Pakistan J. Zool., vol. 54(1), pp 87-97, 2022 DOI: https://dx.doi.org/10.17582/journal.pjz/20200812070843

Detection of Expression Alteration of Cytokines in the Intestine of Balb/c Mice Infected with *Cryptosporidium parvum* using Relative Fluorescence Quantitative PCR Method

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Article Information
Received 12 August 2020
Revised 25 October 2020
Accepted 21 November 2020
Available online 28 April 2021 (early access)
Published 16 November 2021

Authors' Contribution
LC designed the study and drafted the paper. LC and YC validated sequencing data by RT-qPCR. QK conducted the research and analysed data. JHQ and ZL contributed to the discussions and to draft the final version of the manuscript.

Key words
***Cryptosporidium parvum*, Ct value comparison method, Cytokines, Relative expression**

ABSTRACT

Cryptosporidium parvum (C. parvum) is a parasitic protozoan that causes cryptosporidiosis in mammalian intestinal tract. In this study, *C. parvum* infection model was established in Balb/c mice, followed by extraction of total RNA from small intestine of infected mice at 1, 3, 7 and 14 dpi, respectively. The relative expression of IFN-γ, TNF-α, IL-2, IL-4 and IL-6 in the small intestine of Balb/cmice infected with *C. parvum*, were then detected using Ct value comparison method (RT-QPCR$^{2-\Delta\Delta Ct}$). The primers were designed according to the sequence of mouse cytokines in GenBank. The expression levels of the cytokines were normalized to GAPDH gene expression. Fluorescence threshold (Ct value) obtained from RT-QPCR$^{2-\Delta\Delta Ct}$ method with GAPDH standard curve (linearity R^2=1), followed by sequencing analysis showed that all tested cytokines were amplified. The PCR products exhibited consistent melting curves, suggesting reliable specificity of the cytokine primers. Compared with the control group, the expression levels of IFN-γ, TNF-α, IL-2, IL-4, IL-6 of infected group were all up-regulated, demonstrating an important role of cytokines in controlling *C. parvum* infection. Hence, these findings suggest that expression of IFN-γ, TNF-α, IL-2, IL-4 and IL-6cytokines could be used as reference for diagnosis of *C. parvum* infection.

INTRODUCTION

Cryptosporidium parvum is one of the most common diarrhea causing pathogens in calves. *C. parvum*, which is a protozoan parasite is mainly found in the brush edge of epithelial cells of the small intestinal, and can cause atrophic and damage to the villi and further generate a series of dynamic changes in immune indicators of body (Hu *et al*., 2010; Feng and Xiao, 2017; Laurent and Lacroix-Lamandé, 2017). Epithelial cells of the small intestine serve as the main effector cells during Cryptosporidial infection, as they are involved in the regulation of the body's immune system via secretion of various cytokines forming an immunologic network (Zhang, 2016; Drinkall *et al*., 2017). It has been shown that cytokines including IFN-γ, TNF-α, IL-2, IL-4 and IL-6 play a vital role in immune regulation and are capable of hindering the development of disease due to their rapid recruitment after *C. parvum* invasion and infection (Noti *et al*., 2010; Han *et al*., 2011). Hence, detection of expressions of cytokines such as IFN-γ, TNF-α, IL-2, IL-4 and IL-6 allows evaluation of the host immune status and exploration of the underlying pathogenesis, to gain convincing evidence for the mechanisms of host immune responses against Cryptosporidial infection.

Currently, most methods used to detect protein expression (e.g., cytokines, enzymes) include enzyme-linked immunosorbent assay (ELISA) that relies on availability of target-specific antibodies. However, ELISA is costly and difficult to be customized for certain proteins (Tong *et al*., 2014). Quantitative real-time PCR (qPCR) method, which is a highly used molecular method, can quantify the expression of biological macromolecules at the nucleic acid level. The procedure involves quantitative detection of the target gene using fluorescent substances after each PCR cycle. Based on the selection of internal or external references, the qPCR method can be divided into the relative quantitative method and the absolute

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0030-9923/2022/0001-0087 $ 9.00/0

quantitative method. The absolute quantitative method requires use of standard with a known copy number for the construction of calibration curves, whereas the relative quantitative method uses a constant internal reference as the control (Tang *et al*., 2009). The comparative Ct method is a relative quantitative method, which generates amplification curves of target genes and internal reference genes based on the fluorescence signals, rather than the traditional standard curves. In this method, the relative content of initial template can be determined based on the Ct values. Theoretically, the amplification efficiencies of the target genes are similar to the internal reference genes in the same tissue. Therefore, normalization of the target genes can be achieved by using GAPDH gene as the internal reference. Meanwhile, with the copies of uninfected groups as reference samples, the difference (ΔΔCt) between the standardized targets and the reference samples can be calculated and the fold change ($2^{-\Delta\Delta Ct}$) can be obtained. Compared to ELISA, this method is relatively inexpensive, and is a simple method for analysis of the relative changes of gene expression detected by real-time PCR.

In this study, the fluorescence quantitative Ct method (RT-qPCR$^{2-\Delta\Delta Ct}$) was used to detect the expressions of the main effector cytokines, including IFN-γ, TNF-α, IL-2, IL-4 and IL-6, at different phases of *C. parvum* infection in the small intestine of infected mice, with an aim to explore the immunologic modulation by these cytokines in the intestinal epithelial cells.

