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Peroral *Echinococcus multilocularis* egg inoculation in *Myodes glareolus*, *Mesocricetus auratus* and *Mus musculus* (CD-1 IGS and C57BL/6j)

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ABSTRACT

Echinococcus multilocularis transmission predominantly occurs in Europe between the red fox (*Vulpes vulpes*) and various species of rodent intermediate hosts. We infected 3 species of rodent, *Myodes glareolus* ($n = 47$), *Mesocricetus auratus* ($n = 11$) and outbred *Mus musculus* (CD-1 IGS) ($n = 9$) with an *E. multilocularis* egg suspension that contained 100 eggs with viable oncospheres and performed *post mortem* examination 6, 8 (*M. glareolus*) and 10 weeks post inoculation (wpi). C57BL/6j mice ($n = 4$) were used as positive controls as they have been shown to exhibit macroscopic liver lesions 4 wpi. To the best of our knowledge, this is the first study to experimentally assess susceptibility in the ostensibly competent host *M. glareolus*. Lesions were only detected in 2 of 47 *M. glareolus* (4.3%) at 8 and 10 wpi and although both contained protoscolices (1675 at 8 wpi and 88 at 12 wpi) the low percentage of infected animals brings into question their role as transmitters of the parasite. Significant differences were observed between inbred and outbred mice with *E. multilocularis* infection in the former demonstrating increased establishment ($p \leq 0.0001$) and growth ($p \leq 0.0001$). No lesions were found in all 11 *M. auratus*.

1. Introduction

Studies of the fox tapeworm, *Echinococcus multilocularis* have focused on naturally infected human and animal populations for disease mapping and risk assessment whilst experimental work is often conducted in mouse models intended for medical benefit (Dematteis et al., 2003; Eckert and Deplazes, 2004; Vuitton and Gottstein, 2010). Although experimental studies have identified profound differences in the susceptibility of the definitive carnivore hosts (Kapel et al., 2006) very little information exists on experimental infections of these tapeworms in their naturally occurring intermediate hosts, although such studies would clarify which host species play a key role in the transmission, why they are physiologically suited for parasite establishment and growth, and which minimum infectious doses would be required in natural settings across various relevant species. In addition to its ecological value, such data would constitute novel information for risk assessment and prevention.

In Europe, Arvicolidae species of rodents serve as intermediate hosts although the parasite is capable of more or less normal development in small mammal species from several families. Thus, the range of intermediate hosts that may be susceptible seems to be wider as compared to that of the definitive hosts. Even rodents not sympatric with *Echinococcus multilocularis* may establish metacestodes with protoscolices when experimentally inoculated (Thompson and Lymbery, 1995). That said, the growth and persistency of metacestodes varies between species and genus (Ohbayashi et al., 1971) and thus the geographical distribution of intermediate hosts species ought to affect transmission dynamics.

Experimental *E. multilocularis* infection in rodents can be achieved via various routes. Oral inoculation of *E. multilocularis* eggs is referred to as primary infection, whereas secondary inoculation involves the injection of metacestode homogenates or oncospheres intraperitoneally (IP), intrahepatically (IH), subcutaneously (SC) or intravenously (IV). Although secondary inoculation bypasses the early gastrointestinal exposure responsible for oncosphere activation and development, and thus provides a more narrow view of

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E. multilocularis infection dynamics, the majority of experimental studies apply this mode of administration (Matsumoto and Yagi, 2008). SC however does constitute a very sensitive method for testing oncosphere viability (Federer et al., 2015). Primary inoculation is thus more similar to the natural route of exposure, as the inoculated oncospheres have to pass through the gastrointestinal passage prior to liver establishment. However, due to the extensive safety measures required and difficulties in obtaining eggs few laboratories utilise this method (Romig and Bilger, 1999).

Early studies that utilised primary inoculation of rodents (e.g. Yamashita et al., 1956; Yamashita et al., 1958, 1963; Ohbayashi, 1960; Ohbayashi et al., 1971) provided information on metacestode development, but unfortunately inoculation dose varied between infections even in the same experiment. Thus, it is difficult to interpret varying establishment among these rodent species from these experiments.

A meta-analysis of literature on *E. multilocularis* prevalence in both definitive and intermediate hosts (Takeuchi-Storm et al., 2015) demonstrated that genera was a significant factor for parasite prevalence with *Ondatra* having the highest estimate followed by *Arvicola*, then *Microtus* with *Apodemus* and *Myodes* having the lowest. Significant differences were observed in all accept *Microtus* and *Arvicola* and *Apodemus* and *Myodes*. Considering the consensus of various observations (Hanosset et al., 2008; Raoul et al., 2015) it is not surprising that *Ondatra*, *Microtus* and *Arvicola* had the highest odds ratios (OR). However, in a global perspective, it is worth noting that the genus *Myodes* which is widely distributed on all continents where the parasite occurs (Eckert, 1998) was found to be of similar low importance for parasite transmission as *Apodemus*, which are not considered suitable intermediate hosts (Tsukada et al., 2000) however the Japanese field mouse (*Apodemus argenteus*) has been found infected in Japan (Tsukada et al., 2002).

