Succinctus

Early-phase migration dynamics of *Echinococcus multilocularis* in two mouse strains showing different infection susceptibilities

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Abstract

The early-phase migration dynamics of *Echinococcus multilocularis* in the intermediate hosts remain largely unknown. We compared the parasite burden in the intestine, liver and faeces of DBA/2 and C57BL/6 mouse strains using parasite-specific quantitative PCR. Our results indicated that the parasites invaded mainly from the middle segments of the small intestine and completed migration to the liver within 24 hours post infection. DBA/2 mice had higher parasite DNA burden in the intestine and liver but lower in the faeces than C57BL/6 mice, suggesting that parasite invasion into the intestine may be a critical stage regulating *E. multilocularis* infection in mice.

Keywords: *Echinococcus multilocularis*; migration dynamics; oncospheres; primary alveolar echinococcosis; qPCR

1. Introduction

Alveolar echinococcosis is a harmful zoonosis caused by the larval stage of *Echinococcus multilocularis*, a species of tapeworm in the family Taeniidae. The intermediate hosts, primarily rodents and occasionally humans, get infected following oral uptake of the parasite's eggs, which are excreted in the faeces of the definitive hosts. It has been suggested that the *E. multilocularis* eggs hatch in the small intestine of the intermediate hosts, releasing oncospheres that penetrate the intestinal wall following the activation and finally reach the liver via the portal vein (Pater et al., 1998; Huang et al., 2016). These migration dynamics have been observed in the closely related parasites of the Taeniidae family, *Taenia taeniaformis* (Banerjee and Singh, 1969) and *E. granulosus* (Heath, 1971); however, due to the high risk of handling infective *E. multilocularis* eggs, there is limited information on the migration dynamics, such as invasion sites and the time to reach the liver in the intermediate hosts.

Experimental infection of *E. multilocularis* using wild rodents and inbred mice has shown that the susceptibility to parasite infection varies significantly among animal species and mouse strains (Nakaya et al., 1997; Matsumoto et al., 2010; Woolsey et al., 2016). Among these differences, two inbred mouse strains, DBA/2 and C57BL/6, have shown significant differences in their susceptibility to *E. multilocularis* infection. When infective parasite eggs were administered orally to both the strains, DBA/2 mice had a significantly higher number of cysts in the liver than

C57BL/6 mice (Matsumoto et al., 2010). This genetic difference was confirmed by quantitative trait locus (QTL) mapping analysis, which successfully identified the locus (*Emcys1* in chromosome 6) statistically linked to the number of cysts in the liver (Nakao et al., 2011). However, two congenic lines, B6.D2-*Emcys1* and D2.B6-*Emcys1*, where *Emcys1* was introgressed from DBA/2 into C57BL/6 background and *vice versa*, showed no significant differences in the cyst number, suggesting that *Emcys1* does not solely control the cyst establishment in mouse liver but multiple genetic factors are involved in this phenotype (Islam et al., 2018). Therefore, it remains unclear which factors are involved in determining the number of cysts during the early phase of infection. Thus, the comparisons of parasite migration dynamics in these two mouse strains may lead to understanding of the factors responsible for their phenotypic difference.

Quantitative PCR (qPCR) is an effective tool to detect parasite DNA with high sensitivity and specificity, which can be applied for the analysis of parasite migration dynamics in the intermediate host. Several attempts using qPCR have been made to analyse migration dynamics of protozoan parasites such as *Toxoplasma gondii* and *Trypanosoma cruzi*, which are small in size and not suitable for microscopic analyses when infection density is low (Daryani et al., 2014; Dias et al., 2015). Specific primers and probe for qPCR have been developed to quantify the mitochondrial large ribosomal subunit gene (*rrnL*) of *E. multilocularis* (Knapp et al., 2014).

Previous studies successfully detected and quantified parasite DNA from both faeces and environmental samples using this method (Knapp et al., 2014; Lass et al., 2019).

Here, we investigated the migration dynamics of *E. multilocularis* using the parasite-specific qPCR on DNA extracted from the intestine, liver, faeces of mice. Furthermore, the migration dynamics were compared between the two mouse strains showing different susceptibilities against echinococcosis.