MATERIALS AND METHODS

Parasites strain and test animals

The *C. parvum* oocysts were preserved by the parasitism Laboratory in the College of Veterinary Medicine, Hebei Agricultural University (Liu, 2018); 48 three-week-old Balb/c mice of SPF grade were purchased from Vitalriver company, Beijing.

Agents and equipment

The RNA extract kit, RNA reverse transcription kit, 2×Green qPCR SuperMix and pEASY-T1 cloning plasmid were all purchased from TransGen Biotech company, Beijing; DL2000 DNA Marker was obtained from Bao biological company; Gel extraction kit and nucleic acid dyes were purchased from CoWin Biosciences company. Light Cycler®96 real-time fluorescence RT-qPCR device was purchased from Roche company.

Establishment of BALB/c mice model infected with C. parvum

Forty eight female mice (11g-14g) from heathy BALB/c mice aged 3 weeks were divided into four groups: infection (n=2) and control group (n=2). One group was used for intestinal tissue extraction and the other was used for collecting rat feces, weighing and dissecting. After feeding for 3 days, rats' feces were floated in saturated saline and detected by microscopic examination to verify no parasite infection. The 15mg/mL dexamethasone acetate was used to maintain immunosuppressive by adding in oral drinking water for 7 days. *C.parvum* was artificially infected by gavage. The *C. parvum* oocysts stored in 2.5% potassium dichromate were washed with PBS for 3-4 times until colorless. The suspension of oocyst was injected into the blood cell counting plate for counting and diluting to 1.0 x10^5/mL. The oocysts were gavaged into an empty stomach twice with 500uL each time. 40 mg/L gentamicin solution was given to mice every day to prevent secondary infection. Rat feces were collected from the first day after infection and ground as well as screened to remove large fecal particles. The filtrate was centrifuged by 3500g for 10 min, and the supernatant was discarded. We added 15mL saturated sucrose into the precipitate and mixed them well, centrifuged 3500g for 10min. Then the supernatant was diluted 10 times with distilled water and centrifuged 3500g for 10 min. The precipitate we got was washed twice with distilled water, we discarded the supernatant after centrifugation, only leaved a small part of liquid and precipitation, and blow and suck evenly. Finally, we counted oocysts under high power microscope, and then keep it in 2.5% potassium dichromate. On the 1st, 3rd, 7th and 14th days after challenge, the intestinal epithelial cells were isolated, and washed slowly with PBS, and then placed in a 1.5mL centrifuge tube and stored at -80 °C. The apparatus and environment were disinfected thoroughly before the experiment. During the experiment, all mice were fed in a sterile environment. The drinking water was boiled and disinfected as well as changed every day with no drinking limit. Dry heat sterilization was used for feed and bedding material oven.

Total RNA extraction and reverse transcription

The Balb/c mice were divided into uninfected group and infected group. The infected group mice were infected with 10^5 *C. parvum* oocysts after 7-days of immunosuppression with Dexamethasone Acetate at the concentration of 15mg/L. At 1, 3, 7 and 14 dpi, small intestines of three mice from each group were collected, and then ground in a grinder under sterile circumstance. Total RNA was extracted using Trizol Reagent following manufacturer's instructions. After the removal of genomic DNA, the reaction system was prepared on ice. The reaction mixture consisted of 1 μL of 1x gDNA Eraser Buffer, 1 μL of gDNA eraser, 3 μL of RNA Free Water and 5 μL of extracted RNA. Then the mixture was sub-

packed into PCR tubes that were soaked beforehand by DEPC to assure the absence of RNA enzyme. Next, the prepared reaction mixture was placed in a water bath at 42°C for 2 min, and finally preserved on ice for standby. The reverse transcription mixture was set up follows: 10 μL of reaction solution without genomic DNA, 1 μL of Primer Script RT Enzyme Mix, 1 μL of RT Primer Script Mix, 4 μL of 5xPrimer Script buffer and 4 μL of RNA Free Water. The reaction mixture was thoroughly mixed and following amplification program was set up: at 42 °C for 15min and and at 85 °C for 5min. Finally, the reverse transcription cDNAs products were stored at -20 °C until ready to use.

Design and synthesis of PCR primers

The synthetic primers were designed using Primer Premier 5.0 software based on the available cDNA sequences of GAPDH gene, IFN-γ, TNF-α, IL-2, IL-4, IL-6 of Balb/c mice in GenBank. The primers were synthesized by Sangon Biotech (Shanghai) Co., Ltd. The sequences of the primers are shown in Table I.

Real-time RT-qPCR amplification and product sequencing of GAPDH, IFN-γ, TNF-α, IL-2, IL-4, IL-6

A total of 20 μL volume of reaction mixture was applied for q-PCR detection. The mixture contained 2 μL of cDNA of small intestine from uninfected mice group, 10 μL of 2×EvaGreen qPCR SuperMix, 0.4μL each of upstream and downstream primers and 7.2 μL of ddH_2O. The following amplification program was used: pre-denaturation at 95˚C for 10 min, followed by a total of 45 cycles, consisting of denaturation at 95 ˚C for 30s, annealing at 60˚C for 20s, and extension at 72˚C for 20s. After the qPCR reaction, the products were analyzed by electrophoresis on 1.5% agarose gel and the gel recovery products were sent to Sangon Biotech (Shanghai) Co., Ltd for sequencing.

