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ORIGINAL ARTICLE

IL-4 gene expression in adventitial layer (fibrous layer) of hepatic ovine and bovine hydatid cysts

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Abstract Cystic Echinococcosis is a parasitic disease with cosmopolitan distribution caused by the tape worm *Echinococcus granulosus.* Fibrous layer is developed around the cyst as a host immune response reaction. The aim of this study was to evaluate the rate of IL-4 gene expression in fibrous layer of bovine and ovine hepatic hydatid cysts using quantitative technique of Real-Time PCR. In this descriptive study the samples of hydatid cyst fibrous layer were taken from 6 bovine and 6 ovine hepatic hydatid cysts. Samples of normal liver tissue close to the cyst were also taken as controls. Total RNA from each sample was extracted and then converted to cDNA. Afterward, the rate of IL-4 gene expression for each sample was evaluated using real-time PCR technique. Data were analyzed by REST software (version 2.0.13, 2009). In sheep the rate of IL-4 gene expression in the fibrous layer of hepatic hydatid cysts was 1.98 times more than the rate of IL4 gene expression in control samples, but the difference was not significant ($P = 0.561$). In cattle the rate of IL-4 gene expression in the fibrous layer of hepatic hydatid cysts was 9.84 times more than that of control samples which was statistically significant ($P < 0.001$). With high rate of IL4 expression especially in fibrous layer of bovine hydatid cyst, it can be concluded that this interleukin may play an important role in host parasite relationship.

Keywords Hydatid cyst · IL-4 · Gene expression · Real-Time PCR · Fibrous layer

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Introduction

Cystic Echinococcosis (CE) or hydatidosis is a parasitic disease with cosmopolitan distribution, which is caused by the tape worm *Echinococcus granulosus.* This zoonotic infection is a major public health and economic problem in many regions of the world (Fauser and Kern 1997; McManus et al. 2003; Rigano et al. 1999).

Hydatid cyst, which is the larval stage of *E. granulosus*, develops in intermediate hosts viscera, consist of two parasite-derived layers, an inner nucleated germinal layer and an outer acellular laminated layer surrounded by a host-produced fibrous capsule as the consequence of the host immune response (Zhang et al. 2003; Zhang and McManus 2006; Zhang et al. 2011). In fertile hydatid cyst brood capsules and protoscoleces bud off from the germinal membrane (Zhang et al. 2011).

Both humoral and T cell mediated responses seem to play an important role in immunology of hydatid cyst. These responses are considered to be regulated by cytokines (Arai et al. 1990). As to the production of distinct cytokine patterns, immune responses to this parasite can be divided into a Th1-type reaction and a Th2-type reaction (Fauser and Kern 1997; Mosmann et al. 1986). It has been shown that, like other helminthic infections (Allen and Maizels 1996; Finkelman et al. 1991; Lange et al. 1994; Mosmann and Sad 1996; Pearce et al. 1991), hydatid cyst induces two very distinct Th1 and Th2 cytokine secretion

patterns (Zhang et al. 2003). Existence of complex mixture of antigens in hydatid cyst fluid (McManus and Bryant 1995), which probably contain distinct epitopes for each T-cell subset (Zhang et al. 2003) that may explain why Th1 and Th2 interleukins secret simultaneously in hydatid cyst infected hosts. So early Th1 cytokines, which can kill the parasite (Vuitton 2003) shifts to Th2 cytokine response in the chronic stage (Zhang et al. 2012).

IL-4 can affect a variety of target cells in different ways. It has an important role in regulating antibody production, inflammation and the development of effector T-cell responses (Jeong et al. 2004). Production of interleukin-4 (IL-4) is directing the immune system toward Th2-dependent allergic reactivity (Maggi 1998; Mueller et al. 2002; Nelms et al. 1999). It is made in response to immunologic recognition, principally by CD4 T lymphocytes (Ben-Sasson et al. 1990; Zhang et al. 2003). IL-4 is responsible for the production of IgE in mice (Finkelman et al. 1990; Zhang et al. 2003).