2. Materials and methods

Two inbred mouse strains (DBA/2 and C57BL/6) were purchased from Japan SLC (Shizuoka, Japan). All the mice were male and infected with *E. multilocularis* Nemuro strain at 9 weeks old. *Echinococcus multilocularis* eggs were obtained from the faeces of beagle dogs purchased from Kitayama Labes (Nagano, Japan) that were orally inoculated with approximately 500,000 protoscoleces. The faecal materials were collected from the infected dogs on days 33–35 after infection. Parasite eggs were isolated by filtering the faeces with a sieve of 125 µm mesh size. To minimize contamination of free parasite DNA, the filtrate was mixed with a five-fold volume of water and then the eggs in the precipitate were collected. A total of twenty mice of each strain were orally administered with 2,640 parasite eggs under anaesthesia. Five mice of each strain were necropsied at 2, 4, and 24 h p.i. for tissue extraction. At necropsy, the whole intestine was divided into five equal-length segments of the small intestine, the cecum, and two equal-length segments

of the colon according to previously described methods (Bøgh et al., 1990; Woting and Blaut, 2018). The liver was divided into two parts: liver R, consisting of medial right, lateral right, and caudate lobes, and liver L, consisting of medial and lateral left lobes. To prevent contamination by gut contents or blood, the surface of tissue segments was washed with PBS three times. The intestinal segments, except for the cecum, were cut longitudinally, placed on a drinking straw cut in half, and the content was thoroughly washed away with PBS (Supplementary Fig. S1 A, B). For the cecum, a plate made of aluminium foil was used instead of a straw. The scissors, tweezers, and straws were replaced each time to prevent cross contamination. In order to collect faecal samples, five mice per strain were kept independently in cages with drawers at the bottom for faecal collection. All the faeces were collected at 24 h p.i. and used for subsequent DNA extraction and microscopic observation. All procedures were performed in accordance with the Guidelines for Animal Experimentation of the Japanese Association for Laboratory Animal Science, and the protocol for the animal experiments was approved by the ethics committee of the Hokkaido Institute of Public Health (study ID K30-4). The animals were handled within a safety facility (Biosafety Level 2 Enhanced) at the Hokkaido Institute of Public Health.

DNA extraction from tissue samples was performed with the DNeasy Blood and Tissue kit (Qiagen, Hilden, Germany) following the method described previously (Rufener et al., 2018) with some modifications. Briefly, the liver lobes and intestinal segments were homogenised with beads

and lysed with 30 µl proteinase K in 720 and 400 µl buffer ATL, respectively. After overnight incubation, 10 µl of RNase A (100 mg/ml: Nippon Gene, Tokyo, Japan) was added to the liver lysate according to the manufacture's recommendation. The lysate was incubated for 2 min at room temperature, followed by centrifugation at 20,000× *g* for 5 min. The amount of the tissue lysate used for DNA extraction was 80 µl and 40 µl for liver lobes and intestinal segments, which correspond to 4.4-8.4% and 5.4%-14% of the total lysate, respectively. This experimental condition was determined by our preliminary experiment where DNA yield was tested using varying volumes of tissue lysate prepared from mouse tissues mixed with inactivated parasite eggs.

Faecal samples were suspended and mixed well with 5 ml of PBS. Then, 500 µl of faecal suspension was used for DNA extraction. DNA extraction was performed as previously described (Irie et al., 2017). Briefly, 500 µl of faecal suspension was mixed in 450 µl of 1M KOH, 25 µl of 2 M dithiothreitol, and 25 µl of 20% SDS. Then, the mixture was heated at 65 °C for 30 min. After neutralisation with 187.5 µl of 2 M Tris-HCl, pH 8.3, and 62.5 µl of 25% HCl, the mixture was centrifuged at 10,000×*g* for 1 min. Phenol/chloroform/isoamyl alcohol (25:24:1) extraction was performed on 700 µl of supernatant. After centrifugation at 10,000×*g* for 5 min, the supernatant layer was collected. Then, a Gel/PCR Purification kit (Takara Bio Inc., Shiga, Japan) was used to purify DNA from 135 µl of specimen. The resulting DNA was eluted in 30 µl elution buffer.

