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Research Article **Araştırma Makalesi**

Effects of inactivated *Streptococcus iniae*, *Edwardsiella tarda*, and Poly I:C on mRNA Expression Levels of CXCL-10 and CXCL-9 Genes in Japanese Flounder

İnaktive Edilmiş *Streptococcus iniae*, *Edwardsiella tarda* ve Poly I:C'nin Japon Pisi Balığındaki CXCL-10 ve CXCL-9 Geninin mRNA Ekspresyon Seviyeleri Üzerindeki Etkileri

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Abstract: The present study accomplished the successful cloning and sequencing of the *JfCXCL9_L* and *JfCXCL10_L* genes found in the spleen cDNA of the Japanese flounder (*Paralichthys olivaceus*). Additionally, the tissue distribution of these two genes was examined at the zero-hour mark. Through qRT-PCR analysis, it was observed that the expression of *JfCXCL10_L* closely mirrored that of *IL1-β*, displaying an upregulation following the application of Poly I:C (Viral mimic) and formalin-killed *Edwardsiella tarda* (Gram-Negative mimic), while showing a downregulation after the application of formalin-killed *Streptococcus iniae* (Gram-Positive mimic) treatment. This finding strongly suggests a role for *JfCXCL10_L* in the immune response to viral and gram-negative bacterial stimuli. Regarding *JfCXCL9_L*, mRNA levels were found to be significantly downregulated after FKC_{ET} and FKC_{SI} treatments, though to varying extents. Interestingly, at specific time points, *JfCXCL9_L* levels were even lower compared to Poly I:C treatment. These intriguing results shed valuable light on the immune response mechanisms and potential functions of both *JfCXCL9_L* and *JfCXCL10_L* in the context of the Japanese flounder's immune system.

Keywords
- CXC Motif
- Chemokine
- Poly I:C
- *Paralichthys olivaceus*
- Expression

Özet: Bu çalışma, Japon pisi balığının (*Paralichthys olivaceus*) dalak cDNA'sında *JfCXCL9_L* ve *JfCXCL10_L* genlerinin başarılı bir şekilde klonlandığını ve dizilendiğini bildirmektedir. Bu iki genin dokulardaki dağılımları da herhangi bir stimulant uygulanmadan önce (0h) belirlenmiştir. Ayrıca, qRT-PCR analizi, *JfCXCL10_L* ekspresyonlarının *IL1-β* ile benzerlik gösterdiğini ortaya koymuştur. Poly I:C ve formalinle öldürülmüş *Edwardsiella tarda* (FKC_{ET}) uygulamasından sonra artan ekspresyon seviyesi gösterirken, formalinle öldürülmüş *Streptococcus iniae* (FKC_{SI}) uygulamasından sonra azalan ekpresyon seviyesi tespit edilmiştir. Bu durum, *JfCXCL10_L*'nin viral ve gram-negatif bakteriyel uyarılara karşı bağışıklık tepkisinde rolü olduğunu düşündürmektedir. *JfCXCL9_L* mRNA düzeyleri, FKC_{ET} ve FKC_{SI} uygulamalarından sonra anlamlı derecede düşmüştür. Özellikle belirli zaman noktalarında, *JfCXCL9_L* düzeyleri Poly I:C uygulamasına kıyasla daha düşük bulunmuştur. Bu bulgular, *JfCXCL9_L* ve *JfCXCL10_L*'nin Japon pisi balığında bağışıklık tepkisi mekanizmaları ve potansiyel işlevleri konusunda bilgi sunmaktadır.

Anahtar kelimeler
- CXC Motifi
- Kemokin
- Poly I:C
- *Paralichthys olivaceus*
- Gen Ekspresyonu

1. INTRODUCTION

In aquaculture, the successful production and cultivation of a species require specific target traits, such as high disease resistance, rapid growth rate, temperature and salinity tolerance, good meat quality, and high feed conversion efficiency (Sanchez et al., 2008). Among these attributes, disease resistance holds significant importance as diseases pose a serious challenge across all aquaculture species. The Japanese flounder (*Paralichthys olivaceus*) is one of the most important commercial