Determination of amplification efficiencies of primers of GAPDH, IFN-γ, TNF-α, IL-2, IL-4 and IL-6

The gel recovery product was inserted to the vector pEASY-T1, and *E. coli* were transformed with the vector plasmid. Plasmid DNA was extracted by mini-prep and validated by enzymatic digestion. The concentration and purity of plasmids were measured by ultraviolet spectrophotometer and copy numbers were calculated. After dilution to 10^8 copies/μL by solution buffer, the plasmids were preserved at -20 °C until ready for use. Based on the gradient of 1×10^5, 1×10^4, 1×10^3, 1×10^2 and 1×10^1 copies/μL, the extracted plasmids were diluted for the amplification of GAPDH gene, IFN-γ, TNF-α, IL-2, IL-4, IL-6, respectively. The reaction system and amplification conditions were the same as above. After the reaction, the system automatically generated the amplification curve of reaction cycle number and detection fluorescence quantity according to the change rule of fluorescence value, the melting curves and amplification efficiencies were analyzed using Light Cycler 96 SW 1.1 software.

Determination of fold change using real-time RT-qPCR comparative Ct method

After validating the amplification efficiency of six pairs of primers, the cDNAs from infected and uninfected mice were used as templates for gene-specific amplification of GAPDH, IFN-γ, TNF-α, IL-2, IL-4 and IL-6 genes. The average of Ct values of each group was calculated, and the fold changes ($2^{-\Delta\Delta Ct}$) were obtained using the following formulas. The gene expression from uninfected group were set as 1×, based on which the gene expression fold changes of infected group were obtained using the comparative Ct method. The following formula was used:

$\Delta\Delta Ct = \Delta Ct$ (test samples) - ΔCt (control samples)

ΔCt (test samples) = Ct (test samples, target genes) – Ct (test samples, internal reference genes)

ΔCt (control samples) = Ct (control samples, target genes) - Ct (control samples, internal reference genes)

Fold changes of target genes = $2^{-\Delta\Delta Ct}$

RESULTS

Body weight change and mortality of mice

One week after immunosuppression, $1.0x10^5$ *C.parvum* oocysts were artificially infected in 3 week old female BALB/c mice. After infection two weeks, the mice showed poor spirit, disordered hair, decreased skin elasticity, and decreased appetite and activity. Compared with the control group, the weight gain of the experimental group was slower than that of the control group, and the difference was significant ($P<0.05$) as shown in Figure 1. During the whole experiment, the shape of the fecal ball changed from long shuttle shape to round or oval shape in the early stage of infection, and the color gradually changed from normal brown to brown yellow. In the later stage, the mouse dung became soft and shapeless, showing light yellow. There were four mice died in the experimental group, the mortality rate was 16.7% (4/24). The dead mice all had dyspnea, shaking all over, dehydration and emaciation of the body before death. Autopsy showed that the stomach was bloated, the intestinal wall was thin and inflated, and the intestinal chyme in the small intestine was yellow and watery.

Changes of oocyst excretion in mice after infection

After intragastric administration of *C. parvum* oocysts, fresh mouse faeces were collected every day.

Fecal samples were filtered and centrifuged, and the oocyst output was observed by Maxwell's counting method. The overall change was in a wave shape as shown in Figure 2. On 1st day, oocysts were detected from feces, and the output of oocysts was relatively large, due to part of the oocysts being excreted with feces after gavage infection; the number of oocysts increased significantly on the 3rd day. The peak of output was 7-11 dpi, and reached the top on the 9th day. After that, oocysts were continuously discharged until the 14th day.

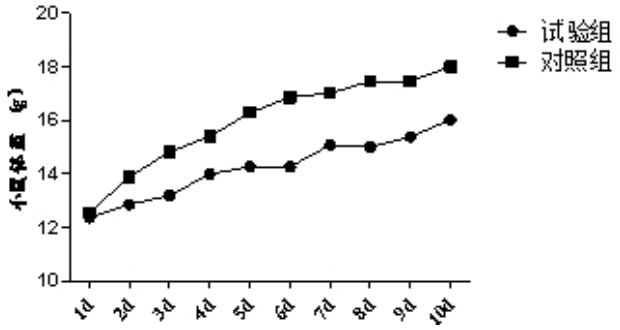

Fig. 1. The electrophoresis result of primers of real-time RT-qPCR. M: DL-1000Marker; 1:IL-2 2:IL-4 3:IL-6 4: TNF-α 5: GAPDH 6: IFN-γ.

Fig. 2. The amplification curves of GAPDH, IFN-γ, TNF-α, IL-2, IL-4 and IL-6. 1-5: The dilution factor is 1×10^{5}-1×10^{1} 6: H_2O.

Detection of real-time RT-qPCR products by gel electrophoresis

The RNA from intestine epithelial cells of mice were extracted for real-time RT-qPCR detection of endogenous gene GAPDH, IFN-γ, TNF-α, IL-2, IL-4 and IL-6. The PCR products were analyzed by electrophoresis on 1.5% agarose gel. The results of electrophoresis are shown in Figure 3. The results suggested that the PCR products were of expected lengths. The lengths of internal gene GAPDH, IFN-γ, TNF-α, IL-2, IL-4 and IL-6 were 140 bp, 153 bp, 192 bp, 168 bp, 165 bp and 120 bp, respectively. The negative control was set under same conditions except for reverse transcription, to eliminate the impact of genomic contamination on PCR system. The results from negative control lane showed absence of amplification products, indicating that the amplification products were synthesized from mRNA as the templates.

