Institut für Parasitologie
der Vetsuisse-Fakultät Universität Zürich

Direktor: Prof. Dr. P. Deplazes

In vivo viability of *Echinococcus multilocularis* eggs in a rodent model after different thermo-treatments

Inaugural-Dissertation

zur Erlangung der Doktorwürde der
Vetsuisse-Fakultät Universität Zürich

vorgelegt von

Karin Federer

Tierärztin
aus Berneck, St.Gallen

genehmigt auf Antrag von

Prof. Dr. P. Deplazes, Referent

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Meiner Familie
und
meinem Partner Colin Schwarzwald

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2. Summary

Echinococcus multilocularis is the causative agent of alveolar echinococcosis, a serious and emerging zoonotic disease in many parts of the northern hemisphere. Humans and other accidental hosts can acquire the infection by the ingestion of eggs excreted by the carnivore definitive hosts.

The goal of this study was to develop a sensitive *in vivo* method to determine the viability of *E. multilocularis* eggs and to establish suitable conditions for inactivation.

The sensitivity of a rodent model was evaluated and, conclusively, C57Bl/6 mice were most susceptible to subcutaneous inoculation of small numbers of sodium hypochlorite-resistant oncospheres, even more than to oral inoculation of mature eggs.

In the second part of the study, various combinations of exposure temperature (45–80°C), times (30–180 min) and relative humidity (70% vs. suspended in water) were tested. After heat treatment in an incubator, the sodium hypochlorite resistance test was used to assess *in vitro* egg viability at the time of inoculation. Subsequently, the infectivity of the oncospheres was evaluated by subcutaneous inoculation in mice.

Eggs exposed to increasing temperatures were more resistant to heat if suspended in water as compared to eggs exposed on a filter paper at 70% relative humidity. Eggs were infectious after heat exposure at 65 °C for up to 120 min, however, no echinococcosis developed after treatment of the eggs at 65 °C for 180 min or at 70, 75 and 80 °C for 7.5, 15 or 30 min.

Key words: *In vivo* viability test, *Echinococcus multilocularis* eggs, heat resistance

3. Zusammenfassung

Echinococcus multilocularis verursacht die alveoläre Echinococcose, welche eine schwerwiegende, zunehmend bedeutsame zoonotische Erkrankung in vielen Teilen der nördlichen Hemisphäre darstellt. Der Mensch und andere Fehlwirte können sich durch Aufnahme von Eiern im Kot von karnivoren Endwirten infizieren.

Das Ziel dieser Studie war die Entwicklung einer sensitiven *in vivo*-Methode zur Beurteilung der Lebensfähigkeit von *E. multilocularis*-Eiern und die Etablierung geeigneter Inaktivierungsmethoden.

In einem ersten Versuch konnte festgestellt werden, dass C57Bl/6 Mäuse bei subkutaner Inokulation mit einer geringen Anzahl an Natriumhypochlorit-resistenten Onkosphären empfänglicher für das Metacestodenwachstum sind, als bei oraler Gabe von reifen Eiern.

Im zweiten Teil der Studie wurde die Behandlung der Eier bei unterschiedlichen Temperaturen (45–80°C) und Expositionszeiten (30–180 min) bei einer bestimmten relativen Luftfeuchtigkeit (70% vs. im Wasserbad) getestet. Nach Hitzebehandlung im Inkubator wurde der Natriumhypochlorit-Resistenztest zur *in vitro*-Bestimmung der Lebensfähigkeit der Eier durchgeführt. Anschliessend wurde die Infektiosität der Onkosphären durch subkutane Injektion bei Mäusen überprüft.

Hitze-behandelte Eier sind im Wasserbad hitze-resistenter als bei 70% Luftfeuchtigkeit. Eier blieben nach Behandlung bei 65°C bis zu 120 min infektiös. Eine Abtötung der Eier konnte bei 65°C nach 180 min oder bei 70, 75 und 80°C nach 7.5, 15 und 30 min erreicht werden.