marine species commonly found along the Japanese coast. Apart from being harvested, it is also cultivated through aquaculture practices. Various research efforts are being conducted, including initiatives to strengthen natural stocks (Sekino et al., 2003; Watanabe et al., 2006), as well as studies on sexual manipulation since the discovery that female individuals reach market size faster (Yamamoto, 1999). With the advancement of aquaculture (Seikai, 2002), comprehensive investigations have been initiated to explore the metabolism (Yagi & Oikawa, 2014; Imai et al., 2020; Kim, 2022), disease (Hirono et al., 2007; Jung et al., 2020; Zhang et al., 2017), genome (Nakamura et al., 2021; Zhao et al., 2021), and immunity (Kondo et al., 2021) of Japanese flounder. The Japanese flounder, during egg production and other breeding processes, is vulnerable to various viral, bacterial, and protozoan pathogens. It is well-known that mortalities are high during the growth phase due to viral, bacterial, and parasitic diseases (Sanchez et al., 2008).

Fish combats diseases through their immune system. The immune system also consists of innate and adaptive immune systems. The first response to disease generally occurs by the innate immune system (Riera Romo et al., 2016). The adaptive immune system acts highly specific to the specific pathogen in case of the continuous presence of pathogens (Alberts et al., 2002). The innate immune system has to provide the first step of protection against many pathogens in living organisms. The innate immune system executes its duty through diverse types of pathogen recognition receptors (PRRs) (Akira et al., 2006). PRRs recognize the pathogens via their conserved molecular structure, and these conserved structures are called pathogen-associated molecular patterns (PAMPs). These patterns show specific differences among gram-negative bacteria, gram-positive bacteria, and viruses (Akira et al., 2006). Therefore, different PAMPs are believed to cause different immune responses. A wide range of PAMPs, such as bacterial cell wall contents and viral nucleotides, are usually ligands for Toll-like receptors (TLR) (Li et al., 2017). The Gram-negative bacteria have an outer membrane covering a thin peptidoglycan inner membrane, and this outer membrane is composed of glycolipids, named lipopolysaccharides (LPSs) (Silhavy et al., 2010). The Gram-positive bacteria do not contain a high amount of LPS in their outer membrane as the gram (-) bacteria; therefore, they have a thicker membrane that contains a high amount of peptidoglycan (PGN) and lipoteichoic acid (LTA) (Silhavy et al., 2010; Pietretti et al., 2014).

The immune system generally senses gram-negative bacteria (Hoshino et al., 1999; Mahla et al., 2013), flagellin-containing bacteria (Kumar et al., 2011; Pietretti et al., 2014; González-Stegmaier et al., 2015), peptidoglycans, (Mahla et al., 2013; Gillis et al., 2008; Zhang et al., 2014), lipoteichoic acid (Pietretti et al., 2014), or soluble extracellular toxins (Tietze et al., 2006), and viral dsRNA (double-stranded RNA) (Pietretti et al., 2014; Alexopoulou et al., 2007; Don et al., 2012; Hu et al., 2017) by various pathways. Recognizing pathogen-associated molecular patterns (PAMPs) by toll-like receptors (TLRs) provides information about the infection type and triggers the immune system to induce the expression of cytokines and chemokines.

Chemokines are chemo-attractants, groups of small cytokines, and they have a crucial role in leukocyte migration in the immune system (Müller et al., 2010; Chen et al., 2013). Chemokines were divided into four sub-family and described in many studies (CC, CXC, CX3C, and XC). Various chemokines were identified in different fish species, and their roles have been characterized to some extent. The role of CXC Chemokine "CXCL10" was newly reported on *Paralichthys olivaceus*, a Japanese flounder (Li et al., 2022). It is revealed that CXCL10 induces immune cell migrations in Japanese flounder as in mammals, such as natural killer cells (NKCs), dendritic cells, T cells, and macrophages (Bonecchi et al., 1998). CXCL9 is secreted by T lymphocytes, Natural Killer (NK) cells, dendritic cells, macrophages, eosinophils, and by some non-immune cells such as liver astrocytes, preadipocytes, thyroid, stromal cells, endothelium, tumor cells, and fibroblasts (Cole et al., 1998; Tensen et al., 1999; Ding et al., 2016; Metzemaekers et al., 2018). The role of CXCL9 is known to accumulate γδT (gammadeltaT) cells in mammals that start an autoimmune response to infections, and it is controlled by CXCL9/CXCR3 axis-dependent mechanisms (Valdés et al., 2022).

In this study, we examined CXCL9 chemokine and CXCL10 chemokine mRNA expression levels on various tissues of Japanese flounder induced by various immunostimulants. Poly I:C was used for the viral mimic, formalin killed cell (FKC) of *Streptococcus iniae* was used for the gram-positive bacterial infection mimic, and FKC of *Edwardsiella tarda* was used for a gram-negative bacterial infection mimic.