In patients with chronic hydatidosis a high level of IL4 has been reported (Rigano et al. 1995a, b, 1996; Zhang et al. 2003) and measurement of serum IL-4 may be a useful marker for the follow up of patients with CE (Zhang and McManus 2006). Mice injected with a vector expressing IL-4 showed higher cyst load than the load in control mice (Al-Qaoud and Abdel-Hafez 2008). So IL-4 may play an important role in hydatid cyst development in the mammalian host (Zhang et al. 2011).

Although many studies have been performed about the IL4 levels in hydatid cyst patients or in animal model, but no study has so far been performed about the IL-4 level in fibrous layer of hydatid cysts where cellular response to the parasite take place. So regarding the role of fibrous layer that controlling the hydatid cyst growth, in this work IL-4 gene expression in fibrous layer of hydatid cyst in comparison with its expression in normal surrounding tissue of the cyst in two well-known intermediate hosts, sheep and cattle, has been investigated using real-time PCR technique.

Materials and methods

Sampling

In this study, hydatid cyst samples were collected from Khomeinshahr slaughterhouse located in Isfahan province, central Iran. Hydatid cysts from 6 sheep and 6 cattle were collected. For every cyst about 2 cm^2 of fibrous layer of ovine and bovine hepatic hydatid cysts was cut, and then each fibrous layer put into a tube containing RNA stabilizer (RNAlater®; Qiagen Inc.). Also for each cyst 2 cm of host normal tissue close to the cyst was also cut and put in RNA later. All samples were then stored at −70 °C until use.

Table 1 The primers used for real-time PCR

Gene name	Primer sequences (forward/reverse) (5′–3′)	Product size (bp)
IL-4 Bovine	TGGCAAGCAAGACCTGTTC	91
	TCCTTCATAATCGTCTTTAGCC	
IL-4 Ovine	GTATGTACCAGCCACTTCGTC	104
	TCCATGCATGAATTCTTTCTC	
YWHAZ Bovine	ACCTACTCCGGACACAGAACA	124
	TTGCTCAGTTACAGACTTCATGC	
YWHAZ Ovine	TGAACTCCCCTGAGAAAGCC	147
	CCGATGTCCACAATGTCAAGT	

RNA extraction

Total RNA was extracted using RNA extraction kit (Jena Bioscience Inc.) according to the manufacturer instructions. Genomic DNA was eliminated with RNase-Free DNase kit (Qiagen Inc.). Total isolated RNA was quantified using Nano Drop® ND-1000 spectrophotometer. Only samples with A260/A280 ratios between 1.80 and 2.00 were analyzed further. Samples were stored at −70 °C until real-time PCR was performed.

cDNA synthesis

cDNA synthesis was performed using cDNA synthesis kit (Thermo Scientific Inc.) according to the manufacturer instructions. For this purpose oligo dT primers were used. Samples were reverse transcribed using 100 µg/ml RNA under conditions of 65 °C for 5 min, 42 °C for 60 min and 70 °C for 10 min. The synthesized cDNA were stored at −70 °C.

Real-time PCR

PCR amplifications were performed using the Quanti Fast SYBR Green PCR Kit (Fermentas) in a total volume of 20 µl. Primers were designed by Gene Runner software. Real-time reverse transcription PCR (RT-PCR) was run on the ABI Step one Plus (Applied Biosystems, ABI, USA). The following cycling conditions were used: initial denaturation at 95 °C for 5 min, followed by 40 cycles of 95 °C for 10 s and 60 °C for 30 s. Melting curve analysis was performed to identify all amplified PCR products. Polymerase chain reactions were performed in duplicate. Tyrosine 3-monooxygenase/tryptophan 5-monooxygenase activation protein, zeta polypeptide (YWHAZ) was used as reference gene in both bovine and ovine samples. Relative gene expression normalized to YWHAZ and calculation was performed using $2^{-\Delta\Delta Ct}$ method. PCR primers that were used for real-time PCR are shown in Table 1. All samples were tested in duplicate.