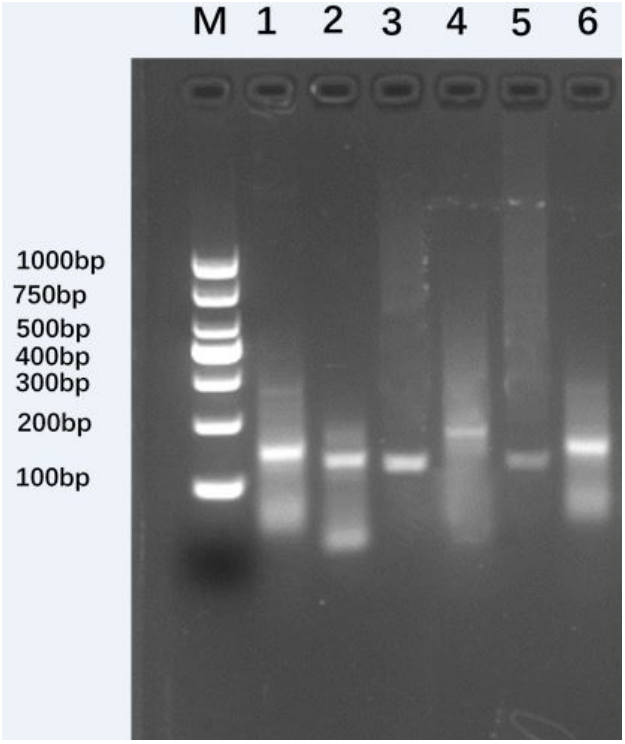

Fig. 3. The melting curves of GAPDH gene, IFN-γ, TNF-α, IL-2, IL-4 and IL-6.

The sequence analysis of real-time RT-qPCR products

The DNA Star software was used to compare the sequencing result of the target gene with the mouse cDNA sequence published on GenBank. The IFN-γ gene sequencing result had the highest homology of 97.92% with (*XM021175069.1*); The TNF-α gene sequencing result had the highest homology of 96.43% with (*U68414.1*); The highest homology between IL-2 gene sequencing results and (*AH001969.2*) was 97.96%; The highest homology between IL-4 gene sequencing results and (*NM021283.2*) was 97.74%; The highest homology between of IL-6 gene sequencing results and (*XM021163844.1*) was 98.84%; The highest homology between GAPDH gene sequencing results and (*XM021191469*) was 92.92%. These results showed that the primers of the quantitative PCR method can accurately amplify the target fragment.

Table I. Design and synthesis of PCR primers.

Primer	Primer sequence	Annealing temperature (˚C)	Predicted length (bp)
GAPDH	5'CGTGCCGCCTGGAGAAACCTG 3'	60	140
	5'AGAGTGGGAGTTGCTGTTGAAGTC3'		
IFN-γ	5 'CCATCGGCTGACCTAGAGAAGA 3'	60	153
	5'CGTGGCACTAACAGCCAGAAA 3'		
TNF-α	5' CTCATGCACCACCATCAAGGA 3'	60	192
	5’ CGTGGCACTAACAGCCAGAAA 3’		
IL-2	5'ATTGACACTTGTGCTCCTTGTA 3'	60	168
	5'TTCCTGTAATTCTCCATCCTGC 3'		
IL-4	5'ATGGATGTGCCAAACGTCCT 3'	60	165
	5' AAGCCCGAAAGAGTCTCTGC 3'		
IL-6	5' CACGGCCTTCCCTACTTC 3'	60	120
	5' ATTTCCACGATTTCCCAGA 3'		

Determination of amplification efficiencies of primers

The extracted plasmids were serially diluted from 1×10^5 to 1×10^1copies/μL. GAPDH gene, IFN-γ, TNF-α, IL-2, IL-4 and IL-6, were amplified at five dilutions. The amplification curve generated from the Roche real-time RT-qPCR system gave Cq and fluorescence values. The amplification curves of GAPDH gene, IFN-γ, TNF-α, IL-2, IL-4 and IL-6 are shown in Figure 4. The results showed that the amplified products were highly stable, and had correlation coefficient of $R^2=1$. The amplification efficiencies of GAPDH, IFN-γ, TNF-α, IL-2, IL-4 and IL-6 were 1.14, 1.16, 1.19, 0.91, 1.07 and 1.08, respectively. Consistently high amplification efficiencies of the internal reference gene and the target genes indicated that the amplification was of good quality.

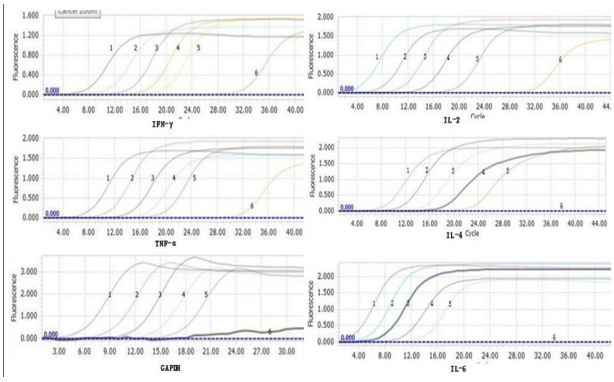

Fig. 4. Relative expression of mouse cytokine TNF-α. *means $P< 0.05$, **means $P< 0.01$, ***means $P< 0.001$.