2. MATERIALS AND METHODS

2.1. cDNA cloning and characterization of *JfCXCL9_L* and *JfCXCL10_L* genes

A sequence was used for structural analysis of the Japanese flounder CXCL10 Like (*JfCXCL10_L*) gene from RNA sequence data generated by NGS. Specific primers shown in "Table 1" were designed for PCR confirmation of coding sequences of this gene. The sequence was amplified using RT-PCR, and the *E. tarda* FKC-treated Japanese flounder spleen cDNA library was used as a template. The sequences were amplified with the following PCR conditions: 5 min denaturation step at 95 °C, followed by 30 cycles of denaturation at 95 °C for 30 s, annealing at 55 °C for 30 s, and 1 min of extension at 72 °C, and final elongation step at 72 °C for 5 min. Two target genes were detected with 1% EtBr agarose gel electrophoresis, and sequences were confirmed by an automated DNA sequencer ABI 3230 X1 (Applied Biosystems, USA). Then fragments were ligated into the pGEM-T Easy vector (Promega, USA). Plasmids were transformed into *E. coli* competent cell JM109 (Wako, Japan) after ligation. The clones containing the full-length insert of *JfCXCL9_L* and *JfCXCL10_L* were amplified by the colony PCR and verified again by the automated DNA sequencer ABI 3230 X1 (Applied Biosystems, USA). These sequences were used for multiple alignments, phylogenetic tree construction, and further analysis. The tool from NCBI "ORF finder" (https://www.ncbi.nlm.nih.gov/orffinder/) was used to determine the open reading frame of cloned DNA fragments, and amino acid sequences were used for the subsequent analysis.

Predicted signal peptides were detected with the program "SignalP 4.1" (http://www.cbs.dtu.dk/services/SignalP). The NCBI tool BLAST (Basic Local Alignment Search Tool) was used to determine amino acid sequence similarities of *JfCXCL9_L* and *JfCXCL10_L* with the other known CXC Chemokine genes (Accession numbers shown in the phylogenetic tree, Figure 3) (https://blast.ncbi.nlm.nih.gov/Blast.cgi). Theoretical molecular weight and isoelectric points were predicted with ExPASy (http://web.expasy.org/ compute_pi/). Unipro UGENE v1.25.0 program (Unipro, Russia, 2017) was used for multiple sequence alignment and constructing a phylogenetic tree with the Neighbor-Joining method (10000 replications of bootstrap). Sequences are accessible on NCBI GenBank via following accession numbers, PP340386 for *JfCXCL9_L* and PP340387 for *JfCXCL10_L*.

2.2. 0h tissue distribution and expression of *JfCXCL9_L* and *JfCXCL10_L* genes

2.2.1. Experimental Fish

Fifty individuals of Japanese flounders with an average total length of 7 cm were reared at the Tokyo University of Marine Science and Technology in Japan. The water temperature was kept at 19 - 20 °C for a week before starting to experiment. Fish were fed *ad libitum* with dry commercial pellets once a day for a week. Four tanks were filled with 200 L of continuously filtered artificial seawater, and 12 fish were placed into each tank to represent different experimental groups. An appropriate amount of aeration was provided for each tank, and salinity was kept at 28 ppt at all tanks.

2.2.2. Preparation of immuno-stimulants and tissue sampling

Formalin-killed cells (FKC) of *S. iniae* strain 02 and *E. tarda* strain 54 were used to stimulate the immune system of Japanese flounders. *S. iniae* FKC and *E. tarda* FKC were prepared according to the previous study (Dumrongphol et al., 2009). Poly I:C was dissolved in DEPC-treated water to obtain a final concentration of 1 mg/1 ml.

All fish were injected intraperitoneally with a dosage of 100 µl per fish. Fish were injected with *S. iniae* FKC ($1x10^{10}$ CFU/ml), *E. tarda* FKC ($5x10^{8}$ CFU/ml), Poly (I:C) (1 mg/ml) and PBS as a negative control.

2.2.3. Tissue sampling

Eight tissues (kidney, spleen, intestine, liver, stomach, muscle, heart gill) were collected from 4 fish to construct a 0h (untreated) cDNA library of Japanese flounder. These tissues were used to investigate the tissue distribution of target genes. Three fish were collected from all stimulated groups at 3 hours, 24 hours, and 72 hours post-injection and were killed for tissue sampling. The spleen of each fish was collected into RNALater to construct the cDNA library.

