Quantification of C-type lectin gene expression during hyperinfection in strongyloidiasis

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Summary

Strongyloides stercoralis is the intestinal nematode in humans, and it infects millions of people worldwide but thrives in warm countries with poor sanitation conditions. Clinical manifestations of the infection may range from asymptomatic to chronic. *Strongyloides* cause hyperinfection syndrome and dissemination in individuals with impaired cell-mediated immunity due to its ability to proliferate within the host that may increase the mortality rate up to 87%. The diagnosis of hyperinfection syndrome is difficult to establish and entails a high level of suspicion. The objective of the present study was to measure the expression level of C-type lectin gene coding to protein biomarker candidates from the excretory/secretory (ES) products of the infective filariform larva that can be used as diagnostic indicators for early hyperinfection syndrome in strongyloidiasis. An experimental study was carried out to induce hyperinfection of L3 larvae of *S. ratti* in experimentally immunosuppressed Wistar rats using prednisolone, a corticosteroid immunosuppressive drug. Prednisolone treatment resulted in a significant increase in the parasitic intensities. Relative semi-quantitative real-time PCR was performed to compare the expression level of the C-type lectin's gene between treated and non-treated groups with this drug. C-type lectin gene showed significantly higher expression levels in the treated samples. The study concluded that C-type lectin expression level was successfully measured and could be used as a diagnostic biomarker during early hyperinfection syndrome in strongyloidiasis.

Key words: C-type lectin, qPCR, hyperinfection, strongyloidiasis

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Introduction

Strongyloides stercoralis is an intestinal parasite infect around 30–100 million humans worldwide through penetrating the exposed skin by filariform larvae (Schar *et al.,* 2013). Once fully developed inside the host, they cause an infection in the gastrointestinal tract that may persist for decades without any symptoms (Krolewiecki *et al.,* 2013). Asymptomatic chronic strongyloidiasis in the immunocompromised individuals may lead to hyperinfection or disseminated syndrome mainly in solid organ transplant recipients and human immunodeficiency virus (HIV), with an estimated mortality rate ranging from 50% to 89% (Mones *et al.,* 2010).

Infection of *Strongyloides stercoralis* is more prevalent among adults due to its unique ability to replicate within the human host; leading to infections that may last for decades if left untreated (Grove, 1996). Abdominal pain, diarrhea, nausea, vomiting, and urticaria are the main symptoms of the chronic

infection with S. stercoralis (Khieu *et al*., 2013 and Forrer *et al.,* 2017). Nevertheless, in immunosuppressed individuals, the autoinfection cycles accelerate and lead to hyperinfection syndrome, a 100% fatal disease in the absence of an effective treatment (Nutman, 2016 and Forrer *et al.,* 2019) .
Corticosteroid drugs are one of the most frequent risk factors for hyperinfection syndrome in strongyloidiasis and may contribute to an adverse outcome in developed countries (Vasquez-Rios *et al.,* 2019). Corticosteroids affect T-helper type-2 response and eosinophil migration to the site of infection. Furthermore, there is evidence suggesting that in nematodes, corticosteroids function as egg molting enhancer, which boosts parasite production and promotes dissemination to other organs (Barros *et al.*, 2014).

The most effective available therapy for strongyloidiasis is ivermectin. Nevertheless, there is no specific recommendation regarding how to monitor therapeutic response (Salvador *et al*., 2014).The C-type lectin (CTL) is an ancient and versatile evolutionary unit among metazoan organisms, a superfamily of both soluble and membrane-bound proteins, that emerged as one of the largest and most widely expressed characteristic carbohydrate recognition domain (CRD) proteins with a 115–130 conserved amino acid(Little *etal*., 2003 and Roth *et al*., 2009).

