EFFECTS OF VARIOUS PHYSICAL AND CHEMICAL FACTORS ON EXCYSTATION OF THE ENCYSTED METACERCARiae OF ECHINOSTOMA CAPRONi

Bernard Fried and Robert C. Peoples
Department of Biology, Lafayette College, 204 Kunkel Hall, Easton, Pennsylvania 18042. e-mail: friedb@lafayette.edu

ABSTRACT: Various physical and chemical factors were studied to determine their effects on the viability of encysted metacercariae of Echinostoma caproni. Viability was equated with chemical excystation in an alkaline trypsin–bile salts (TB) medium. Control cysts showed excystation percentages of >90% in TB. Excystation proved to be a more reliable criterion of cyst viability than observations by light microscopy. Isolated cysts and cysts left in the snail (in situ cysts) were studied. Generally, in situ cysts proved more resistant to various physical and chemical treatments than did isolated cysts. Cysts stored for 7 days at 28 C in a Locke’s 1:1 solution showed 97% excystation, suggesting that cysts of this species would survive postal delays during shipment. Of numerous marinades tested, the one that was most harmful to isolated and in situ cysts was vinegar. Isolated and in situ cysts were killed by boiling (100 C) for 1 or 3 min, but freezing at –10 C did not kill all isolated or in situ cysts after 24 hr. Concentrations of potassium permanganate ranging from 300 to 1,200 mg/L killed most isolated cysts within 5 min, but in situ cysts survived these concentrations for 24 hr. Concentrated solutions of NaCl and sucrose had no effect on the viability of isolated and in situ cysts, suggesting that their use in food preparations for molluscs would not be effective in killing echinostomatid cysts in tainted snail tissues.

Recent work has been concerned with food-borne trematode infections and their transmission to humans and animals by encysted metacercariae in various second intermediate hosts, such as fish, crustaceans, insects, and mollusks, as well as transmission of encysted metacercariae to humans and animals by plants (see review in Fried et al., 2004). Attempts to block transmission of these cysts to the definitive hosts by use of chemical and physical means that kill metacercariae have been relatively few. Ashrafi et al. (2006) described ways of killing fascioloid cysts using various chemical and physical agents. They assessed the effectiveness of their methods on killing cysts by the use of chemical excystation studies and by infecting experimental hosts with treated cysts. The latter authors also provided a review of the relatively few other studies on killing fascioloid and nonfascioloid cysts, mainly worked on heterophyids and other digeneans that encysted in fish and crustaceans. Their review did not mention studies that attempted to kill echinostomatid and brachylaimid metacercariae found in snails. Interest on echinostomatids in snails is of concern because certain ones are infective to humans following the consumption of tainted freshwater snails (Garczyk and Fried, 1998); likewise, interest in brachylaimids exists because metacercariae of these digeneans infect humans following the ingestion of tainted land snails (Butcher et al., 1996). Thus, from an economic and medical point of view parasitologists and malacologists are interested in factors that kill metacercariae in snails.

As mentioned in several recent reviews (Toledo and Fried, 2005; Toledo et al., 2007), the E. caproni–Biophalaria glabrata model is a favorable one for studying various aspects of trematode–host parasite relationships. In this model, cysts of E. caproni can be obtained directly from experimentally infected B. glabrata snails or following prolonged storage of encysted metacercariae in Locke’s solution at 4 C. Moreover, the encysted metacercariae are relatively easy to excyst in vitro with the use of an alkaline trypsin–bile salt Earle’s medium at 41 C. The percentage of excystation in this solution is usually >90% (Rossi et al., 2001). Chemical excystation is a good indicator of the viability of E. caproni cysts, because encysted metacercariae are infective to mice (Rossi et al., 2001). A recent study (Fried and Peoples, 2007) has shown that chemical excystation of E. caproni metacercariae is a more reliable index of cyst viability than judgment by light microscopy alone. In view of the above considerations, we describe herein some of the physical and chemical factors that kill encysted metacercariae of E. caproni. We first examined cysts isolated from the kidney/pericardium of experimentally infected B. glabrata snails (hereafter referred to as isolated cysts) and then studied cysts within the snail host (hereafter referred to as in situ cysts). Our tests include chemical, physical, and storage studies.