2.2.4. Construction of Japanese flounder cDNA library

RNAiso Plus (Takara Bio, Japan) was used to extract total RNA from all samples, and RNA was purified with an RNeasy® Mini Kit (Qiagen, USA). Purified RNA concentration was measured with Nanodrop Lite Spectrophotometer (Thermofisher Scientific, Japan), and all samples were adjusted to 1000 ng per 20 µl of reaction. A high-capacity cDNA Reverse Transcription Kit (Applied Biosystems,

USA) was used to reverse transcribe the RNA samples. All procedures were performed following the manufacturer's instructions.

2.2.5. RT-PCR, Quantitative Real-Time PCR (qRT-PCR), and statistical analysis

qRT-PCR experiments started after the target length of amplicons was confirmed with RT-PCR. Briefly, *JfCXCL9_L* and *JfCXCL10_L* genes amplified from constructed spleen cDNA library of Japanese flounder with P1 and P2 primer sets, respectively. P3, P4, P5, and P6 primer sets were used for qPCR analysis (Table 1), while P5 and P6 were used for control purposes. SYBR Green PCR master mix (Applied Biosystems) used for qPCR on StepOnePlus™ Real-time PCR system (Applied Biosystems) according to manufacturer's protocols. EF1α was used as an internal control, and all target genes' mRNA expression levels were normalized with EF1α mRNA expression levels. Afterward, the $2^{-\Delta\Delta Ct}$ method was used to calculate all mRNA expression levels. Differences in expression levels between the untreated (0h) and the other groups (3h, 24h, and 72h) were determined with a t-test. Statistical analysis was done with R Studio. Differences were considered significant, while p-values were smaller than 0.05.

Table 1. Oligonucleotide Primers used in this study.

	Primer Names	Forward primer (5 - 3)	Reverse primer (5 - 3)
P1	*JfCXCL9_like*	GAACGGCTCAGAGTCAACGC	GGTGGCCTGGAGTTAAACACC
P2	*JfCXCL10_like*	CTACCCCTCACAGTGCTCTG	TCACTGAAGGTGCTTTCGTTAG
P3	*JfCXCL9_L_qPCR*	ATGCAGCTTTACCCCCAGTC	TCTTTCAGGTGCCCTCTGATTC
P4	*JfCXCL10_L_qPCR*	TCAAAGTGCTCCTGCTCCTG	CAGAAATTGGTTGCCGGGTAG
P5	*JfIL1β_qPCR*	CAGCACATCAGAGCAAGACAACA	TGGTAGCACCGGGCATTCT
P6	*JfEF1α_qPCR*	CTCGGGCATAGACTCGTGGT	CATGGTCGTGACCTTCGCTC

3. RESULTS AND DISCUSSION

3.1. cDNA Cloning and characterization of *JfCXCL9_L* and *JfCXCL10_L* genes

cDNA sequence of *JfCXCL9_L* and *JfCXCL10_L* genes were successfully amplified with designed primers and cloned into the competent cell. The cloned cDNA sequence of *JfCXCL9_L* showed that it has a 539 bp length, including a 5'-untranslated region (UTR) of 36 bp, 405 bp of ORF encoding 134 amino acids, and a 3'-UTR of 98 bp. The cloned cDNA sequence of JfCXCL10 showed a 495 bp length, including a 5'-UTR of 122 bp and 285 bp of ORF encoding 94 amino acids acid and a 3'-UTR of 91 bp. These sequences were used for the following bioinformatical analysis. Amino acid sequences derived from the NCBI BLAST database showed that CXCL9 and CXCL10 chemokines have a predicted signal peptide at the N terminus. *JfCXCL9_L and JfCXCL10_L* genes have 28aa and 20aa lengths of a predicted signal peptide, respectively. Predicted isoelectric points of *JfCXCL9_L and JfCXCL10_L* genes were 10.71 and 9.62; molecular weights were 15.36401 and 10.28315 kDa, respectively.