The large ribosomal subunit gene (*rrnL*) of the *E. multilocularis* Nemuro strain was amplified from DNA of protoscoleces purified from intraperitoneally infected mice using previously reported primers Em-rrn forward 5'-CTGTGATCTTGGTGTAGTAGTTGAGATTT-3' and Em-reverse 5'-GGCTTACGCCGGTCTTAACTC-3' (Knapp et al., 2014). The PCR fragment was ligated into the pMD20-T-vector (Takara Bio Inc.) using a 10×A-attachment mix (Toyobo) and Ligation Mighty mix (Takara Bio Inc.). The ligated product was transformed into DH5α cells (Takara Bio Inc.), and the cells were cultured on LB plate medium. The transformed single colony was grown in culture overnight. The plasmid DNA were purified from overnight culture using NucleoSpin® Plasmid EasyPure (Takara Bio Inc.). The purified plasmid DNA was digested with *PciI* (New England BioLabs Inc., Ipswich, MA, USA). The digestion of plasmid DNA at a single site was confirmed by agarose gel electrophoresis and the resulting linearized plasmid DNA was purified from the gel using a Gel/PCR Purification kit. The concentration of purified plasmid DNA was measured using a Qubit 3.0 fluorometer (Thermo Fisher Scientific Inc., Waltham, MA, USA). The number of plasmid DNA copies was calculated using the following equation:

$$X \text{ copies}/\mu l = (Y \text{ ng}/\mu l \text{ DNA}/[\text{Plasmid length nucleotide} \times 660]) \times 6.023 \times 10^{14}$$

The plasmid DNA was stored at -30 °C until qPCR reaction.

qPCR was performed using the pair of primers described above and the Em probe 5'-TGGTCTGTTCGACCTTTTTAGCCTCCAT -3' (Knapp et al., 2014) labelled fluorescently with a 6-carboxy fluorescein reporter and a black hole quencher 1. The reaction mixture consisted of 10 µl THUNDERBIRD® Probe qPCR Mix (Toyobo), 6.00 pmol of rrnL primers, 0.40 pmol of Em probe, 0.04 µl of 50× ROX reference dye, 6.36 µl water, and 2.00 µl of extracted DNA or plasmid DNA. For absolute quantification, plasmid DNA was diluted by 10-fold serial dilution and included in each qPCR run with concentrations ranging from 1.0×10^{0} to 1.0×10^{7} copies per reaction. The qPCR reaction was run on a 7500 Fast Real-Time PCR system (Applied Biosystems, Foster City, CA, USA). The program consisted of a 20 s initial denaturation at 95 °C followed by 45 cycles of 3 s denaturation at 95 °C and 30 s extension at 60 °C. The quantitative results were expressed by the DNA copy numbers and corrected using the following equation:

DNA copies in whole tissue/faeces (copies) = DNA copy number obtained by qPCR (copies/µl) ×(Total sample weight (mg)/sample weight used for DNA extraction (mg)) × Total volume of extracted DNA (µl)

Differences in qPCR results between the two mouse strains were evaluated with the Mann-Whitney *U* test using GraphPad Prism software 3.0 (GraphPad Software, San Diego, CA, USA). In all statistical analyses, a significant difference between groups was defined by $p < 0.05$.

Faecal samples suspended in PBS described above were used to search for unhatched parasite eggs. Five hundred microliters of the faecal suspension (approximately 9.2%-12% of total faecal suspension) was mixed with 10 ml PBS and dispensed into 6-well culture plates. The plate was then examined under a stereo microscope.

3. Results

The copy numbers of the *E. multilocularis rrnL* gene in the intestinal wall and the liver were examined by parasite-specific qPCR at 2, 4, and 24 h after oral administration with 2,640 eggs. Regardless of mouse strain, at 2 and 4 h p.i., the parasite DNA was detected in the intestine and liver, while there was little DNA detected in the intestine at 24 h p.i. (Fig. 1). The copy number of parasite DNA detected in the intestine at 24 h was less than 10% of that detected at 2 h. More than 75% of the parasite DNA detected in the whole intestine was obtained from the second to fourth segments of the small intestine in both strains at 2 and 4 h p.i. (Fig. 1). In the liver, the copy number of parasite DNA increased with time in DBA/2 and the median reached its maximum at 24 h p.i.