The melting curves of gene amplification are shown in Figure 5. No undesired peaks were noted in the melting curves of GAPDH, IFN-γ, TNF-α, IL-2, IL-4 and IL-6, indicating the absence of nonspecific amplification such as due to primer dimer. Hence, this suggests that the PCR amplifications reactions were reliable.

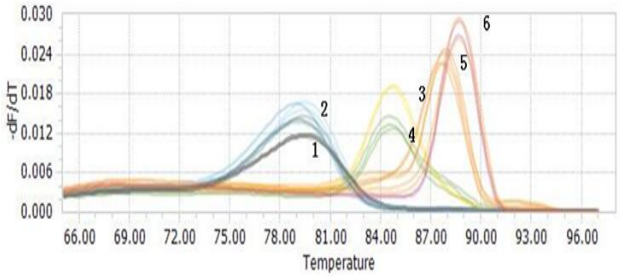

Fig. 5. Relative expression of mouse cytokine TNF-γ. *means $P< 0.05$, **means $P< 0.01$, ***means $P< 0.001$.

Gene copies calculation

Cytokine expressions in the test group and the control group at 1, 3, 7 and 14 dpi were determined three times. The results are shown in Table II. The stable amplification cycle CQ value indicated good reproducibility and accuracy of this method. Based on the calculated -ΔΔCt from the Cq values of the infected group and the uninfected group, each cytokine expression in the two groups diverged at 1, 3, 7 and 14 dpi.

The fold changes ($2^{-\Delta\Delta Ct}$) were calculated based on the average Ct values of the infected group and the uninfected group. The gene expressions of target genes from the uninfected group were set as 1×. Then the relative fold changes of target mRNA expressions of infected group to uninfected group were obtained using the comparative Ct method. The expression of internal reference GAPDH gene

was relatively stable. The IFN-γ expression reached to a fairly high level at 1 dpi, and significantly increased to a maximum (10.74-fold to day 1) at 14 dpi ($p<0.05$); TNF-α expression exhibited sustained increment at 1, 3, 7 and 14 dpi, with the infected group showing 2.56-fold, 6.19-fold, 19.02-fold, 21.70-fold compared to the uninfected group, respectively; The expression of IL-2 slightly increased at 1, 3 and 7 dpi with no significant difference compared to the uninfected group ($P>0.05$). However, at 14 dpi, the expression of IL-2 elevated to a maximum level, which was 3.81-fold compared to the control group with significant difference ($P<0.05$); The IL-4 expression showed marginally increasing trend during 1, 3 and 7 dpi, which were insignificant to the control group ($P>0.05$). However, similar to the trend of IL-2 expression, the expression of IL-4 at 14 dpi elevated to the highest, and was 5.04-fold compared to the uninfected group. The expression of IL-6 was the highest on the 14th day, which was 5.04 times compared to the control group. The expression of IL-6 during experimental period was in a stable trend. At 1, 3 and 7 dpi, the expression of IL-6 exhibited significant difference compared to that of the control group ($P<0.05$). Although the expression of IL-6 at 14 dpi showed no significance due to slight decline($P>0.05$), it was still higher than that of the control group.

The mRNA expression levels of TNF-α in intestinal

The mRNA expression level of TNF-α in intestinal was detected by RT-qPCR, and the results were shown in Figure 6. The expression of TNF-α increased significantly and continued to increase after infection. On the 7th day, the expression of TNF-α in the infection group increased

Table II. The Cq values of the infected group and the uninfected group.

Cytokines	Infection time (d)	Amplification cycle Cq values		ΔCtvalues (Tested group-Control group)	- ΔΔCtvalues ΔCt-ΔCt group-
		Tested group	Control group		
GAPDH	1	22.59±0.15	21.53±0.03	1.06	0
	3	22.43±0.21	22.25±0.09	0.18	0
	7	23.02±0.09	22.10±0.03	0.97	0
	14	23.46±0.07	21.88±0.03	1.58	0
IFN-γ	1	23.87±0.09	25.77±0.06	-0.90	1.96
	3	23.28±0.09	25.60±0.07	-2.32	2.5
	7	25.72±0.20	27.03±0.31	-1.31	2.28
	14	25.31±0.22	26.64±0.05	-1.33	2.91
TNF-α	1	12.73±0.05	12.42±0.07	0.29	0.77
	3	13.77±0.11	14.72±0.05	-0.95	1.13
	7	13.63±0.11	15.84±0.28	-2.21	3.18
	14	13.44±0.12	16.37±0.27	-2.93	4.51
IL-2	1	18.74±0.10	18.19±0.04	0.55	0.51
	3	19.31±0.11	19.57±0.28	-0.26	0.44
	7	18.50±0.29	17.77±0.10	0.73	0.24
	14	18.27±0.08	18.61±0.08	-0.34	1.92
IL-4	1	22.77±0.15	22.55±0.10	0.22	0.84
	3	22.61±0.02	23.57±0.15	-0.96	1.14
	7	23.49±0.05	23.75±0.11	-0.26	1.23
	14	22.06±0.12	22.82±0.04	-0.76	2.34
IL-6	1	25.31±0.16	26.18±0.06	-0.93	1.99
	3	24.74±0.08	26.82±0.05	-1.08	1.26
	7	25.65±0.10	27.10±0.21	-1.35	2.32
	14	26.85±0.49	28.37±0.55	-1.52	3.1