The structural classification of Chemokines is based on their cysteine residues in amino acid sequences. Typically, chemokines comprise four conserved cysteine residues in mammalians, and the first two of these residues specify the subfamily of chemokines. Multiple alignments of several fish species and mammalians showed conserved cysteine residues (Figure 2). Six cysteine residues were found in the *JfCXCL9_L* gene, which is conserved among other fish species, but the mammalians have four conserved cysteine residues (Figure 1). The highest ident match of 100% was observed with the predicted nucleotide sequence of "Predicted: *Paralichthys olivaceus C-X-C motif chemokine 10-like (LOC109629332), mRNA*" (XM_020087031.1, NCBI, 2023). On the other hand, the cloned sequence was also matched (99.75%) with "*Paralichthys olivaceus chemokine CXCL9 (CXCL9) mRNA, complete cds*" (KU821020.1). Hence, further characterization analysis of this sequence revealed that this mRNA is coding the CXCL9 gene in Japanese flounder.

JfCXCL10_L gene in Japanese flounder was found to have four cysteine residues, and they were conserved among the other fish species and mammalians (Figure 2). A BLAST search for nucleotide sequence resulted in the highest ident of 100% was observed with the predicted sequence of "*Paralichthys olivaceus C-X-C motif chemokine 10-like (LOC109624870), mRNA*" in NCBI database (XM_020079790.1, NCBI, 2023).

The phylogenetical analysis of amino acid sequences of *JfCXCL9_L* and *JfCXCL10_L* resulted in phylogenetically distinguished clusters between fish and mammalian's CXCL9 and CXCL10 genes (Figure 3).

Figure 1. Multiple sequence alignment of known CXCL9 chemokines with Muscle alignment algorithm by using Unipro UGENE v1.25. All amino acid sequences are derived from the NCBI database with accession numbers shown in the figure. Sequence with underlines indicates sign.

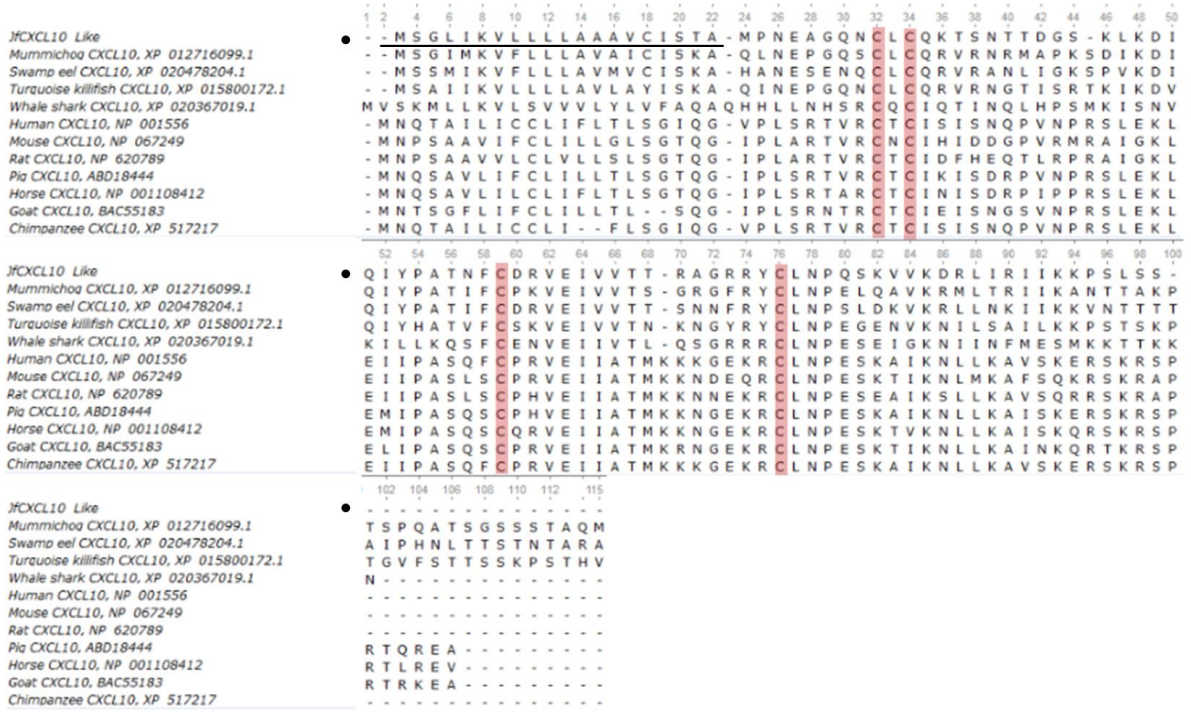

Figure 2. Multiple sequence alignment of known CXCL10 chemokines with Muscle alignment algorithm by using Unipro UGENE v1.25. All amino acid sequences are derived from the NCBI database with accession numbers shown in the figure. Sequence with underlines indicates sign.