MATERIALS AND METHODS

Maintenance and use of the cysts

Encysted metacercariae (cysts) of E. caproni were dissected from the kidney/pericardium of experimentally infected B. glabrata snails as described in Rossi et al. (2001). The cysts were stored about 500 per 10 ml of Locke’s 1:1 solution at 4 C and used within 10 wk poststorage. Cysts removed from the snails were referred to as “isolated cysts” to distinguish them from those within the snails, which are referred to as “in situ cysts.” Prepared as described in the last paragraph of this section. Isolated cysts treated with various chemical and physical agents were excysted in an alkaline trypsin–bile salts (TB) medium described by Fried and Roth (1974) for the excystation of Parorchis acanthus. The TB medium has been used for numerous studies on E. caproni (Ursone and Fried, 1995; Rossi et al., 2001; Fried and LaTerra, 2002). Previous studies (Rossi et al., 2001; Fried and LaTerra, 2007) showed >90% excystation following treatment of E. caproni cysts in TB for 2 hr at 41 C. During the chemical and physical tests used in this study, trials on approximately 500 isolated cysts stored for 2–10 wk at 4 C and treated in TB for 2 hr at 41 C showed 93% excystation, that is, 4/12 of 308 cysts released active larvae into the medium. Therefore, in the present study, chemical and physical treatments that yielded excystation results of 90% or higher were not considered detrimental to the viability of E. caproni cysts.

Although most tests were done on isolated cysts, some were also done on in situ cysts. To obtain in situ cysts, juvenile B. glabrata snails, about 5–7 mm in diameter, were exposed individually in multwell chambers to about 25–50 cysts of E. caproni as described in Schnick and Fried (2004) to obtain about 12–25 encysted metacercariae per snail. Confirmation of the number of cysts per snail was made when the chemical and physical tests were done on in situ cysts. These studies were done 2–14 days postinfection of the snails.

Storage treatment

To simulate possible conditions that cysts may undergo during shipment by first-class mail, 3 samples each of 50–100 isolated cysts were stored at 23, 28, or 41 C in shell vials containing 10 ml of Locke’s...
Table I. Effects of physical treatments on excystation of isolated and in situ cysts.*

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Time</th>
<th>Isolated cysts</th>
<th>In situ cysts</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Total number</td>
<td>Number excysted</td>
</tr>
<tr>
<td>Temperature</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>-10 C</td>
<td>24 hr</td>
<td>74</td>
<td>23</td>
</tr>
<tr>
<td>-10 C</td>
<td>48 hr</td>
<td>48</td>
<td>0</td>
</tr>
<tr>
<td>-60 C</td>
<td>2 hr</td>
<td>11</td>
<td>0</td>
</tr>
<tr>
<td>-60 C</td>
<td>24 hr</td>
<td>50</td>
<td>0</td>
</tr>
<tr>
<td>100 C</td>
<td>1 min</td>
<td>12</td>
<td>0</td>
</tr>
<tr>
<td>100 C</td>
<td>3 min</td>
<td>16</td>
<td>0</td>
</tr>
<tr>
<td>100 C</td>
<td>5 min</td>
<td>19</td>
<td>0</td>
</tr>
<tr>
<td>Water content</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Aestivation</td>
<td>24 hr</td>
<td>19</td>
<td>0</td>
</tr>
<tr>
<td>Aestivation</td>
<td>48 hr</td>
<td>12</td>
<td>0</td>
</tr>
<tr>
<td>Desiccation</td>
<td>24 hr</td>
<td>32</td>
<td>0</td>
</tr>
<tr>
<td>Desiccation</td>
<td>48 hr</td>
<td>76</td>
<td>0</td>
</tr>
</tbody>
</table>

* The number of isolated cysts used per trial ranged from 11 to 76. The usual number of in situ cysts per trial ranged from 12 to 25.
† Indicates the use of a higher number of in situ cysts than in a typical trial.
‡ Indicates the use of a lower number of in situ cysts than in a typical trial.

