell eggs following temperature treatments. A dramatic enhancement in loss was observed between 65 and 75°C. That loss was associated with disintegration of the onchospheres, which was directly observed by microscopy. Earlier research reported similar detrimental effects of 1% NaOCl on *T. pisiformis* eggs after heated at 65°C for 2 hrs (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 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.

A primary interest in this investigation was to identify *in vitro* methods that could reliably assess viability of taeniid eggs. Our results indicate that use of vital dyes to stain onchospheres under relatively low and higher temperatures poses a problem in the accurate assessment of onchosphere viability. If this situation applies equally to other kinds of treatments, then use of vital dyes to assess taeniid egg viability would produce significant inaccuracies in estimates of those treatment effects. Further, inclusion of 0.5% NaOCl as exshelling agent in methods that assess egg viability may further compromise the accuracy of the viability estimates due to degeneration of a portion of eggs/onchospheres. Additionally, the increased destruction of

eggs/onchospheres by NaOCl following high heat treatment presents limitation on the use of NaOCl in viability studies that assess heat effects. From these conclusions, methods that incorporate 0.5% NaOCl and/or vital dyes for accurately assessing anti-taeniid egg treatments are not currently available.

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CHAPTER III

PRELIMINARY *IN VIVO* STUDIES

3.1. In vivo assay using mouse model

3.1.1. T.taeniaeformis eggs

Gravid proglottids were obtained from the feces of naturally infected cattle. The eggs were isolated from the proglottids as mentioned in 2.1, except that the eggs used in experiment 2 were stored in PBS containing Penicillin (100 IU/ml), streptomycin (100IU/ml). All the eggs were used within 4 weeks from the date of proglottid collection.

3.1.2. Animals

5-6 weeks old, 25 male C_3H/He, 16 male CB-17 scid/scid mice (Taconic breeders, Massachusetts, USA) or 8 female NCRNU-M-M-Tac:cr: (Ncr)-Foxn1<nu> (Derek Mc Lean's lab, Pullman, WSU) were received and housed in EALB (Washington State University, Pullman). All mice were fed commercial mouse food (Harvalans Inc, USA) and water ad libitum.

3.1.3. Infection

Infection (*T. taeniaeformis* eggs in phosphate buffer saline) was given to mice orally using 2" curved 18G blunt feeding needles (Harvard Apparatus) as stomach tube (Mitchell et al, 1980).

3.1.4. Necropsy

The mice were euthanized using CO_2 inhalation technique after 3-5 weeks post infection (p.i) and the entire carcass was screened for the presence of cysts. Additional emphasis was placed on the liver by slicing it into 2mm pieces using scalpel.

3.1.5. Experimental design

The experimental design was a Completely Randomized Design with mouse within a cage as the experimental unit.

3.1.6. Thermal treatment of T. taeniaeformis eggs

In experiment 1, the two treatments were infection of mice with 300 eggs in phosphate buffer saline (180 µl) after heat treatment at 55^{o} C or 75^{o} C for 10 minutes to each of the 5 male C_3H/He mice. Necropsy was conducted at 33 days p.i.

In experiment 2, the only treatment was the infection with 600 eggs in 180 µl per mice after thermal exposure in water bath at 65^{o}C for 10 minutes, to each of the 4 CB-17 *scid/scid* male mice or 4 female NCRNU-M-M-Tac:cr: (Ncr)-Foxn1<nu> mice. The positive control comprised of 4 mice each of both strains that received 600 eggs, while the negative control included single CB-17 *scid/scid* male mice fed 180 µl PBS that had no eggs. A single mouse that received 180 µl PBS with out any eggs was used as a negative control. Necropsy was conducted at 21 days p.i.

In experiment 3, three groups of 3 male, 10-12 week old, CB-17 scid/scid mice each were infected orally as mentioned above with either 2000 intact *T.taeniaeformis eggs*, 700 hypochlorite exshelled onchospheres or 700 hypochlorite exshelled onchospheres obtained from

eggs treated at 65^{o}C for 10 minutes, per mice (suspended in150 µl PBS). These mice were initially received at 5-6 weeks of age. But, due to 5 week delay in procurement of samples, they were infected at 11-12 weeks of age. Necropsy was performed at 21 days post infection.

3.2. Ensilage of T. taeniaeformis eggs in potato slurry

Eggs collected from proglottids were dispensed into several veraspor (3µm) filter bags that were sealed with heat and either stored at 4^{o}C in PBS (control) or ensiled in with potato slurry (21% DM) in mini silo containers (250 ml) for 28 days, when the final p^{H} was 4.48. Then the eggs were collected from the filter bags into PBS and 2 groups of 5 male C_3H/He mice each were infected orally with either 200 control eggs or 300 ensiled eggs. The final dry matter of the potato silage was 29.3%. Necropsy was conducted at 33 days p.i.

Experiments that infected mice did not yield any cysticercii larvae, when the mice were examined after necropsy. Even the control group mice in any of the above experiments showed no infection and hence were unable to generate the data.

APPENDIX

Table 2. *T. taeniaeformis* egg numbers isolated directly from cat feces using sugar floatation and the two-step method

Sample No	Sucrose floatation test (Glass slide)		Two step Method	
	Wt of sample (g)	No of Eggs found Per g	Total sample wt (g)	Total eggs Expected/recovered
1	3	2	50	100/380
2	3	10	40	400/1680
3	3	100	40.4	4040/6000
4	3	10	50.3	503/1300