significantly, which was 9.91 times that of the control group, with a significant difference (P <0.05); The expression level continued to increase at 14 days, which was 24.19 times that of the control group, with an extremely significant difference (P <0.01). It shows that *C. parvum* infected BALB / c mice can induce the body to produce high levels of TNF-α to balance the relationship between intestinal cytokines, maintain and regulate the stability of the intestinal mucosal environment because TNF-α is an important factor affecting inflammation and injury.

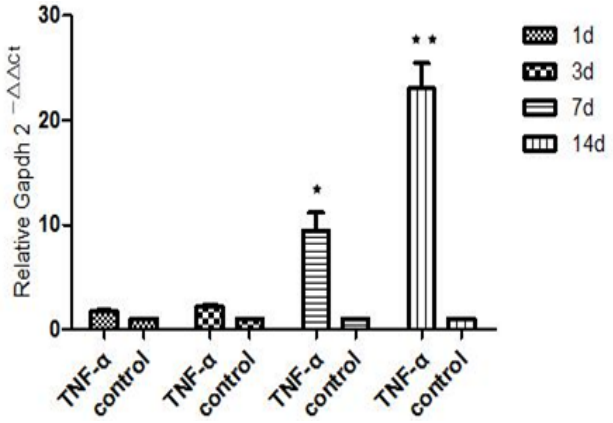

Fig. 6. Relative expression of mouse cytokine IL-2. *means *P*< 0.05, **means *P*< 0.01, ***means *P*< 0.001.

The mRNA expression levels of IFN-γ in intestinal

The mRNA expression level of IFN-γ in intestinal was detected by RT-qPCR, and the results were shown in Figure 7. IFN-γ was detected at a high level on the first day of infection, and the expression level reached a maximum of 10.74 times at 14 days, with a significant difference (P <0.05); During the infection, the expression of IFN-γ decreased, and the expression was the lowest on the 7th day, but it still showed higher expression than the control group, which was about 4.92 times that of the control group. IFN-γ was up-regulated throughout the test, indicating that the body resisted *C. parvum* infection through a regulatory mechanism that depends on IFN-γ.

The mRNA expression levels of IL-2 in intestinal

The mRNA expression level of IL-2 in intestinal was detected by RT-qPCR,and the results were shown in Figure 8. The expression of IL-2 increased slightly at 1d, 3d, and 7d in the infection group, while there was no significant difference compared to the control group (P > 0.05). The expression level of IL-2 increased at 14 days after infection, and the expression level was the highest, 3.81 times that of the control group, which was significantly difference compared with the control group (P <0.01). It is indicated that *C. parvum* can induce the increase of the body's IL-2 level and play a regulatory role in the later stages of infection.

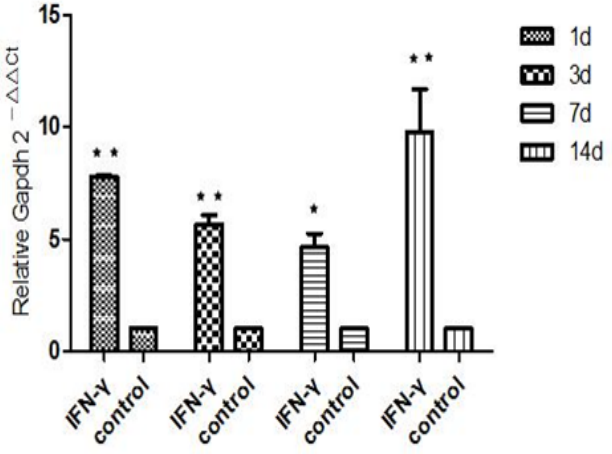

Fig. 7. Relative expression of mouse cytokine IL-4. *means *P*< 0.05, **means *P*< 0.01, ***means *P*< 0.001.

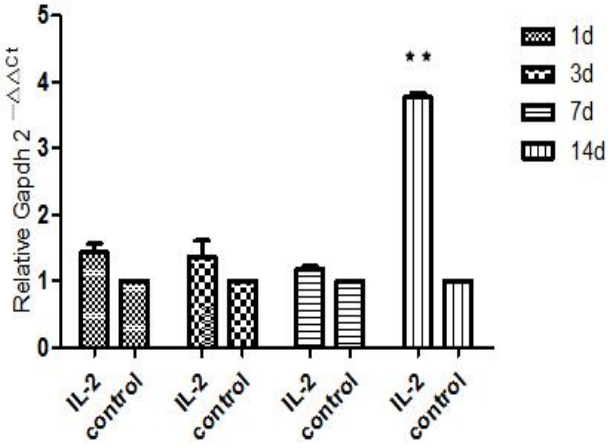

Fig. 8. Relative expression of mouse cytokine IL-6. *means *P*< 0.05, **means *P*< 0.01, ***means *P*< 0.001.