Considering the low OR for *Myodes* and the key role played in *E. multilocularis* transmission by *Myodes rufocanus* in Japan (Saitoh and Takahashi, 1998) it was deemed appropriate to experimentally assess the susceptibility of *Myodes glareolus* in an effort to elucidate their potential role as transmitters of the parasite in Europe. Although it was not possible to obtain *Apodemus* spp., outbred *Mus musculus* (CD-1 IGS) was inoculated as murid representative. This species has been found naturally infected (Leiby and Kritsky, 1972; Pétavy et al., 1990). The Syrian hamster (*Mesocricetus auratus*) was also included. This species is capable of harbouring the adult worm in its intestine after immunosuppression (Kamiya et al., 1991; Nonaka et al., 1996) however, post infection with approximately 40 eggs the Alaskan strain of the parasite, did not result in establishment (Yamashita et al., 1958). As this represents the only study found and due to the relatively low inoculum of eggs used it was deemed appropriate to attempt experimental infections with a European strain of the parasite. C57BL6/j mice were used as positive controls for egg viability as they have been shown to demonstrate macroscopic lesions after 4 wpi (Matsumoto et al., 2010).

2. Materials and methods

2.1. Experimental inoculation

The *E. multilocularis* eggs used for inoculation were isolated from worms in naturally infected foxes from the city of Zurich and the surrounding area, during the official Swiss hunting season. Eggs were tested for viability by the sodium hypochlorite (s-h) resistant test (Deplazes et al., 2005). In brief, the percentage viability of the eggs was determined to be the number of eggs with intact oncospheres after the s-h solution had been applied.

Animals were anesthetized with isoflurane and the egg suspension containing approximately 100 viable *E. multilocularis* eggs was administered via gavage. This was calculated as follows: the total number of eggs per ml divided by the percentage viability (via s-h resistant test) to determine the percentage of viable eggs per ml. The number of viable eggs per ml was then used to calculate the volume of egg suspension that would contain 100 viable eggs. Animals were inoculated on different days with the s-h test conducted prior to each inoculation round and the volume of egg suspension adjusted accordingly. During the period of inoculation eggs were stored at 4 °C to maximise viability (Veit et al., 1995) in 1% penicillin-streptomycin solution.

Animals were housed in a safety facility (Biosafety Level 2++ approved by the Danish Working Environment Authority, Journal no. 20120014119/21) at the Department of Plant and Environmental Sciences (University of Copenhagen, Denmark), under experimental license no. 2012-15-2934-00150. All animals were imported under permission from the Danish AgriFish Agency (CVR: 29979812, No. 1013624417).

Four species/strains of rodent were experimentally inoculated with 100 viable *E. multilocularis* eggs:

- *Myodes glareolus*

Female ($n = 23$) and male ($n = 24$) *M. glareolus* were obtained from Institute of Environmental Sciences, Jagiellonian University Kraków, Poland. All animals were 56 days old at inoculation (DAI). Animals were euthanized at 6 wpi (Female $n = 4$, Male $n = 4$), 8 wpi (Female $n = 15$, Male $n = 16$) and 10 wpi (Female $n = 4$, Male $n = 4$). The animals euthanized at 8 wpi were a control group of a separate study investigating *E. multilocularis* infection in relation to basal metabolic rate (BMI). The conditions that these rodents were exposed to (housing, nutrition, and *E. multilocularis* infection) were precisely the same as the 6 and 10 wpi animals and were thus included. All animals were inoculated between 20/04/2015 and 29/04/2015.

- *Mus musculus* (CD-1® IGS)

Male ($n = 3$) and female ($n = 6$) CD-1 animals were obtained from Charles River Germany. Animals were 56 DAI. All animals were euthanized 6 wpi. The original study design was for 12 animals to be inoculated but a shortage of eggs meant that it was not possible for 3 male animals to be inoculated. Animals were inoculated 1 month after the *M. glareolus* and C57BL/6j mice on 26/05/2015. As such it was intended to also inoculate an additional two C57BL/6j mice. The shortage of eggs precluded this but in the interest of the 3 R's (Russell et al., 1959) it was decided to proceed with the inoculations.

- *Mesocricetus auratus*

Female ($n = 5$) and male ($n = 6$) *Mesocricetus auratus* were obtained from Charles River France.

Animals were 56 DAI. These animals were euthanized 6 wpi (Female $n = 3$, Male $n = 3$) and 10 wpi (Female $n = 2$, Male $n = 3$). Animals were inoculated 07/04/2014. These animals were inoculated in the same period as the Woolsey et al., 2015b study that demonstrated heavy *E. multilocularis* infection in *Microtus arvalis* (which were inoculated with the same egg suspension spanning dates before and after 07/04/2014).

- *Mus musculus* (C57BL/6j)

Female ($n = 4$) *M. musculus* were obtained from Charles River Germany in March 2015. Mice were 42 DAI. All animals were inoculated 20/04/2015.