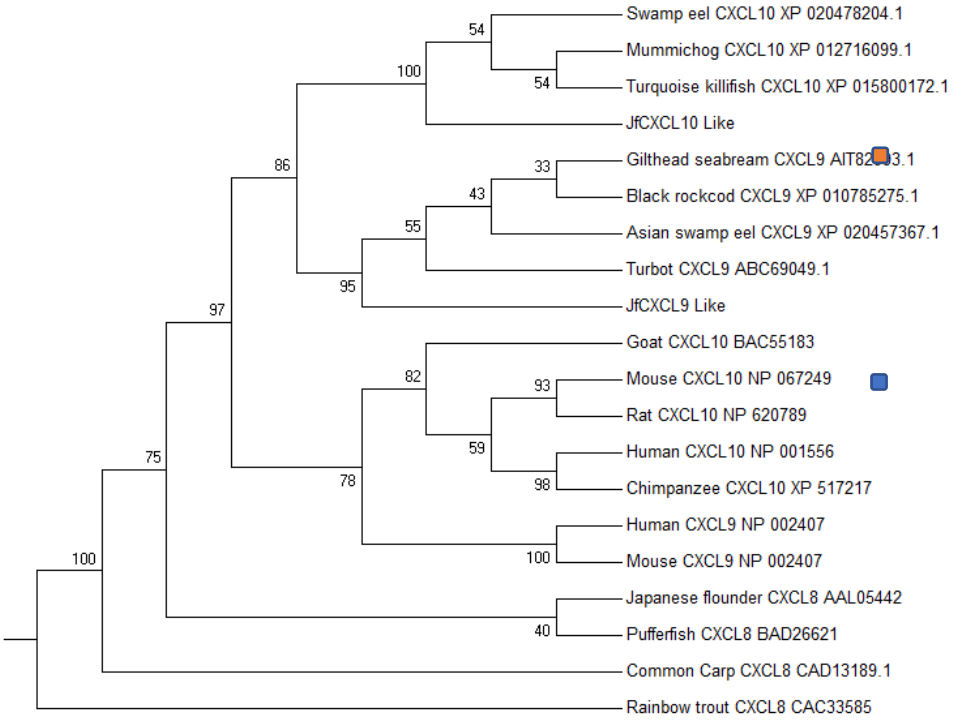

Figure 3. Neighbor-joining phylogenetic tree of CXC chemokines belongs to distinct species (bootstrap: 10000 replications). The numbers at the forks indicate the bootstrap value. Sequences used in the phylogenetic tree are derived from the NCBI database.

3.2. Expression analysis of JfCXCL9_L and JfCXCL10_L genes

3.2.1. RT-PCR analysis of 0h tissue distribution

Japanese flounder kidney, spleen, intestine, liver, stomach, muscle, heart, and gill cDNA libraries from 4 untreated fish were used as 0h templates for RT-PCR assay to determine 0h tissue distribution. Internal control gene *JfEF1α* was detected in all tissues. *JfCXCL9_L* mRNA was detectable at 0h in most of the tissues. Very low brightness was observed in stomach tissues, and muscle tissues showed no band. We must mention that *JfCXCL9_L* mRNA was the brightest in the spleen tissues, indicating that this gene's basal level is possibly related to the immune system. Generally, high basal expression of mRNA levels of a gene in the spleen suggests that the gene is actively transcribed and translated into a protein in the tissue. Given the RT-PCR results, we can assume that *JfCXCL9_L* is related to the passive immune response as mRNA expression is relatively higher in stress axis organs such as the kidney, spleen, and thymus at no-pathogen exposure (Sayed et al., 2022). It is also mentioned that this gene is downregulated after exposure to a pathogen on carp (Pijanowski et al., 2019) and Atlantic salmon (Boison et al., 2019). *JfCXCL10_L* was not detectable in all tissues at 0h by RT-PCR (Figure 4). Conversely to *JfCXCL9_L*, undetected *JfCXCL10_L* mRNAs at 0h suggest that it is part of an active immune response, as it is reported by Li et al (2022).