To compare the total amount of parasite DNA in tissues and faeces of DBA/2 and C57BL/6 mice, the total parasite DNA copy numbers were calculated by summing the numbers detected in all segments of the small intestine and each liver lobe (Fig. 2A, B). The parasite DNA copy number in the small intestine was significantly higher in DBA/2 than in C57BL/6 mice at 4 h p.i. ($p < 0.05$),

while no significant differences were found at 2 and 24 h p.i. (Fig. 2A). The parasite DNA in the liver was significantly higher in DBA/2 than in C57BL/6 mice at 4 and 24 h p.i. but not at 2 h p.i. (Fig. 2B, $p < 0.01$). In contrast, the parasite DNA in the faeces collected during 24 h after oral administration was significantly higher in C57BL/6 mice than in DBA/2 mice (Fig. 2C, $p < 0.01$)

Microscopic examination of the faeces demonstrated that there were no non-hatched eggs in the faeces collected within 24 h after oral egg administration in either strain (data not shown).

4. Discussion

Experimental infection using *E. multilocularis* eggs serves as a model for natural infection in intermediate hosts; however, the risk of infection to humans and the necessity of safety equipment are the bottlenecks for conducting experiments using the infective parasite eggs (Pater et al., 1998; Matsumoto et al., 2010). Alternatively, experimental infection by intraperitoneal, intravenous or intrahepatic inoculation of tissue homogenates containing the larval parasite (termed secondary AE) is frequently used because of its easiness and safety. As a result, knowledge of the early phase of primary AE is limited; for instance, the migration dynamics of *E. multilocularis* after the administration of eggs are not clear. This basic information is essential for the analysis of host-parasite interactions in the early phase of the infection, which should be critical to prevent infection. In this study, we aimed to reveal the migration dynamics of *E. multilocularis* using parasite-specific

qPCR and to elucidate the stages that regulate host susceptibility/resistance during the early phase of infection.

In both DBA/2 and C57BL/6 mouse strains, many DNA copies were detected in the second to fourth segments of the small intestine at 2 and 4 h p.i. (Fig. 1). These results suggested that these segments were the main invasion sites for the oncospheres. This is generally in line with previous reports investigating other closely related cestodes, *T. taeniaformis*, and *E. granulosus*, which seldom invaded the upper segment of the small intestine but more often the middle segments (Heath, 1971; Bøgh et al., 1990). It has also been reported that the eggs of *Taenia* spp. and *E. granulosus* do not hatch only by incubating with artificial gastric fluid, but egg hatching followed by oncosphere activation can occur when incubated with artificial intestinal fluid (Heath, 1971). Taken together, it is strongly suggested that *E. multilocularis* eggs were hatched and activated by intestinal fluid in the upper part of the small intestine, and then, the oncospheres invaded the downstream part i.e., the second to fourth segments of the small intestine.

The migration dynamics of *E. multilocularis* oncospheres to the liver have rarely been described, except for a few studies dealing with the histological examination of the metacestode at the early stage of infection (Ohbayashi, 1960; Ishige et al., 2006). These studies showed spherical or ovoid parasites in the liver of mice 24 h p.i. However, it was unclear when these parasites arrive at the liver and when the migration from the intestine to the liver is completed. In the present study,

the parasite DNA was detected in the liver at 2 h p.i. (Fig. 1). Furthermore, there were few DNA copies in the small intestine at 24 h p.i. in both mouse strains. These data indicated that some parasites reached the liver within at least within 2 h p.i. and that migration to the liver was mostly completed by 24 h p.i.

C57BL/6 mice had significantly lower parasite DNA than DBA/2 mice in the small intestine at 4 h and in the liver at 4 and 24 h p.i. Conversely, the parasite DNA detected in faeces collected during 24 h after inoculation was significantly higher in C57BL/6 mice. These observations suggested that some factors that prevent the penetration of oncospheres exist in the small intestine or that elimination of the parasite occurs in the early phase of infection in resistant C57BL/6 mice. One possible factor is intestinal immunity. Administration of immunosuppressive drug (Armua-Fenandez et al., 2016; Joekel and Deplazes, 2017) and depletion of granulocytes (Joekel et al., 2020) resulted in the parasite establishment in the liver of Wistar rats, which are completely resistant to *E. multilocularis* infection, suggesting that innate immunity has critical roles for infection defence. In the study by Joekel et al. (2020), the authors concluded that the interaction of oncospheres with granulocytes may take place only in blood stream since any pathological changes or neutrophil influx into mucosa were not observed in rat intestine at 16-17 h p.i. However, considering the findings that the parasite invasion into intestine occurs as early as at 4 h p.i. and its migration into liver completes within 24 h p.i., further immunohistological