Regulation of host immune response isthe proposed role of CTLs in some parasitic nematodes such as *Onchocerca volvulus*, *Trichostrongylus colubriformis*, *Teladorsagia circumcincta* and *Haemonchus contortus*(Kim *et al*., 2010 and Zhang, *et al*., 2018).Other parasite lectins seem to play physiological roles, such as lectins of *Ancylostoma ceylanicum*(Brown *et al*., 2007).These functional Glc-NAc-binding lectins highly expressed in male gonads and sperm, suggesting a function in worm reproduction. The origin of the secreted lectins is to be determined, and it is important to differentiate between products released in the digestive tract of nematode and those directed for secretion into the host environment. (Harcus *et al*., 2009).

Nematode CTLseems to be the most abundant family in all nematodes. (Bauters *et al*., 2017). During nematode migration in host tissues, important functions are introducedof CTL as tissue recognition involving in evading from the immune system. Some inconsistent existence in the grouping of sequences may become resolve by identifying more CTL sequences within different nematodes order (Etebar *et al*., 2018).

A real-time qPCR is a great tool and the most widely applied technique for the analysis and quantification of mRNA. Relative expression is widely used where the expression of a target gene is normalized by an unregulated reference gene (Pfaffl *et al.*, 2002).The aim of the current study was to apply the RT-qPCR techniqueto quantifyand analysis of the expression of C-type lectin geneduring strongyloidiasis hyperinfection.

Materials and Methods

Helminth free Wistar rats (3-4 weeks old and weighing 100g) were commercially purchased. The experiments were approved by the Institutional Animal Care and Use Committee (IACUC) of the Universiti Putra Malaysia (UPM), Malaysia. *Strongyloides ratti* was isolated from the wild brown rat, *Rattus norvegicus*; preliminary identified by conventional parasitological techniques and confirmed using molecular methods. The parasite was then established and maintained by serial passage in Wistar rats in the animal house, Faculty of Medicine and Health Sciences, Universiti Putra Malaysia (UPM). All infected animals were housed under standard laboratory conditions.

Infection of Wistar rat with *S. ratti*
Fecal pellets were collected and cultured for harvesting a new generation of infective larvae. To establish the infection, ~1,500 freshly harvested infective filariform larvae were washed several times in phosphate buffer saline (PBS), concentrated in 1ml of PBS and inoculated by subcutaneous injection at the dorsum of the neck (Shimizu, 2012).

Experimental design and Drug administration
The Wistar rats were divided into two groups, 10 rats each. Infected animals in the first group were treated with oral inoculation of 3mg/kg body weight prednisolone solution (Mediphar Laboratories,

Lebanon) daily until the end of the experiment. In the second group (control group), animals were infected with *S. ratti* without receiving prednisolone. The rats were monitored daily for signs of abnormalities such as discomfort and illness, body weights were checked regularly. Filariform larvae from both groups were isolated at the same time in order to quantify C-type lectin gene expressionusing a semi-quantitative PCR method.

RNA Extraction

Fifty thousand freshly harvested filariform larvae from both groups, treated and non-treated with Prednisolone were thoroughly washed in DEPC-treated water (HiMedia, India) several times, and then the larvae were pelleted in a bottom of 15ml conical tube by centrifugation at 5000x g. The tissue of larvae was ruptured with the aid of rapid freezing in liquid nitrogen and subsequent mechanical forces application for grinding and homogenization using glass mortar and pestle (Tazir, 2009).RNA extraction was performed by using the RNeasy Mini Kit (Qiagen, Hilden, Germany) according to the manufacturer's protocol. Purified RNA was aliquoted and stored at -80°C until further use.

Measurement of RNA concentration, purity, and integrity

Totally isolated RNA was quantified and the purity was evaluated using NanoDrop® ND-1000 UV-VIS spectrophotometer (ThermoFisher Scientific, USA). The integrity of the extracted RNAs was assessed using agarose gel electrophoresis and further confirmed by measuring the 28S:18S ribosomal ratio on an RNA picoChip using the Agilent 2100 Bioanalyzer (Agilent Technologies, USA).