Schlüsselwörter: *In vivo* Vitalitätstest, *Echinococcus multilocularis*-Eier, Hitzeresistenz

4. Manuscript

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In vivo viability of *Echinococcus multilocularis* eggs in a rodent model after different thermo-treatments

Karin Federer[a,b,1], Maria Teresa Armua-Fernandez[a], Stefan Hoby[b], Christian Wenker[b], Peter Deplazes[a,*]

[a]Institute of Parasitology, University of Zurich, Winterthurerstrasse 266a, 8057 Zurich, Switzerland

[b]Zoo Basel, Binningerstrasse 40, 4054 Basel, Switzerland

Karin Federer: karin.federer@walterzoo.ch

Maria Teresa Armua-Fernandez: mtarmua@vetparas.uzh.ch

Stefan Hoby: hoby@zoobasel.ch

Christian Wenker: wenker@zoobasel.ch

Peter Deplazes: deplazesp@access.uzh.ch

*Corresponding author: Peter Deplazes, Institute of Parasitology, University of Zurich, Winterthurerstrasse 266a, 8057 Zurich, Switzerland

Phone: +41 (0) 44 635 85 02; Fax: +41 (0) 44 635 89 07

[1] Present address: Walter Zoo AG Gossau, Neuchlen 200, 9200 Gossau, Switzerland

Abstract

Echinococcus multilocularis is the causative agent of alveolar echinococcosis, a serious and emerging zoonotic disease in many parts of the northern hemisphere. Humans but also primates and other accidental hosts can acquire the infection by the ingestion of eggs excreted by the carnivore definitive hosts, e.g. after hand contact with egg-contaminated environments or by consumption of contaminated food or beverages. The goal of this study was to develop a sensitive *in vivo* method to determine the viability of *E. multilocularis* eggs and to establish suitable conditions (optimal temperature, exposure time and humidity) for their (prophylactic) inactivation. The sensitivity of a rodent model was evaluated and, conclusively, C57Bl/6 mice were most susceptible to subcutaneous inoculation of small numbers of sodium hypochlorite-resistant oncospheres, even more than to oral inoculation of mature eggs.

In the second part of the study, various combinations of exposure temperature (between 45 °C and 80 °C), times (between 30 min and 180 min) and relative humidity (70% vs. suspended in water) were tested. After heat treatment in an incubator, the sodium hypochlorite resistance test was used to assess *in vitro* egg viability at the time of inoculation. Subsequently, the infectivity of the oncospheres was evaluated by subcutaneous inoculation in mice. Eggs exposed to increasing temperatures were more resistant to heat if suspended in water as compared to eggs exposed on a filter paper at 70% relative humidity. As survival of eggs in water droplets on the vegetables cannot be excluded, further experiments were performed with eggs suspended in water only. Eggs were infectious after heat exposure at 65 °C for up to 120 min, however, no echinococcosis developed after treatment of the eggs at 65 °C for 180 min or at 70, 75 and 80 °C for 7.5, 15 or 30 min.

Key words: *in vivo* viability test, *Echinococcus multilocularis* eggs, heat resistance

1. Introduction

Alveolar echinococcosis (AE) is considered a rare, but serious and emerging zoonotic disease in many parts of the northern hemisphere (Eckert et al., 2011). Wild canids (e.g. *Vulpes vulpes*, *Alopex lagopus*, *Canis latrans*, *Nyctereutes procyonoides*) are the main definitive hosts maintaining the *Echinococcus multilocularis* cycle. However, domestic dogs can also contribute to contaminate the environment with infectious eggs (Hegglin and Deplazes, 2013). Intermediate hosts (small mammals, mostly rodents) and accidental hosts, including humans, can acquire the infection by accidentally ingesting eggs present in the environment (e.g. soil, vegetation, vegetable food) or, in case of humans, possibly also by oral contact with egg-contaminated hands.

AE is also relevant in animal health (Deplazes and Eckert, 2001). Various animal species such as domestic pigs, wild boars, beavers, chinchillas, dogs, horses and various primate species can be affected (Deplazes and Eckert, 2001, Eckert et al., 2011). Severe cases of AE were diagnosed in seven western lowland gorillas (Gorilla gorilla) at the Basel Zoo from 1999 to 2011 (Wenker and Hoby, 2011). Five of the apes died of the disease and two of the clinical cases are currently being treated. At Basel Zoo, direct contact with the definitive host, the red fox, can be excluded, and it is assumed that the eggs of this tapeworm passed through contaminated feed to the gorillas (Wenker et al., 2008).

Previous studies have shown that taeniid eggs have a high tenacity in the environment and are resistant especially to lower temperatures (Colli and Williams, 1972, Veit et al., 1995). On the other hand, desiccation and high temperatures are the two most important factors for the inactivation of *Echinococcus* eggs. Eggs of *E. granulosus* are killed within 5 min at 60-80 °C and immediately at 100 °C (Colli and Williams, 1972). Williams (1963) showed that eggs of *Taenia pisiformis* were no longer infectious to rabbits after immersion for 5 seconds in boiling water.