The mRNA expression levels of IL-4 in intestinal

The mRNA expression level of IL-4 in intestinal was measured by RT-qPCR, and the results were shown in Figure 9. The expression of IL-4 increased slowly at 1, 3 and 7 dpi, and there was no significant difference compared with the control group (P > 0.05); the highest expression level was found at 14 dpi, which was 5.04 times that of the control group. Compared with the control group, the difference was significant (P < 0.01). IL-4 was produced by Th2 cell subsets in mice, suggesting that Th2 type immunity plays a role in resisting and clearing *C. parvum* infection.

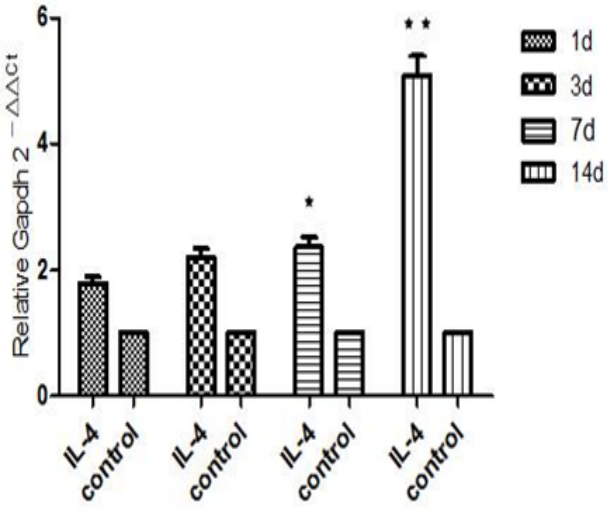

Fig. 9. Relative expression of mouse cytokine IL-4. *means P< 0.05, **means P< 0.01, ***means P< 0.001.

The mRNA expression levels of IL-6 in intestinal

The mRNA expression level of IL-6 in intestinal was detected by RT-qPCR, the results are shown in Figure 10. The expression of IL-6 showed a stable trend, and the expression levels were significantly different from those of the control group on the 1st, 3rd and 7th days after infection ($P < 0.05$), which were 2 to 3 times that of the control group; The expression level of IL-6 decreased slightly after 14 dpi, which was not significantly different from the control group ($P > 0.05$), but still higher than that of the control group. IL-6 mainly induced B cell differentiation in *C. parvum* infection, indicating that *C.parvum* infection can induce a cellular immune response in the body.

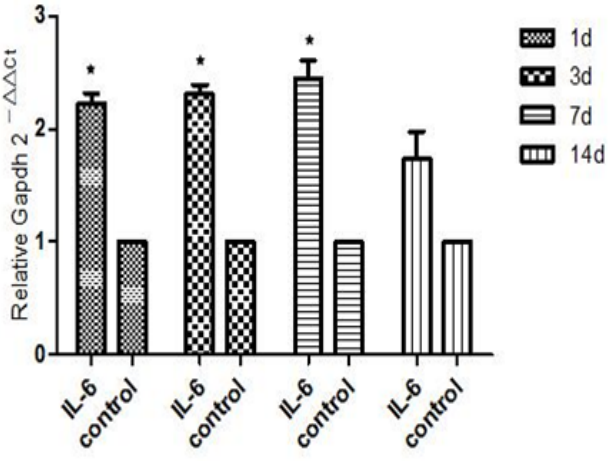

Fig. 10. Relative expression of mouse cytokine IL-6. *means P< 0.05, **means P< 0.01, ***means P< 0.001.

DISCUSSION

C. parvum was artificially infected according to the mouse infection model. One week after immunosuppression, 1.0×10^5 *C. parvum* oocysts were artificially infected in 3 week old female BALB/c mice, The clinical symptoms, mortality and ovulation sac of the experimental group and the control group were observed. In the late stage of infection, the mice showed poor spirit, disordered hair, decreased skin elasticity, and decreased appetite and activity. Compared with the control group, the weight gain of the experimental group was slower than that of the control group, and the difference was significant ($P<0.05$). During the whole experiment, the shape of the fecal ball changed from long shuttle shape to round or oval shape in the early stage of infection, and the color gradually changed from normal brown to brown yellow. In the later stage, the mouse dung became soft and shapeless, showing light yellow. Four mice died in the experimental group, and the mortality rate was 16.7%. Some of mice may be accompanied by bacterial or viral infection after immunosuppression, and there is needle-like necrotic spots on the surface of liver and lung. After filtration and centrifugation of fecal samples, the law of ovulation sac was observed by acid fast staining, it was found that the peak of ovulation was appeared on 7-11dpi. According to the death of mice in the experimental group and the control group, it was concluded that the death of mice mostly occurred at the peak of ovulation sac. The injury of intestinal chorionic membrane in mice during the peak period of ovulation was more serious and the resistance was decreased. After the peak of ovulation, the death phenomenon was not obvious. It is suggested that the nutrition of mice was plundered during the peak period of ovulation sac, which may cause mechanical damage to intestinal mucosa, such as villus atrophy, follicular proliferation, inflammatory cell infiltration and other inflammatory reactions, which can directly or indirectly affect nutrient absorption.