Complementary DNA (cDNA) Synthesis

Single stranded cDNA was synthesized using RevertAid first strand cDNA synthesis kit (Fermentas Life Science, USA) according to the manufacturer's instructions.

Endogenous Control for Relative Quantitation

The selection of Sr-GAPDH as an endogenous gene was based on the result of a previous study thatshowed a stable and constant expression level of this gene in all parasitic and free-living life cycle stages of *S. ratti* (Younis, 2011).

Primer Design and its Specificity for RT-qPCR

The sequence of target DNA of C-type lectin was selected and the primer set was designed from the database of the National Center for Biotechnology Information (NCBI). CTL and GAPDH specific primer sets were ordered from Integrated DNA Technologies (IDT, Singapore) (Table 1).

Table 1: Primer sets of C-type lectin and GAPDH reference gene used for qPCR**.**

Primer	Sequence 5-3	Size	Reference
C-type lecin	ACCCCTGTTCCATTACCAACAA TTTGGGATAGCCTGGGGAGG	117	Present study
Sr-GAPDH	GTACCACTAACTGTTTAGCTCC GCACCTCTTCCATCTCTCC	154	Younis, 2011

Semi-quantitative real time PCR

Quantitative PCR run was performed on Mastercycler ep Realplex (Eppendorf, Germany) using SensiFAST™ SYBR® No-ROX Kit (Bioline Reagents Ltd, USA) according to the manufacturer's instructions in triplicate under test. The baseline and threshold for each of the three runs were set identically and the Ct values recalculated in order to compare between the run data to be made.

Analysis of semi-quantitative data

The raw Ct value for a target gene and the Sr-GAPDH (endogenous control) gene for non-treated (control) and treated samples with corticosteroids were exported from the Mastercycler analysis program into relative expression software tool REST© (Qiagen Group, Microsoft® Corporation, Germany) for gene expression profile analysis. The regulation means to factor in the expression of a particular gene reflected the cDNA level between two different samples, non-treated and treated (Pfaffl *et al,* 2002).

Statistical analysis

A standalone tool, REST 2009 software was used for gene expression analysis of data from the qPCR experiment. The analysis or quantitation of relative gene expression used reference gene expression to normalize the expression level of the gene of interest in treated and control samples (Pfaffl *et al*, 2002).

Results

Primer efficiency for RT-qPCR

A primer designed to target the C-type lectin gene in the current study were used for RT-qPCR and real time software of Mastercycler ep Real plex (Eppendorf, Germany) and gave approximately -3.159 and 0.96 for curve slope and correlation coefficient respectively. Fig.(1) shows in detail the standard curve analysis, which contains reaction efficiency and related slope, correlation coefficient, and Y-intercept for C-type lectin and GAPDH genes.