investigation at earlier time points is necessary. Indeed, in the closely related parasite *E. granulosus*, microarray analysis at 4 h p.i. has shown that intestinal innate immunity is strongly activated upon invasion of the oncospheres into the intestinal wall, suggesting that the innate immune response, including NK cells, mast cells, and macrophages, may play an important role in the very early stages of infection (Hui et al., 2015). Therefore, it is also possible for *E. multilocularis*, innate immunity in the intestine is induced as a response to oncosphere invasion and the subsequent elimination of the parasite might occur in resistant C57BL/6 mice. Further studies focusing on the intestinal responses in the early phase of infection are required to test this hypothesis.

We found no unhatched parasite eggs in the faeces of either DBA/2 or C57BL/6 mice, suggesting that all inoculated eggs hatched in the intestine. Several previous *in vitro* studies have successfully activated oncospheres using hog or dog bile (Deplazes and Gottstein, 1991; Huang et al., 2016), which are aberrant intermediate hosts of the parasite (Deplazes and Eckert, 2001; Deplazes et al., 2005). From our data and those of previous studies, egg hatching and oncosphere activation in the intestine are not likely possible stages that determine host susceptibility/resistance in mice.

In this study, we performed *E. multilocularis*-specific qPCR on DNA extracted from mouse faeces and tissues within 24 h p.i. to analyse parasite migration dynamics. To the best of our knowledge, this is the first study to use the qPCR method for migration dynamics analysis of taeniid

cestodes. To date, histopathological examination and direct microscopic observation of oncospheres have been commonly used to study the migration dynamics in intermediate hosts (Heath, 1971; Turner and McKeever, 1976; Bøgh et al., 1990; Ishige et al., 2006). However, these methods require a very large number of eggs, and only a small number of parasites can be analysed at one time, which is unsuitable for quantitative analysis of parasites in whole organs. The qPCR primers and probe used in this study have been shown to have excellent sensitivity and specificity (Knapp et al., 2014; Maksimov et al., 2017; Lass et al., 2019). Indeed, our qPCR assay was able to detect parasite DNA when tested with DNA samples extracted from the small intestine segment 1 or liver R experimentally mixed with 10 parasite eggs (data not shown). In addition, qPCR has the advantage of that even a small number of parasites in a wide search range can be measured quantitatively (Dias et al., 2015). However, it should be noted that the gene copy number obtained by qPCR can be affected by several factors such as DNA extraction methods. In addition, it has been suggested that Taeniid family cestodes change their morphology and cell number during the early-phase migration (Heath, 1971; Ishige et al., 2006), which may affect the copy number of mitochondrial gene in a single oncosphere. These limitations make it difficult to make a direct comparison of copy numbers obtained from different tissues and/or extraction methods (e.g., tissues and faeces in this study).

In conclusion, this study provided fundamental data on the migration dynamics of *E. multilocularis* in the early phase of infection in intermediate hosts. Furthermore, the comparison of these dynamics between the two mouse strains suggested that the factors that regulate host susceptibility against cyst establishment in mice may be at a stage of invasion. Our findings and methods can contribute to further understanding on host-parasite interaction in the early phase of infection, leading to the development of novel strategies for the prevention and control against echinococcosis.

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Conflicts of interest

The authors declare no conflict of interest.

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Figure Legends

Fig. 1. Amount of parasite DNA in different mouse tissues

The quantity of parasite DNA detected in five small intestinal segments (1–5), caecum (Cc), two colonic segments, and the left (L) and right (R) lobes of the liver of DBA/2 (black squares/dots) and C57BL/6 (white squares/dots) mice at 2, 4, and 24 hours post inoculation (h p.i.). are shown. Each dot represents the data from one mouse. The copy number of the parasite DNA is indicated on the left side of the panel. The horizontal lines indicate the median values.

Fig. 2. Comparison of the parasite DNA amount in tissue and faecal samples

The numbers of parasite genes obtained from (A) whole small intestine, (B) whole liver, and (C) whole faeces samples from DBA/2 (black squares) and C57BL/6 (white squares) mice at 2, 4, and 24 h p.i. are shown. * and ** indicate $p < 0.05$ and $p < 0.01$ determined by non-parametric Mann-Whitney *U* test, respectively. The copy number of the parasite DNA is indicated on the left side of each panel. The horizontal lines indicate the median values.