In a previous study, eggs of *E. multilocularis* freshly isolated from gravid proglottids lost infectivity after exposure to a temperature of 45 °C for 3 hours at a relative humidity of 85-95% and after treatment at 43 °C for 2 hours at a relative humidity of 15% (Veit et al., 1995). *Echinococcus multilocularis* eggs have a high resistance to freezing and only temperatures of -83 °C for 48 hours or -196 °C for 20 hours resulted in egg inactivation (Veit

et al., 1995). In the Central European environment the maximum duration of survival of *E. multilocularis* eggs at temperatures between -15 °C to 27 °C was shown to be as high as 240 days (Veit et al., 1995).

The aim of this study was to develop a sensitive method to determine the viability of *E. multilocularis* eggs and to evaluate short and gentle heat treatment conditions (temperature, exposure time and humidity) to inactivate eggs in order to provide *E. multilocularis*-free food for highly susceptible people such as immunosuppressed patients (Vuitton et al., 2015), or apes in Zoos in the endemic areas.

2. Materials and methods

2.1 Ethical issues

The usage of the animals in this trial was approved by the Cantonal Veterinary Office of Zurich, Switzerland (permission 145/2012). All mice were housed and handled according to the Swiss regulations for animal experimentation.

2.2 Animals

Female, 6-8 week-old C57Bl/6 mice purchased from Charles River GmbH, Germany, were used for inoculation with *E. multilocularis* eggs.

2.3 Parasite isolates

Adult *E. multilocularis* stages were harvested by the sedimentation and counting technique (SCT) from the intestines of naturally infected red foxes (*Vulpes vulpes*) shot by hunters during the official hunting season. Adult worms containing gravid proglottids were washed with PBS and homogenised by pushing through a 100 µm mesh. This solution containing the eggs was further sieved through 41 and 21 µm with nylon meshes as previously described (Deplazes et al., 2005). The purified egg suspension was stored at 4 °C in PBS with penicillin, streptomycin and fungizone (1:100) (Life Technologies, Switzerland) for a maximum of 6-7 months.

The percentage of mature eggs in suspension was determined in triplicates on the day of the subsequent experiments for each egg-batch by assessing the sodium hypochlorite (s-h) resistance of the oncospheres as previously described Deplazes et al., (2005). The triplicate measurements were averaged and only egg batches with s-h resistances above 30% were used for the pre-trial and for the main experiment.

2.4 Parasite inoculation pre-trial

To assess the sensitivity of a rodent viability model, 10 groups of 4 mice each were used in a pre-trial. Five groups received combined intraperitoneal (i.p.) / subcutaneous (s.c.) inoculations. Meanwhile, the other 5 groups only received a peroral (p.o.) inoculation. Aliquots of the same egg-batch containing 20, 100, 500, 1000 or 2000 s-h resistant (= mature) eggs or oncospheres were prepared in 0.4 mL PBS. For the s.c. and i.p. inoculations, the embryophores were disintegrated using 2% sodium hypochlorite solution as previously described (Deplazes and Eckert, 1988a). After the embryophore disruption, the oncospheres were washed 3 times and resuspended in sterile PBS before inoculation. Twenty mice (5 groups) were inoculated both i.p. and s.c. with oncospheres and another 20 mice (5 groups) were inoculated p.o. with eggs. Intraperitoneal inoculations were made in the area of the left groin and subcutaneous injections in the left dorsum, at the level of the last thoracic vertebra. The oral inoculation was performed with a sterile gavage.

2.5 Exposure of eggs to thermo-treatments

In the first part, 40 different conditions were tested. *Echinococcus multilocularis* eggs were exposed to different temperatures (45, 50, 55, 60, and 65 °C), time intervals (30, 60, 120 and 180 min) and at a relative humidity of 70% or in a water suspension (Table 1).

Treatments at 70% relative humidity were done in a furnace (Heraeus vacutherm, Thermo Fisher Scientific Inc. Switzerland). Temperature and humidity in the interior of the furnace were monitored with a sensing probe (HC2-S, Rotronic AG, Switzerland). Eggs were applied to a 21 µm nylon mesh (Lanz-Anliker AG, Rohrbach, Switzerland) with a pipette and recovered after incubation by washing them through the mesh and re-suspended in sterile PBS.

The egg/water suspension was incubated in a water bath in an Eppendorf tube. Temperature was controlled and adjusted with a thermometer in a second tube that was filled with the same amount of water.