Table 2 The expression rate of IL 4 in ovine and bovine hydatid cyst fibrous layer

Isolated cyst	No	IL 4 expression rate	Std. error	95 % CI	*P*
Sheep					
Control	6	1	0.047–56.698	0.003–1, 116.511	0.561
Test	6	1.980			
Cattle					
Control	6	1	3.451–31.740	2.028, 95.533	<0.001
Test	6	9.842			

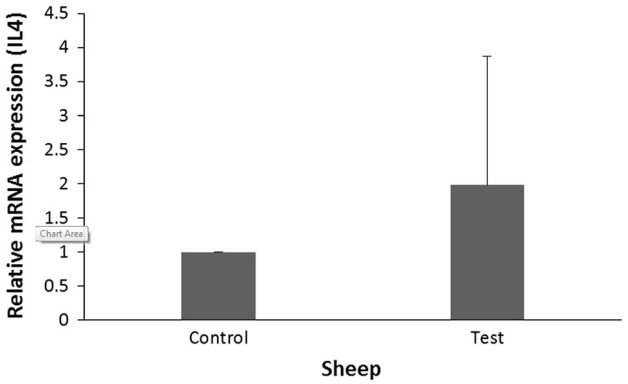

Fig. 1 IL4 gene expression in fibrous layer of ovine hydatid cyst (test group) in comparison with the rate of IL4 gene expression in normal liver tissue close to hydatid cyst location (control group)

Data analysis

Data were analyzed by REST software (version 2.0.13, 2009).

Results

In sheep the rate of IL-4 gene expression in the fibrous layer of hepatic hydatid cysts was 1.98 times more than that of control group (normal liver), but the difference was not significant ($P = 0.561$) (Table 2). The results of IL4 gene expression in fibrous layer of sheep has been shown in Fig. 1.

Bovine IL-4 gene expression in test group samples (fibrous layer of hepatic hydatid cysts) was 9.84 times more than that of control group (normal liver) which was statistically significant ($P < 0.001$). The results of IL4 gene expression in fibrous layer of bovine hydatid cyst has been shown in Fig. 2.

Discussion

Results of this investigation revealed that IL4 gene expression in fibrous layer of hydatid cyst was significantly

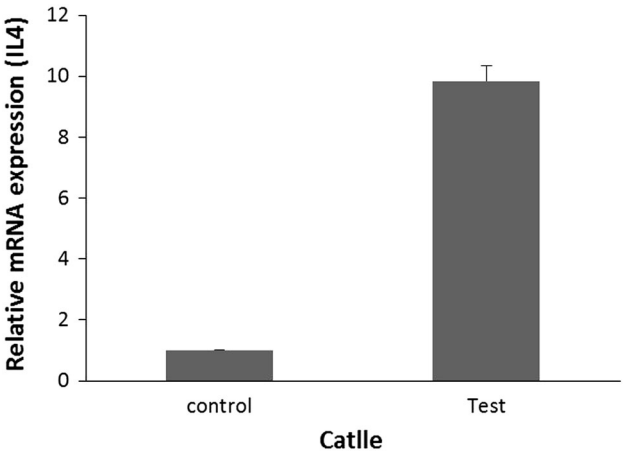

Fig. 2 IL4 gene expression in fibrous layer of bovine hydatid cyst (test group) in comparison with the rate of IL4 gene expression in normal liver tissue close to hydatid cyst location (control group)

higher than the rate of expression of gene of this interleukin in normal liver close to the hydatid cyst. Also the rate of IL4 gene expression in fibrous layer of hydatid cyst in cattle was much higher than that of sheep. Regarding the role of IL4 in hydatid cyst infection, it has been shown that in peripheral blood mononuclear from hydatid cyst patients stimulated with this parasite antigens, IL4 increased significantly (Rigano et al. 1995a). Also a high level of IL4 has been reported in patients with chronic hydatid cyst (Rigano et al. 1995b; Zhang et al. 2003; Rigano et al. 1996) Fibrous layer develops by host as a protective response to hydatid cyst. Expression of high level of IL4 in fibrous layer shows that this cytokine may play a key function in regulation of immune response in favor or against the parasite.