1:1. These cysts were subjected to chemical excystation following 7 days of storage.

Physical tests

Both isolated and in situ cysts were subjected to various physical tests. Boiling and freezing of cysts were done to simulate possible conditions to which the cysts would be subjected during food preparation. Isolated cysts or whole snails containing cysts were placed directly into a 30-ml beaker containing 25 ml of boiling deionized water (DI) for 1 or 3 min. For tests on freezing, isolated cysts and snails with cysts were maintained in petri dishes, 6 mm in diameter, each with 10 ml of DI; the dishes were maintained at −10 C for either 24 or 48 hr. To test the effects of a colder temperature, isolated and in situ cysts were maintained in DI at −60 C for either 2 or 24 hr. Both aeration (24 C and 98 % relative humidity) and desiccation (23 C and 45 % relative humidity) treatments were done on cysts to determine the effects of water loss on cysts over time. Desiccation is a more severe form of water deprivation than is aeration. Isolated and in situ cysts subjected to aeration were kept on moist filter paper placed in a covered finger bowl for either 24 or 48 hr. Cysts subjected to desiccation were left in open petri dishes for either 24 or 48 hr.

Potassium permanganate treatment

Because of its use as a helmint larvicide, its relatively cheap cost, and apparent effectiveness in detaching and limiting the viability of fasciolid cysts (Asghafi et al., 2006), potassium permanganate (KMnO4) was tested to determine its effect on E. caproni cysts. KMnO4 was diluted with DI (1:1) to achieve concentrations of 300, 600, and 1,200 mg/L as done by Ashrafi et al. (2006). Isolated cysts were treated for either 5 min or 1 hr, washed 3 times with DI water, and then subjected to chemical excystation in TB. Because in situ cysts showed a high percentage of excystation in all concentrations of KMnO4 after 1 hr, treatment time was increased to 3 hr.

Chemical treatments

The effects of various chemicals on E. caproni cysts were determined based mainly on the work of Ashrafi et al. (2006) using ammonium hydroxide, acetic acid, hydrochloric acid, and formaldehyde at reagent strength or following dilution with DI (v/v) to obtain concentrations of 25, 50, 25, 12.5, 6.3, and 3.1%. Absolute ethanol was only tested at full strength. Isolated cysts were treated for 1 hr and then washed 3 times prior to treatment in TB. Because consumption of snails treated with hazardous chemicals poses a health risk, chemical tests on in situ cysts were considered not applicable to simulate possible modes of metacercariae infections to humans; such tests were not done.

Treatment with food marinades

Cysts were treated with various sauces and marinades for 1 hr to mimic conditions that cysts would possibly experience during food preparation. The following marinades were used: vinegar (Shop Rite, Elizabeth, New Jersey), lemon juice and Italian salad dressing (GIANT Foods, Landover, Maryland), soy sauce (I is Choy, Lincoln, Nebraska), and Worcestershire sauce (French's Co., Rochester, New York). Sodium chloride and succrose solutions were also used because of their prominent role in human diets; they were tested at concentrations (w/v) of 20, 10, and 5% with DI as the diluant. Following treatment in the marinades, isolated cysts and snails containing cysts were washed 3 times with DI water prior to TB treatment.

RESULTS

Storage of cysts at various temperatures

Isolated cysts stored at either 23 or 28 C for 7 days showed excystation percentages of >90%, whereas those stored at 41 C for 7 days showed a marked decline (<40%) in excystation. The results at 23 C were 55 (91.6%) of 60 excysted; at 28 C, 142 (96.6%) of 147 excysted; and at 41 C, 19 (39.6%) of 48 excysted.