Based on the first set of results showing a higher survival rate in water, in the second part of the experiment, 7 additional conditions were tested only in water suspension

(summarized in Table 2). The critical temperature of 65 °C was checked again at exposure times of 60 and 120 min. Furthermore, temperatures of 70° at exposure times of 30 and 60 min, 75° at exposure times of 15 and 30 min, and 80 °C at an exposure time of 7.5 min, respectively, were tested.

2.6 In vivo viability test

In the first part of the *in vivo* viability test, a total of 160 mice divided in 40 groups were used to test the viability of eggs after exposure to the different temperature conditions. The route of inoculation and the number of injected oncospheres was based on the results of the pre-trial. Hence, the 4 mice in each group were injected subcutaneously in the region of the left dorsum. Based on egg counts and sodium hypochlorite (s-h) resistance test performed prior to heat exposure, aliquots containing approximately 500 mature eggs were prepared for each animal. Immediately after the thermo-treatment, the embryophores were dissolved with sodium hypochlorite (as described above) and resuspended in 0.4 mL PBS (the actual number of oncospheres injected was dependent on efficacy of heat treatment). Seven mice served as controls and were injected with 500 untreated oncospheres in 0.4 mL PBS.

Based on the results of the first part, a second viability experiment including 28 mice inoculated subcutaneously with oncospheres produced from eggs treated in water suspension at higher exposure temperatures (from 65-80 °C) and at shorter exposure times (7.5-120 min) was performed. To further confirm that eggs are not more heat resistant when inoculated p.o., 4 animals were per orally inoculated with eggs exposed to 65 °C for 60 min in water suspension. Three mice each were inoculated with untreated oncospheres s.c. and untreated eggs p.o., respectively, and served as controls. Animal groups and exposure conditions of the eggs of both experiments have been summarized in Fig. 1 (C and D).

2.7 Detection of E. multilocularis infections

Dissection of all the animals was carried out 8 weeks after the inoculation to detect subcutaneous infections. To confirm visible infections, DNA was extracted from the lesions

using a commercial kit (Qiamp DNA mini kit, Qiagen, Germany), according to the manufacturer's instructions and an *E. multilocularis*-specific PCR was run (Stieger et al., 2002). A subset of lesions representing different exposure groups were analysed by histopathology with PAS staining.

2.8 Statistical analysis

Data was analysed by using the statistical software IBM® SPSS® Statistics, Version 22. The outcomes of the experiments were the number of infected mice and the proportion of s-h resistant *E. multilocularis* eggs. The proportion of s-h resistant eggs was not normally distributed; thus, it was transformed by an arcsin square root transformation. A general linear model (GLM) analysis was performed evaluating the effects of humidity (water vs. 70% relative humidity), temperature (45, 50, 55, 60 and 65 °C) and the exposure time (30, 60, 120 and 180 min). Interactions between these 3 variables could not be considered because of the small sample size.

3. Results

3.1 Parasite inoculation pre-trial

Inoculations with 500, 1000 and 2000 mature eggs/oncospheres (p.o./s.c., i.p.) resulted in *E. multilocularis* infections in all animals. Inoculations with 100 mature eggs or oncospheres resulted in infections in all s.c. (4/4) and i.p. (4/4) and in half of the p.o. (2/4) inoculations. When only 20 mature eggs or oncospheres were administered, *E. multilocularis* metocestode growth was detected only in the s.c. inoculations (4/4).

3.2 Thermo-treatment experiment

Table 1 shows the proportion of mice developing metacestodes after inoculation with viable oncospheres treated at different temperature regimes in a water suspension or on a filter at 70% relative humidity.

The GLM analyses revealed significant effects of all three factors on the s-h resistance of the *E. multilocularis* eggs. Treatments in water, under higher temperatures and during longer periods were significantly more effective than treatments in 70% r.h. (Wald chi-square=45.1, df 1, $p<0.001$), under lower temperatures (Wald chi-square=70.9, df 1, $p<0.001$) and during shorter periods (Wald chi-square=4.5, df 1, $p<0.05$), respectively. Similarly to the s-h resistance investigations, treatments with higher temperatures (Wald chi-square=43.7, df 1, $p<0.001$) and longer durations (Wald chi-square=10.8, df 1, $p<0.0.01$) significantly reduced the probability that mice got infected. When eggs were exposed in water to the thermo-treatment only the highest temperature of 65 °C and exposures of 120 min or longer completely prevented cyst development. In 70% r.h. eggs were inactivated already with incubation at 65 °C for 30 min and at 55 °C for 180 min. However, the differences between the treatments in water and in 70% r.h. were not significant for the portion of infected mice (Wald chi-square=2.2, df 1, $p=0.14$). *E. multilocularis* infections were confirmed by PCR in all the samples and 5 samples also underwent histopathologic examination, which confirmed the presence of *E. multilocularis* cysts in all cases.