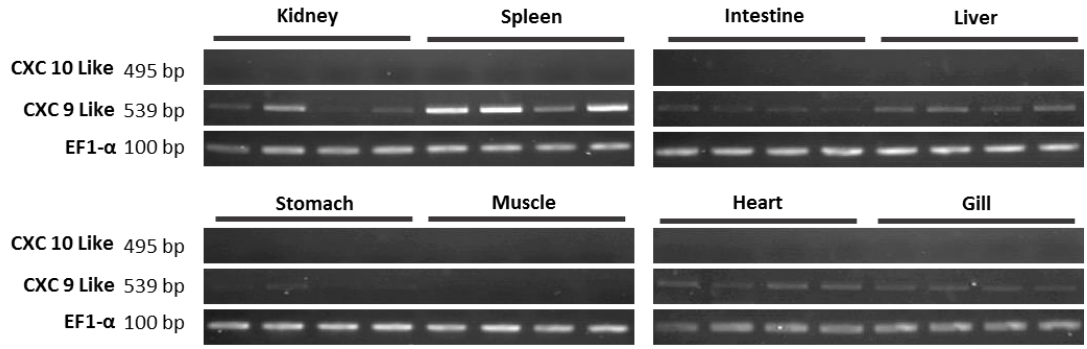

Figure 1. mRNA levels of jfCXCL9_L, jfCXCL10_L, and EF1α genes in different untreated (0h) Japanese flounder tissues. Four individuals of fish were used for each tissue.

3.2.2. qRT-PCR analysis

Spleen cDNAs of Japanese flounders were used to conduct qRT-PCR experiments. Interleukin-1 beta (*IL1-β*) mRNA levels were used to compare expression levels of *JfCXCL9_L* and *JfCXCL10_L* mRNAs. mRNA expression levels of three genes at 0h, 3h, 24h, and 72h in the spleen samples were investigated after injection of PBS, Poly I:C, *E. tarda* FKC (FKC_{ET}), and *S. iniae* FKC (FKC_{SI}) treatments by qRT-PCR.

IL1-β is a cytokine that plays an important role in innate immune response in teleost. *IL1-β* is reported to be primarily secreted by monocytes/macrophages, and the immune response usually includes inflammation, phagocytosis, and the activation of other immune cells. It was reported that the *IL1-β* is upregulated in teleost monocytes/macrophages after Lipopolysaccharide (LPS) stimulation, mimicking gram-negative bacterial infections. Upregulation of *IL1-β* against gram-negative bacteria *E. tarda* and gram-positive *S. iniae* was the expected treatment outcome (Kondo et al., 2014). *IL1-β* upregulation after Poly I:C treatment is also reported in Japanese flounder (Zhou et al., 2014). This study examined *JfIL1-β* mRNA expression levels to determine if the innate immune response was activated in all treated groups. There was more than 5-fold upregulation after all treatments, and upregulation was rapid in Poly I:C and FKC_{ET} treatment. However, FKC_{SI} treatment has resulted in relatively slow upregulation (Figure 5). These results showed that our treatments were successfully performed in this study.

