Experimental Parasitology 189 (2018) 61–65

Contents lists available at ScienceDirect

Experimental Parasitology

journal homepage: www.elsevier.com/locate/yexpr

Utilization of real time PCR for the assessment of egg burden in the organs of *Schistosoma japonicum* experimentally infected mice

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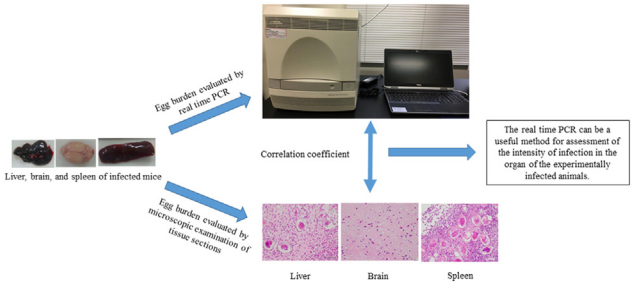
HIGHLIGHTS

- Assessment of egg burden in mouse's organs using qPCR and tissue microscopy.
- Strong correlation between egg burden assessed by microscopy and qPCR in the liver.
- qPCR is more sensitive in assessing the egg burden in the brain and spleen.
- qPCR is useful in evaluating *S. japonicum* egg burden in various organs.

GRAPHICAL ABSTRACT



ARTICLE INFO

Article history:
Received 8 September 2017
Received in revised form
10 March 2018
Accepted 13 April 2018
Available online 18 April 2018

Keywords:
qPCR
Real time PCR
Schistosomiasis
S. japonicum

ABSTRACT

Schistosoma japonicum, causing zoonotic intestinal schistosomiasis, is found in China, the Philippines and parts of Indonesia. Severe disease manifestations are basically due to the deposition of eggs in some vital organs such as the liver, spleen and brain. Traditionally, histopathological microscopic examination of the egg burden was used to evaluate the intensity of infection in the affected organs. However, this technique is laborious, time-consuming and requires trained personnel. In this study, real time PCR targeting the mitochondrial NADH dehydrogenase I gene was used to compare with microscopic examination of tissue sections in evaluating the egg burdens in different affected organs. Livers, spleens and brains of the *S. japonicum* infected mice after 8 and 18 weeks post-infection (p.i) were harvested and examined. Results showed that there were statistically significant correlations between the egg burden evaluated by tissue section examination, and the Ct values of the real time PCR of livers with heavy egg burden at 8 ($r = -0.81$) and 18 ($r = -0.80$) weeks p.i. Furthermore, a correlation ($r = -0.56$) between the egg burden assessed by the microscopic examination and Ct value of the real time PCR of spleens with moderate egg burden after 18 weeks p.i and not 8 weeks p.i was also observed. Brains with low egg burden showed no schistosome eggs in the microscopic examination, however one sample tested positive by real time PCR. These results suggested that real time PCR is useful in evaluating schistosome egg burden in the organs of

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https://doi.org/10.1016/j.exppara.2018.04.014

the experimentally infected mice model that will give further insights into the pathology of schistosomiasis.

1. Introduction

Schistosoma japonicum infection is a parasitic disease still of public health importance in China, the Philippines and parts of Indonesia (Balen et al., 2007; Leonardo et al., 2002). Disease manifestations vary from being asymptomatic to severe organ involvement. It is well known that schistosome eggs, and not the adult worms induce the morbidity among infected individuals (Burke et al., 2009). The number of eggs in the tissue plays an important role in determining organ damage. The schistosome eggs can be deposited in the liver and intestine of mammalian hosts where they promote formation of inflammatory granulomas, the main pathology of schistosomiasis. Aside from being deposited in the organs associated with the hepatic portal vein where they can cause serious ectopic lesions (Andrade Filho Jde et al., 1998; Vargas et al., 2013), the schistosome eggs can sometimes be seen affecting the nervous system (Carvalho, 2013; Harter et al., 2014; Vale et al., 2012).

Understanding the disease pathology has been crucial in the management of infected individuals and in the control of schistosomiasis. Experimental infection of the parasite in mice has been widely used in investigating disease pathology (Wang et al., 2015) and the use of animal models provides a better understanding on the effects of the schistosome parasites inside the hosts (Hurst et al., 2000; Zhang et al., 2009). One of the pathological assessments being performed in experimental animals is by determining the presence of the eggs and their pathological effects in the affected organs through histopathological examination. However, this traditional technique has some drawbacks such as laborious protocols, and lengthy time required for the microscopic examination of the samples.