Table 1: *Proportions of mice with macroscopically detected cysts 8 weeks after subcutaneous inoculation of Echinococcus multilocularis thermo-treated oncospheres. Treatment conditions varied according to temperature, exposure time, and humidity. Temp, temperature; Water, suspended in water; 70% r.h., exposed to 70% relative humidity.*

	30 min		**60 min**		**120 min**		**180 min**	
Temp	**Water**	**70% r.h.**	**Water**	**70% r.h.**	**Water**	**70% r.h.**	**Water**	**70% r.h.**
45 °C	3/3	4/4	4/4	3/4	4/4	4/4	4/4	3/4
50 °C	3/4	4/4	4/4	4/4	1/4	4/4	3/4	3/4
55 °C	3/4	4/4	2/4	3/4	1/4	0/4	0/4	0/4
60 °C	0/4	1/4	3/4	0/4	3/4	0/4	2/4	0/4
65 °C	2/4	0/4	1/4	0/4	0/4	0/4	0/4	0/4

Based on the results of this first experiment, a second exposure experiment was conducted with higher temperatures. The proportions of mice with macroscopically detected cysts 8 weeks after subcutaneous injection of E. multilocularis thermo-treated oncospheres are summarized in Table 2.

As compared with the first trial, 65 °C and 60 min exposure did not result in infections; however, exposure at the same temperature for 120 min resulted in 2 of 4 infections after subcutaneous inoculation (confirmed by histology or PCR). Peroral inoculation with 500 thermo-treated eggs exposed at 65 °C for 120 min was negative in all 4 mice.

Table 2: *Proportions of mice with macroscopically detected cysts 8 weeks after subcutaneous injection of E. multilocularis thermo-treated oncospheres. Treatment conditions varied according to temperature and exposure time. All eggs were suspended in water during thermo-treatment.*

Temp	**7.5 min**	**15 min**	**30 min**	**60 min**	**120 min**
65 °C	not done	not done	not done	0/4	2/4
70 °C	not done	not done	0/4	0/4	not done
75 °C	not done	0/4	0/4	not done	not done
80 °C	0/4	not done	not done	not done	not done

The s-h resistance has been shown to be a useful parameter to document egg maturity, but its use as a parameter for viability has not been investigated for *E. multilocularis*. The s-h resistance of the temperature exposed eggs is summarized in Table 3. Compared to the *in vivo* viability results, the s-h resistance of the temperature exposed eggs in water was lower than the eggs treated in 70% humidity.

Table 3: *Percentage of sodium hypochlorite-resistant oncospheres detected after thermo-treatment of eggs. Treatment conditions varied according to temperature, exposure time, and humidity. Temp, temperature; Water, suspended in water; 70% r.h., exposed to 70% relative humidity.*

	30 min		**60 min**		**120 min**		**180 min**	
Temp	**Water**	**70% r.h.**	**Water**	**70% r.h.**	**Water**	**70% r.h.**	**Water**	**70% r.h.**
45 °C	11.5	13	12	8.2	13.5	15.5	10	10.3
50 °C	9.5	12.6	10.5	13.5	9	10.7	6	13.3
55 °C	1.4	16	0.4	8.3	0	9	0	11.8
60 °C	0	8.2	0	6.4	0	6.4	0	0.7
65 °C	0	8.3	0	7.3	0	3.2	0	0.7

4. Discussion

There is an urgent need for a reliable method to assess *E. multilocularis* egg viability *in vitro*. Effective precautions, including decontamination of potentially contaminated food, must be established to counteract the emergent increase of echinococcosis in highly susceptible immunocompromised human patients (Vuitton et al., 2015). Furthermore, the optimisation of safety strategies to protect highly susceptible animal species such as captive primates in endemic areas (Wenker et al., 2008) is crucial.