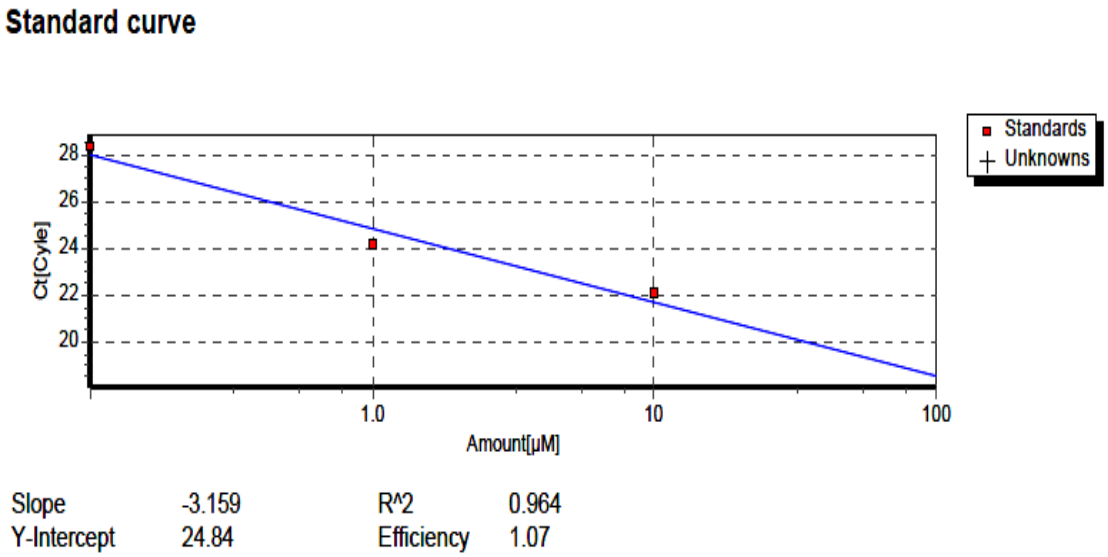

Fig. 1: Standard curve of the C-type lectin primer efficiency test.

Semi-quantitative PCR for C-type lectin

CTL gene from the treated sample with corticosteroid and non-treated (control) wasanalyzed for relative fold changes in gene expression by qPCR (Fig. 2). Evaluation of quantitative PCR expression profiles demonstrated that the CTL genewas up-regulated in the treated sample in comparison to the non-treated one (control) by a mean factor6.256. (Table 2, Fig. 3).

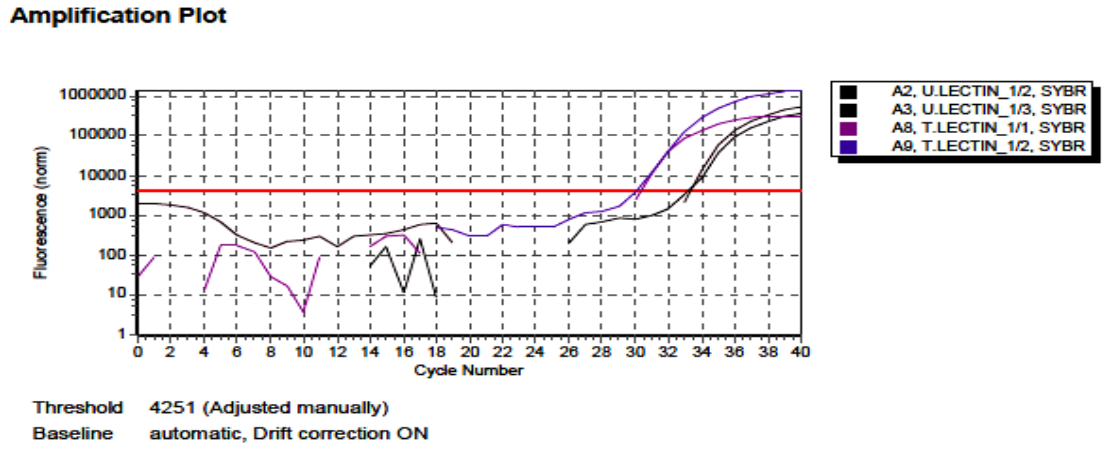

Fig. 2: Amplification Plot for C-type lectin Semi-quantitative PCR.

Table 2: Relative C-type lectin gene expression values of treated and non-treated samples.

Gene	Type	Reaction Efficiency	Expression	95% C.I.	P(H1)	Result
GAPDH	REF	0.91	1.000			
CTL	TRG	1.07	6.256	4.604 - 8.502	0.000	UP

P (H1): Probability of alternate hypothesis that difference between sample and control groups is due only to chance.TRG: Target REF: Reference

Boxplot

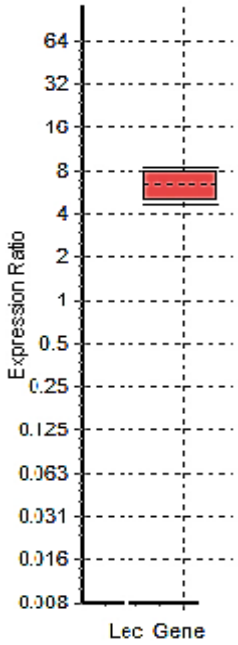

Fig. 3: Relative gene expressions fold of *CTL*. The dotted lines represent the median gene expression. The whiskers represent the upper and lower observations. The graph was generated using REST software.