Physical tests

The results of the physical tests are summarized in Table I. Isolated and in situ cysts showed >30% excystation following treatment at −10 C for 24 hr. No cyst excysted after 48 hr treatment at −10 C. Freezing at −60 C for 2 or 24 hr killed all cysts, as did boiling cysts (100 C) for 1 or 3 min. These results were the same for isolated or in situ cysts. Isolated cysts did not excyst following aeration or desiccation, whereas in situ cysts excysted at approximately 90% or higher levels (Table I).

Potassium permanganate treatment

The 5-min KMnO4 test based on Ashrafi et al. (2006) resulted in 0 excystation (about 50 isolated cysts per trial) when cysts were treated with 600 mg/L or 1,200 mg/L of KMnO4. Isolated cysts treated in 300 mg/L of KMnO4 for 5 min showed 9.0% excystation. All cysts (about 50 per trial) were killed in the

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above-mentioned concentrations when treated for 1 hr. Whole
snails (in situ cyst tests) treated with KMnO₄ for 24 hr showed
high excystation percentages as follows: in 300 mg/L, 25 of 30
(83.3% excysted); in 600 mg/L, 45 of 48 (93.8% excysted); and
in 1,200 mg/L, 27 of 32 (84.4% excysted).

**Chemical tests**

All chemicals mentioned in the Materials and Methods killed
isolated cysts except for 6.3 and 3.1% HCl, which yielded 55.0
and 55.5% excystation, respectively. Cysts treated with 3.1%
formaldehyde showed 14.3% excystation, whereas cysts treated
with absolute ethanol yielded 10.4% excystation.

**Treatment In marinades, sodium chloride, and sucrose**

Results of marinades, sodium chloride (NaCl), and sucrose
on excystation of isolated and in situ cysts are summarized in
Table II. Excystation did not occur following 1 hr treatment for
both isolated and in situ cysts in vinegar. All concentrations of
NaCl and sucrose solutions did not interfere markedly with ex-
cystation of isolated or in situ cysts. However, marinades such
as Italian salad dressing and lemon juice had a marked effect
on isolated cysts, but not as dramatic an effect on in situ cysts.

**DISCUSSION**

This is the first study that has examined physical and chemi-
cal factors that kill digenean metacercariae that encyst in snails
in a manner similar to that of Ashrafi et al. (2006) for fasciolids
that encyst on vegetation. In our study, we equated viability
with the ability of the cyst to undergo chemical excystation and
release a live juvenile worm. This approach allows for mean-
ningful observations on cyst viability without having to use in-
fection experiments in animal models. Previous studies on E.
caproni have indicated that the ability of this organism to ex-
cyst is highly correlated with the infectivity of the cyst in ani-
mal models (Chien et al., 1993; Rossi et al., 2001).

Some differences are apparent in our echinostomatid study
compared with that of Ashrafi et al. (2006) on fasciolids. The
hardiness of fasciolid cysts is shown by their ability to survive
treatment with KMnO₄ for 5 min at concentrations of 300–
1,200 mg/L (Ashrafi et al., 2006), whereas isolated cysts of E.
caproni were killed at these concentrations after a 5-min treat-
ment. Apparently, cysts such as fasciolids that encyst in the
open on vegetation or other surfaces have cyst walls and other
unknown factors that are more protective to KMnO₄ than the
cyst walls of E. caproni that encyst in snails. Species of Echi-
nostoma cercariae in the 37-collar-spined _revolutum_ group oc-
casionally encyst ectopically or on the shells of molluscs (Fried
and Graczyk, 2004). Based on observations in our study, treat-
ment of mollusc surfaces with KMnO₄ should kill cysts on the
shells.

Cysts within the pericardial/kidney region of _B. glabrata_
infected with _E. caproni_ were generally more resistant to both
chemical and physical treatments than isolated cysts, suggesting
that the intact snail provides some protection to the cysts.
Chemical and physical treatments of intact snails with echi-
nostomatid cysts must be more harsh to kill in situ cysts than
similar treatments on isolated cysts.