The animal experiments performed in this study followed the requirements of the Veterinary Office and the 3R principles, thus the three consecutive experiments contributed to minimised animal numbers. In the first experiment we optimised the inoculation method demonstrating, that embryophore free, but not enzymatically activated *E. multilocularis* oncospheres, developed subcutaneously or intraperitonealy. Indeed, the ability of *Echinococcus* spp. oncosphere's to hatch without intestinal passage has been demonstrated by intra-tracheal *E. granulosus* egg inoculation in sheep with further hydatid cyst development in the lungs (Thompson, 1995). Moreover, sodium hypochlorite treatment of the eggs (previous s.c. or i.p. inoculations) is an elegant method to kill bacterial and fungal contaminants and using this procedure, no complications were seen in the animals involved. Strong subcutaneous development of *E. multilocularis* metacestodes was observed years ago by s.c. injection of metacestode material in *Meriones unguiculatus* (Eckert and Burkhardt, 1980). Recently, s.c. inoculation was applied for an infection model facilitating the treatment assessment of secondary alveolar echinococcosis in mice (Küster et al., 2013). The main advantage is that the infections can be monitored easily by eye without any invasive handling of the animals. In our experiments, subcutaneous growth of the parasite could be seen as first as 29 days after inoculation.

The results of this study document that a temperature exposure in water for 120 min at 65 °C did not completely inactivate the *E. multilocularis* eggs. For complete inactivation, at least 180 min at 65 °C was required. Veit et al., (1995) reported complete inactivation of *E. multilocularis* eggs at much lower temperatures. This pioneer study on *E. multilocularis* egg resistance, however, was based on low standardized egg isolation (using eggs of 10 proglottids per mouse without information about the number of mature eggs). Therefore, the higher survival rates of eggs at higher temperatures observed in our study might be the

result of a much higher sensitivity of the *in vivo* viability test enabling the detection of few viable oncospheres.

Interestingly, the fact that the humidity is of great importance has previously been described (Matsumoto and Yagi, 2008, Veit et al., 1995). As with heating, dehydrogenation also leads to denaturing of the colloidal structure of the inner embryonic membrane (Laws, 1968).

Apart from the clear results in the treatment of the eggs at 70% relative humidity, there are also three questionable results with the eggs treated in water. After temperature exposure at 55 °C for 180 min and at 60 °C for 30 min parasite development was not documented despite the fact that conditions at 60 °C for 60 min and 60 °C for 120 or 180 min were not lethal for the oncospheres resulting in infections in mice. One cause could be a technical problem. As we know from other studies, parasite eggs can adhere to the cannula's wall; therefore different numbers of oncospheres could have been injected. Another cause could be attributed to the limited number of infected mice per group. Following to the 3R principle, the number or animals was reduced to the minimum. Another difficulty derived from animal welfare reasons was that the experiment time was limited to 8 weeks. If for unknown factors, the metacestode development was slower or delayed in the thermo-treated eggs, the short incubation period could critically affect the detection of the cysts by eye and we cannot exclude that small cysts could have thus been overlooked. Küster et al., (2013) observed that even after a longer time span, no mobility impairment was observed in the subcutaneously inoculated mice in contrast to i.p. inoculation. Based on these and our observations, we propose that a longer incubation period (12-14 weeks) in further experiments to investigate the viability of the subcutaneously inoculated oncospheres would be more adequate without concerns of animal welfare.

An *in vitro* viability test for the validation of optimal criteria to kill *E. multilocularis* eggs would be of high value. Therefore, we tested whether the s-h resistance test could replace the *in vivo* testing in mice. This test is useful to quantitatively determine the amount of mature eggs after mass isolation from gravid worms. It is striking that the s-h resistance for the eggs treated in water is lower than the eggs treated with 70% relative humidity. Deplazes and Eckert, (1988b) documented that the s-h resistance did not correlate with the *in vitro* activation rate of *Taenia hydatigena* oncospheres after long-term preservation. In

the presented study, the s-h resistance test did not reflect the *in vivo* results. In some cases, no resistant oncospheres were observed (previous inoculation) while *in vivo* the parasite development was evidenced. On the other hand, in many samples some resistant oncospheres were observed after the exposures that did not result in parasite development. Therefore, according to our findings, the s-h resistance test cannot be used as a reliable alternative to *in vivo* viability testing.

5. Conclusion

We developed a highly sensitive *in vivo* viability test for *E. multilocularis* eggs. This study revealed that a treatment of *E. multilocularis* eggs in water suspension at 65 °C for 120 min did not effectively kill the eggs. This finding demonstrates that temperature exposure below 70 °C in a liquid phase is not suitable for the decontamination of raw vegetables as salads, for example, would no longer keep the nutritional properties after this high temperature treatment.

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