It has been reported that molecular methods have shown high sensitivity and specificity for the detection of DNA of various parasites including *Schistosoma* from clinical samples such as stool (Mejia et al., 2013; Suzuki et al., 2006). Its high sensitivity allows the diagnosis of schistosomiasis when no eggs can be detected by microscopy during the acute phase of the infection and in cases of infection with light intensity (Espirito-Santo et al., 2014). Real-time PCR method has been evaluated and compared with traditional microscopic examination for the detection and quantification of *S. mansoni* and *S. haematobium* eggs in an endemic setting showing better results (ten Hove et al., 2008). Moreover, real-time PCR has successfully been applied to assess the egg burden in the stool of *S. japonicum* infected individuals (Lier et al., 2006; Lier et al., 2009).

This study aims to develop a more reliable and easier test that can be applied for assessing the parasite egg burden in the organs of experimentally infected animal model. The performance of real time PCR was evaluated using several vital organ samples collected from the infected mice and compared with microscopic examination of tissue sections at different time points of infection.

2. Materials and methods

2.1. Ethical statement

Animal care and all animal experimentation were carried out according to the guidelines for the Care and Use of Laboratory Animals established by Obihiro University of Agriculture and Veterinary Medicine. This study was approved by the Committee for Animal Experimentation of Obihiro University of Agriculture and Veterinary Medicine (Approval no. 28–32).

2.2. Animals and parasites

Thirty-one male ICR mice (4 weeks-old) were used for experimental schistosome infection. *Oncomelania hupensis nosophora* snails harboring *S. japonicum* cercariae were obtained from the Department of Tropical Medicine and Parasitology, Dokkyo Medical University, Japan. Mice were infected percutaneously with 30–60 cercariae. Infected mice sacrificed include 12 mice at 8 weeks post-infection (p.i.) and 19 mice at 18 weeks p.i. Serving as negative controls, non-infected mice were also sacrificed at each time point (2 and 3 respectively). Tissue samples were taken from the spleen, brain and left lateral lobe of the liver and then divided into 2 parts. One part was preserved in 10% neutral buffered formalin for tissue sections and the other part was stored at −30 °C for real time PCR.

2.3. Microscopic examination of tissue sections

After fixing in 10% neutral buffered formalin, the organ tissues were embedded in paraffin and stained with hematoxylin and eosin (H&E) prior to microscopic examination. Three sections were examined for each sample. To estimate the schistosome egg burden in the sample, 30 fields from the three sections were examined. Egg number in each field was counted using 40X magnification. Average egg count for 30 fields was calculated and the number was used as the value representing the egg burden in each sample.

2.4. Real time PCR

Primers and TaqMan probe for the real time PCR were designed based on the mitochondrial NADH dehydrogenase I sequence of *S. japonicum* (GenBank accession no. AF215860) (Lier et al., 2009). These were 5′-ACTGGTTATGGTTTGTTGATGTTAGGT-3’ (forward primer), 5′-AGCCACACGAACAGCACTAATC-3’ (reverse primer) and 6-FAM-5′- AGGTTCTTGGAAAAAGTAT-3′-MGBNFQ (TaqMan probe), respectively. The expected length of the amplification product was 75 bp. Template DNA was extracted from the liver, spleen and brain samples using the Qiagen tissue kit (Qiagen, Germany). Twenty milligrams of the tissue samples were used for DNA extraction and the DNA was eluted in 50 μl of distilled water. Twenty microliters of reaction mixture contained 1 μl of template DNA, 10 μl of TaqMan Universal PCR Master mix (Applied Biosystems, CA., USA), 300 nM of each primer, 250 mM of probe (Applied Biosystems), and distilled water to give a final reaction volume of 20 μl. The conditions for real time PCR were as follows: 50 °C for 2 min, 95 °C for 10 min, and 50 cycles at 95 °C for 15 s and 60 °C for 1 min (Lier et al., 2006). Real-time PCR was performed in MicroAmp optical 96-well reaction plates using 7300 Real-Time PCR Systems v. 2.0 (Applied Biosystems). Standard curve was prepared using serially diluted target gene cloned plasmid DNA samples. Copy number of the target gene in each sample was determined based on the standard curve and threshold cycle (Ct) value (Table S1).