Our literature search did not find evidence of humans feeding
on _B. glabrata_ snails with or without _E. caproni_ infections. We
have not seen reports of human infections with _E. caproni_ and
this trematode in the wild mainly parasitizes various avian and
aquatic mammalian hosts (Fried and Huffman, 1996). However,
various molluscan species serve as natural second intermediate
hosts of echinostomatid trematodes and humans feeding on raw
or improperly cooked tainted gastropods or bivalves are pos-
sible definitive hosts of these food-borne trematodes. Based on
our experiments with the _E. caproni-B. glabrata_ model, boiling
of such infected snails at 100 C for as little as 1 min may be
effective in killing cysts, whereas freezing at 10 C for 24 hr
does not kill all _E. caproni_ cysts in _B. glabrata_. Our observa-
tions, at least based on this model, suggest that the use of cer-
tain marinades are ineffective in killing echinostomatid cysts
and that NaCl or sucrose treatments may not be effective in
killing them. Treatments using various marinades, salts, and
sugars may be used by humans in preparing molluscian dishes
for consumption. It is to be emphasized that such treatments do
not protect humans from the possibility of contracting echino-
stomiasis from tainted molluscan foods.

Among the food preservatives tested, the salad dressing had
little effect on _E. caproni_ cysts (with the exception of Italian).
Several popular food agents used commonly in countries with

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**Table II. Effects of 1-hr treatment of various marinades, sodium chloride, and sucrose on excystation of isolated and in situ cysts.**

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Isolated cysts</th>
<th>In situ cysts</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Total number</td>
<td>Number excysted</td>
</tr>
<tr>
<td>Lemon juice</td>
<td>30</td>
<td>23</td>
</tr>
<tr>
<td>Soy sauce</td>
<td>30</td>
<td>0</td>
</tr>
<tr>
<td>Worcestershire sauce</td>
<td>32</td>
<td>0</td>
</tr>
<tr>
<td>Vinegar</td>
<td>30</td>
<td>0</td>
</tr>
<tr>
<td>Italian salad dressing</td>
<td>25</td>
<td>4</td>
</tr>
<tr>
<td>5% NaCl</td>
<td>93</td>
<td>90</td>
</tr>
<tr>
<td>10% NaCl</td>
<td>56</td>
<td>52</td>
</tr>
<tr>
<td>20% NaCl</td>
<td>43</td>
<td>39</td>
</tr>
<tr>
<td>5% sucrose</td>
<td>62</td>
<td>56</td>
</tr>
<tr>
<td>10% sucrose</td>
<td>76</td>
<td>74</td>
</tr>
<tr>
<td>20% sucrose</td>
<td>95</td>
<td>88</td>
</tr>
</tbody>
</table>

* The number of isolated cysts used per trial ranged from 25 to 95. The usual number of in situ cysts per trial ranged from 10 to 16.
† Indicates the use of a higher number of in situ cysts than in a typical trial.
food-borne zoonotic trematode problems were quite effective on isolated cysts, e.g., soy sauce, vinegar, even Worcestershire sauce. Although the in situ cysts may be shielded in the case of *E. caproni*, metacercariae of other zoonotic trematodes, e.g., species of *Fasciola*, *Paragonimus*, *Clonorchis*, and *Opisthorchis*, might be vulnerable to such treatments.

One of us (B.F.) often supplies cysts of *E. caproni* to colleagues who request this echinostome for teaching or research purposes. Whenever possible, the cysts are sent by express mail and hopefully reach the recipients within a few days of mailing. Occasionally cyst deliveries have been delayed and not reached their recipient for 7–10 days, the viability of such cysts was always in question. Based on the present observations, we are confident that cysts maintained in Locke’s 1:1 in transit for 7 days at temperatures as high as 28 °C retain viability.

Finally, we have not used morphology as a guide to *E. caproni* cyst viability because light microscopic observations are not good indicators of such viability, as documented recently by Fried and Peoples (2007). Cysts that appear nonviable by light microscopy will often excyst when treated in the TB medium. More critical morphological studies by light and transmission electron microscopy are needed to correlate the morphological events that occur during the death of this cyst.

**LITERATURE CITED**