The comparative Ct method is the most commonly used method for relative quantitative analysis in molecular biology (Lin *et al*., 2013). This method is considered a facile and convenient method that does not require standard curves. In this study, the primers were designed based on stringent criteria used of primer design. The primers were chosen to result in consistent amplification length products, to ensure similar amplification efficiencies between target genes. To verify amplification efficiencies, the plasmids were serially diluted and a standard curve was generated by PCR analysis software. The amplification efficiency results showed uniformity of the amplification efficiencies of target genes and the internal reference gene, indicating

reproducibility and precision of this method. The $2^{-\Delta\Delta Ct}$ method could reduce experimental error by normalization of the target genes with an internal gene as reference. Based on this method, the fold changes were compared between the infected group and the uninfected group. To obtain the same amplification efficiency between the six primers, the amplification conditions were continuously optimized, and the primers were designed by using strict construction parameters and amplification lengths. It can be inferred from this experiment that the comparative Ct method could be used to detect the dynamic alternation and relative quantitative expression of IFN-γ, TNF-α, IL-2, IL-4 and IL-6 with marked accuracy.

Epithelial cells infected by *C. parvum* are able to recognize the parasite's somatic surface antigens through TLRs, which can induce cytokines and chemokines production, which can then activate the innate and acquired immune system (Wang *et al*., 2013). Upon antigen stimulation, $CD4^+$ T cells play an important role in *C. parvum* infection via secretion of abundant cytokines, such as IL-2, IFN-α, IFN-γ secreted by Th1 cells and IL-4, IL-6 secreted by Th2 cells. During *C. parvum* infection, IFN-γ can be expressed by Th1 cells in the intestinal tract to resist invasion of pathogens and to exert immunoregulatory effect. As a result, the immune cells are primed for antiviral defense (Yi *et al*., 2018; Zhu *et al*., 2018). Similarly, IFN-γ can enhance macrophages phagocytosis, accelerate the proliferation of T lymphocytes and promote the antiviral function of monocytes and macrophages (Li *et al*., 2017). TNF-α is also a pro-inflammatory factor of high importance, which exhibits the capacity to destroy and inhibit tumor cells. At the same time TNF-α can also induce enhancement of neutrophil phagocytosis and provide synergistic pro-inflammatory effect through the promotion of other inflammatory cytokines or inhibition of anti-inflammatory cytokines (Xia *et al*., 2007). IL-2 is a autocrine growth factor for all T cells, and can be regarded as a typical cytokine gene for transcription regulation during T cell activation. As a cytokine of the chemokine family, IL-4 can stimulate and activate the proliferation of B cells and T cells and plays a key role in the regulation of humoral and adaptive immunity (Mc Donald *et al*., 2004). IL-6 can stimulate the proliferation and differentiation of cells involved in immune response to improve their functions (Li *et al*., 2018; Lacroix S *et al*., 2001).

In this study, the Ct method was used to detect the relative amounts of mRNA in the intestine of mice at 1, 3, 7, and 14 dpi *C. parvum*. The jejunal expressions levels of IFN-γ and TNF-α at 1, 3, and 7 dpi significantly increased, as detected by qPCR ($P<0.05$). IL-2 expression occurred at a later time, of which expression was highest at 14 dpi. The expression of IFN-γ, IL-2 and IFN-α also elevated during this study, indicating that Th1-type immune response also played an important role to fight against *C. parvum* infection. The expression of TNF-α increased continuously, suggesting that TNF-α is possibly involved in the elimination of *C. parvum* (Maillot *et al*., 2000; Robinson *et al*., 2001). By inducing macrophage apoptosis and inducing endothelial cell permeability, TNF-α could activate neutrophils and lymphocytes, promote the synthesis and release of other cytokines, maintain and regulate the environmental equilibrium of intestinal mucosa. TNF-α mediates this effect by mutual balance with IL-6 in the intestinal tract, suggesting that TNF-α is important for intestinal inflammation and injury. The results showed that Th1-type immune response was related to the immune defenses of hosts against *C. parvum* infection and cytokines played a vital role in the process of worm expulsion. The expression of IL-4 was stable during the infection process and exhibited no significant difference compared with the control group at 1, 3, and 7 dpi ($P> 0.05$). This finding revealed that Th2 type immune response participated in the regulation of inflammation during the late stage of parasite elimination to maintain the body's immune balance. The analysis of results showed that the expressions of IFN-γ and TNF-α significantly increased, indicating that they participated in the host defense against *C. parvum*, and restricted further infection. On the other hand the expression of IL-2, IL-4 and IL-6 at the late stage of *C. parvum* infection suggests that these cytokines have an important role in immune-regulation. As IFN-γ, TNF-α and IL-2 are the main cytokines of Th1-type immune response, Th1 type immune response is inferred as the primary mode of host defense against *C. parvum* infection. Similarly, IL-4 and IL-6 were the main cytokines responsible for Th2 type immune response, which also showed the importance of these cytokines in resistance and elimination of *C. parvum* infection (Mc Donald, 2000).

ACKNOWLEDGMENT

This research was supported by the Research and demonstration of key technologies for ecological dairy farming and disease prevention and control, Tangshan City's 2020 Science and Technology Research and Development Plan Project (20150203C). The Dairy cow innovation team construction of Hebei modern agricultural industry technology system prevention and control of cow disease (HBCT2018120205). Key R&D project in Hebei Province Comprehensive prevention and treatment technology for multi-pathogenic infectious diarrhea of calves in dairy farms (20326603D).

Ethical approval

All animal studies have been reviewed by the appropriate ethics committees.

Statement of conflict of interest

The authors have declared no conflict of interest.

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