Discussion

Quantitative real-time PCR is a reliable, sensitive and accurate method most commonly used for the detection of specific mRNAs in various samples using less RNA template and less labor-intensive. This technique is regarded as a useful tool to quantify mRNA expression in biotechnology, molecular medicine, microbiology, and diagnostics. However, the cost is the main disadvantage of this method, whereas the necessary equipment and chemistries are expensive (Bustin, 2000; Wong and Medreano, 2005). RT-qPCR combines reverse transcription with qPCR and is currently the method of choice for amplification and detection of low levels of mRNA gene expression might be performed as a one-step or two-step approach in any biological matrixes. The expression levels in many different samples for a limited number of genes can be measured using this application (Bustin *et al.*, 2009).

In this study, RT-qPCR was used as an assay for the relative quantification of the expression of CTLgene from control and treated with corticosteroid. For data analysis, REST-2009 software was used. This software tool uses statistical whisker-box plots in which the area of the box includes 50% of all observations, spotted lines represent the median of the samples and the whiskers represent the outer

50% of the observations (Pfaffl, 2006). The purpose of using this software tool in the present study was to detect the change in target gene expression standardized by a non-regulated reference geneand to check and assess any significant difference if present in gene expression profile between *S. ratti* filariform larvae samples exposed to corticosteroid and samples without treatment as controls. Little is known about the relative quantification of the expression of this gene at the transcriptomic level and all available information is not related to hyperinfection syndrome.

CTL has been identified from parasitic worms. The function of this protein involves in various biological functions, including impede host immune system by interfere host-parasite interaction (Loukas and Maizels, 2000), antibacterial function (Mallo and Kurz, 2002), binding to host ligands (Loukas and Mullin, 1999), and interfere withthe mucin that secreted in response toinfections with gastrointestinal nematodes (Soga *et al*., 2008).The proteomic analysis revealed that there are fewer proteins with CTL domain in parasitic nematodes modified according to their life cycles, due to their role in parasite-host interface (Yoshida *et al*., 2012; Ganji *et al*., 2014).

Surface and/or secreted CTLs have been characterized in helminths and hypothesized to play a role in immunomodulation. However, their suggested interaction with immune cells of the host remains speculation (Loukas and Maizels, 2000).CTLs of infective larvae released during theparasitic cycle haveled to hypothesize that they play an important role in an offense capacity, possibly due to lectin–glycan interactions and interfering with normal of the host immune system (Figdor *et al*., 2002).

The expression level of the tested CTL gene of the control sample and sample after induction with corticosteroid in *S. ratti* was analyzed by qPCR. The expression level of this gene in non-treated parasites was extremely low in comparison with that of treated ones. Finding significant regulation mean factorof 6.256 in the tested gene when comparing these two samples becomes far more important in the light of the importance of this gene as a potential biomarker in strongyloidiasis hyperinfection.

In conclusion, the current study represents an attempt to employ a transcriptomic analysis to identify the relative expression of gene-encoded CTL identified from ES products of *S. ratti* hyperinfective filariform larvae in response to corticosteroid treatment.The results indicated that the qPCR method has successfully monitored the transcriptional development of this gene. Moreover, the current results showed that corticosteroid treatment can stimulate the expression of CTL, as it overexpressed in the samples treated with this drugover the control samples. Further analysis is essential to detect the effects of prednisolone treatment on CTL gene expression in infective filariform larvae and to investigate the effects of some other immunosuppressive drugs and their doses on the immune system in *Strongyloides*-infected individuals.

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