Supplementary Fig. S1. Graphical image and photograph of the washing method used in this study.

Intestinal segments (A, orange one) were cut longitudinally open and placed on a drinking straw cut into half-pipe shape (A, blue one), and the content was thoroughly washed away with a wash bottle of PBS (B).

Fig.1

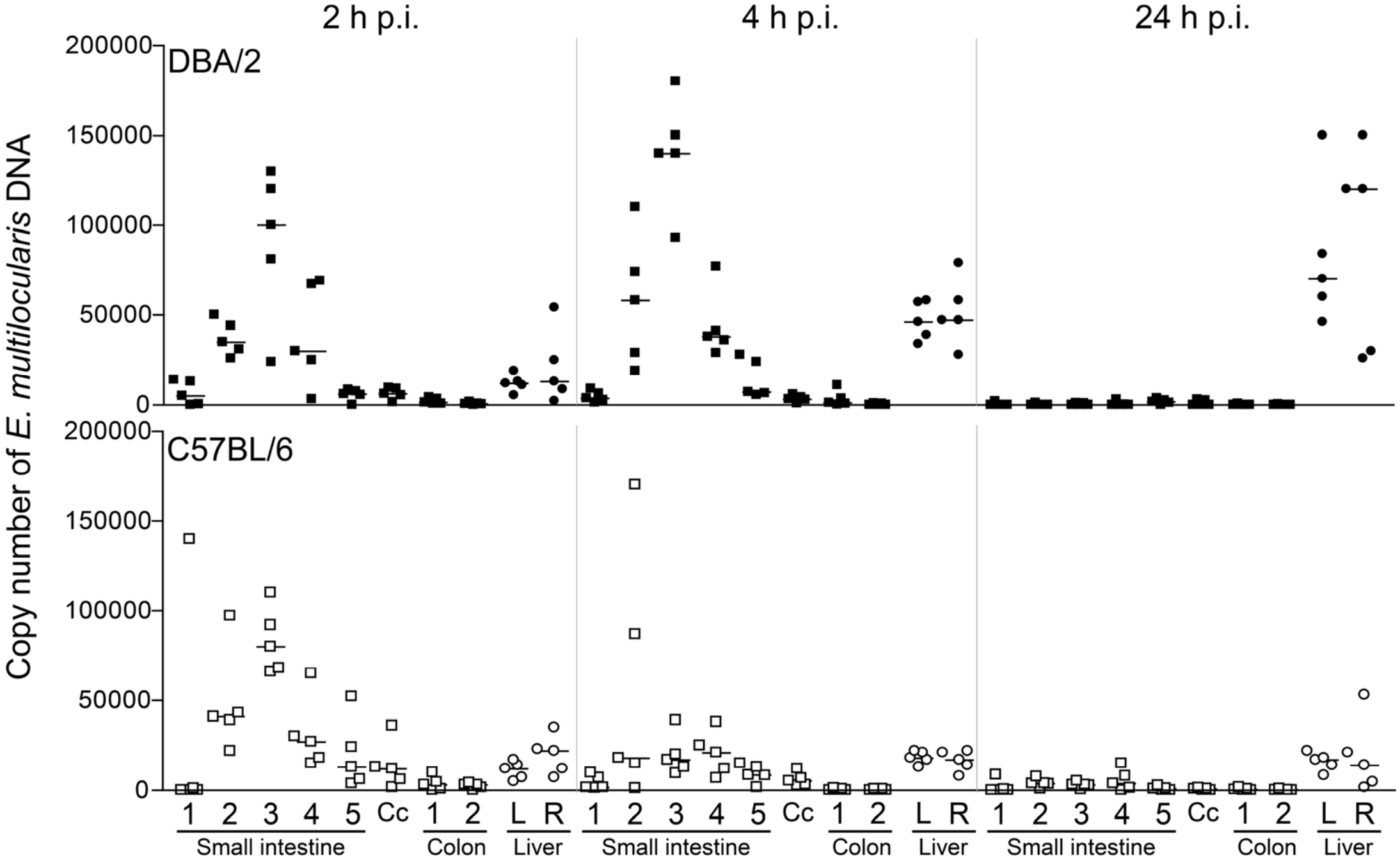
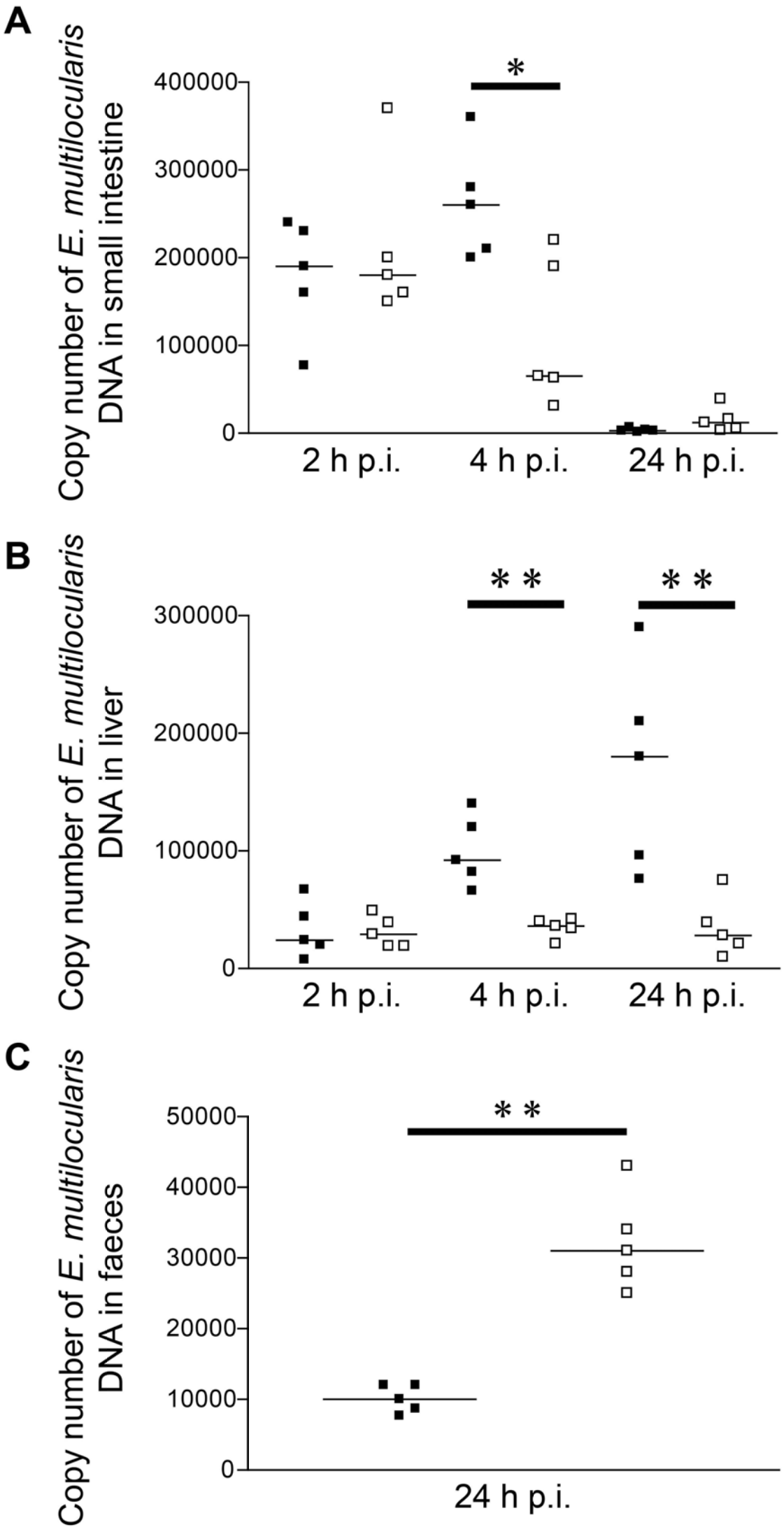
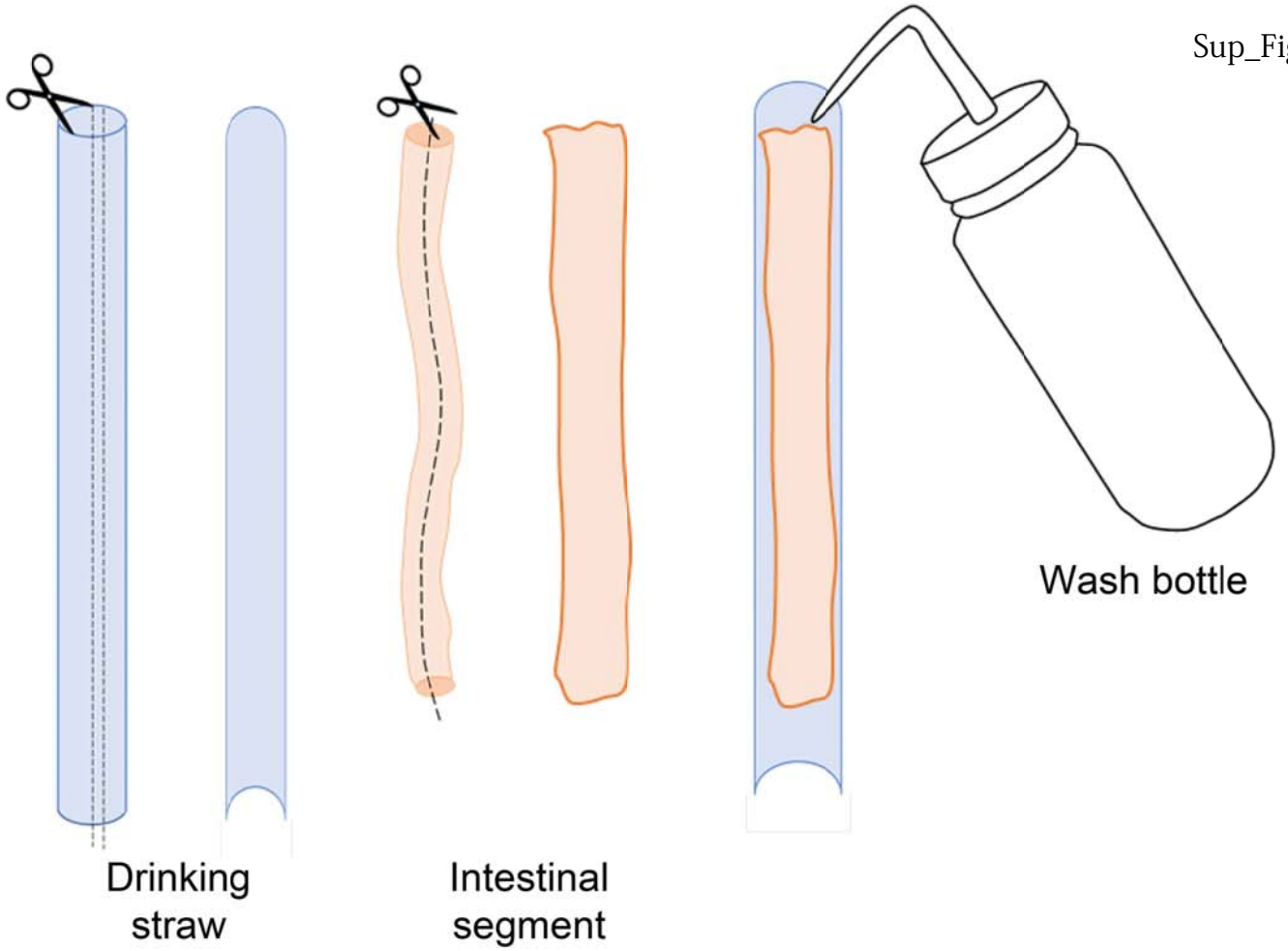

2 h p.i.
4 h p.i.
24 h p.i.
DBA/2
C57BL/6
Copy number of E. multilocularis DNA
200000
150000
100000
50000
0
1 2 3 4 5 Cc 1 2 L R
Small intestine
Colon
Liver

Fig.2


A
Copy number of E. multilocularis DNA in small intestine
400000
300000
200000
100000
0
*
2 h p.i.
4 h p.i.
24 h p.i.
B
Copy number of E. multilocularis DNA in liver
300000
200000
100000
0
* *
* *
2 h p.i.
4 h p.i.
24 h p.i.
C
Copy number of E. multilocularis DNA in faeces
50000
40000
30000
20000
10000
0
* *
24 h p.i.

A



B