In hydatid cyst infection early Th1-polarized cytokine production, which can kill the parasite at the initial stages of development (Vuitton 2003; Zhang et al. 2008), shifts to a predominant Th2 cytokine response in the later chronic stage. It has been shown that Patients with chronic hydatid cyst generate both Th1 and Th2 responses (Baz et al. 2006; Zhang et al. 2008). As Th1 and Th2 cytokines usually down-regulate each other (Zhang et al. 2008; Pearce and MacDonald 2002), this is likely due to echinococcal antigens containing distinct epitopes for each T cell subset (Fraize et al. 2005; Ortona et al. 2003; Zhang et al. 2008). In this study we used fertile cyst containing protoscoleces, so all of the cyst used in this investigation were in chronic stage with Th2 cytokine response.

It seems that the size and developmental stage of hydatid cyst is correlated with cell response in the fibrous layer. In this context it has been shown that in most cattle having larger mature hydatid cysts, CD8 cells were predominant in the pericystic adventitial layer, which also had fewer CD4 cells and a few B cells.

However, in cattle having intensive regressive and complicated hydatid cysts, lymphocytes infiltrating the adventitial layer were mostly composed of CD4 cells. Also it has been shown that the smallest unilocular hydatid cysts were surrounded by an epithelioid cell layer composed of mononuclear cells bound to a laminated layer. These cells have usually been considered to be derived from macrophages (Sakamoto and Cabrera 2003). So variation in rate of IL4 expression in fibrous layer of hydatid cyst observed in our study may be related to the developmental stages of the cysts.

Severe infiltration and accumulation of numerous eosinophils and infiltration of lymphocytes and macrophages were seen in the fibrous layer surrounding hydatid cysts. So eosinophils infiltrating and their products such as IL4 in the fibrous layer may have an important role in formation of hydatid lesions in cattle (Sakamoto and Cabrera 2003).

IL4 is secreted by eosinophils, so the presence of these cells in fibrous layer may be an indication of liver tissue damage (Goh et al. 2013) or may be related to cellular immune response to the hydatid cyst. In this context different studies have shown a potential role for eosinophils in tissue repair (Goh et al. 2013; Heredia et al. 2013; Rosenberg et al. 2012). Also it has been shown that IL-4 secreted by eosinophils has been implicated in both wound repair and liver regeneration (Anthony et al. 2007; Chen et al. 2012; Gallagher et al. 2007; Goh et al. 2013; Murray and Wynn 2011; Palm et al. 2012). So high level of IL4 gene expression in fibrous layer of hydatid cyst observed in this work may also related to tissue repair.

Hydatid cysts have a different behavior in sheep and cattle. In this context Himonas et al. showed that 64 % of hydatid cyst in sheep were fertile while only 16 % of hydatid cyst in cattle were fertile (Himonas et al. 1987). So this difference may be considered as an explanation for difference in the rate of IL4 expression in cattle and sheep observed in this study.

In conclusion, this work is the first report about the level of IL4 gene expression in the fibrous layer surrounding hydatid cysts. With high rate of IL4 expression especially in fibrous layer of bovine hydatid cyst, it can be concluded that this interleukin may play an important role in host parasite relationship. For better understanding the function of IL4 gene expression in fibrous layer of hydatid cyst, it is recommended that the profile of other interleukin genes expression in this layer is determined.

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Conflict of interest The authors have no conflict of interests.

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