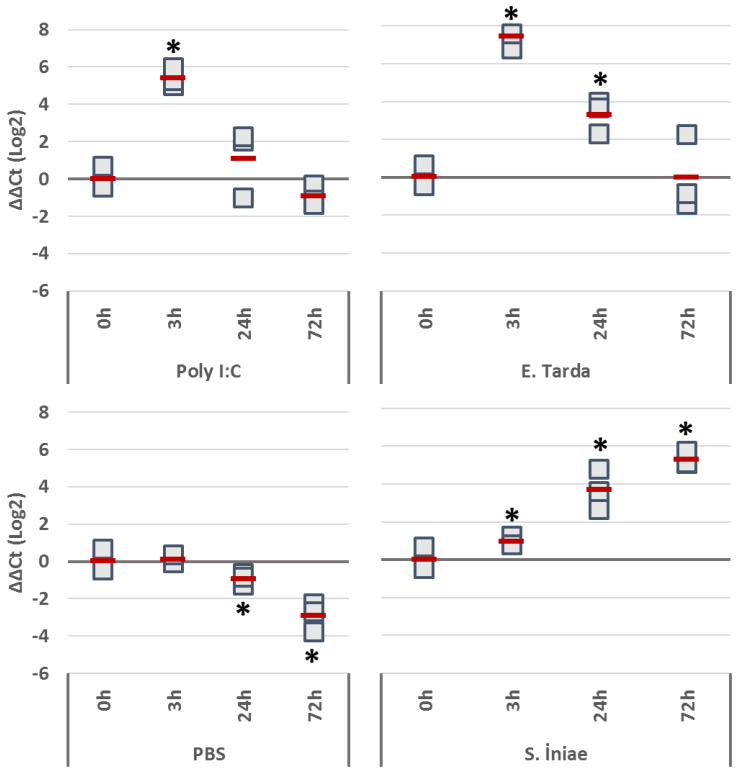

Figure 5. Quantitative RT-PCR results represent mRNA expression levels of ***IL1 β*** under different conditions. Lines on the treatment groups represent the average values of groups. Asterisk (*) indicates statistically significant differences ($p<0,05$) against 0h mRNA expression levels.

JfCXCL10_L mRNA levels were similar to *IL1- β* expressions after Poly I:C and FKC_{ET} stimulations. FKC_{SI} stimulation has resulted in downregulated Jf*CXCL10_L* mRNA of the Japanese flounder. Zhu et al. (2021) reported various TLR (*PoTLR*) gene mRNA expression levels stimulated by FKC_{ET}. It was shown that *PoTLR* expressions were dependent on tissue and time. *PoTLR1, 5S, 5M, 14, 21,* and *22* mRNA expression levels were significant in the spleen. Moreover, the *PoTLR22* mRNA expression pattern seems similar to our JfIL1- β and JfCXCL10_L results. Apparently, the significance of upregulation against only Poly I:C and FKC_{ET} reveals that *JfCXCL10_L* is possibly included in the immune response to viral and gram-negative bacterial stimulations in Japanese Flounder.

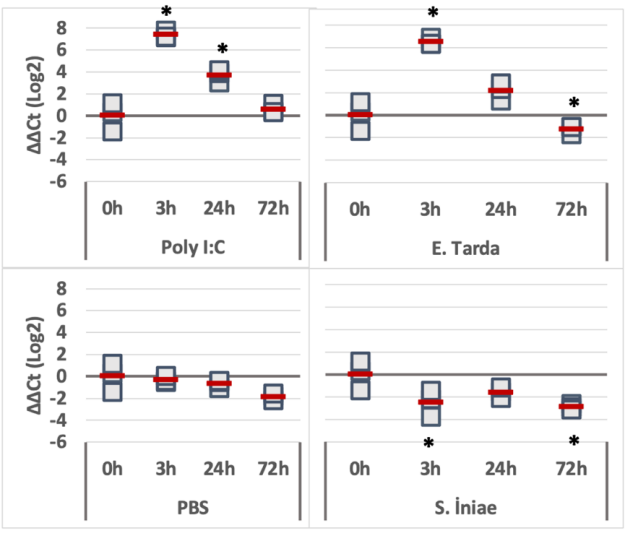

Figure 6. Quantitative RT-PCR results represent mRNA expression levels of ***JfCXCL10_Like*** under different conditions. Lines on the treatment groups represent the average values of groups. Asterisk (*) indicates statistically significant differences (p<0,05) against 0h mRNA expression levels.

JfCXCL9_L mRNA expression levels were significant only against FKC_{ET} and FKC_{SI}. In both cases, *JfCXCL9_L* mRNA was downregulated to some extent. They were also significantly low after 24h at FKC_{ET} and 72h at FKC_{SI}. However, we have found that at the time points of 3h and 24h, mRNA levels after PBS, FKC_{ET}, and FKC_{SI} groups showed slightly lower levels of mRNA than the Poly I:C treatment. All groups showed similar expression patterns at the 72h post-injection. However, *JfCXCL9_L* was also detectable at one of three 0h cDNA libraries by RT-PCR. This gene was found to contribute to antimicrobial protection of the gut during infection by *Citrobacter rodentium* in mice (Reid-Yu et al., 2015). They demonstrated the role of CXCL9 in protecting gut mucosa was not dependent on an adaptive immune response or immunological receptor, CXCR3. These findings also explain the downregulation and 0h tissue distribution of *JfCXCL9_L*. Downregulation of genes generally occurs after they finish their duty or after they bind to correlated receptors (CXCR3) (Fulkerson & Rothenberg, 2006). Another approach that can be made is the suppression of *JfCXCL9_L* (Reid-Yu et al., 2015). In both cases, our results suggest that *JfCXCL9_L* chemokine in Japanese flounder needs further studies for advanced characterization.

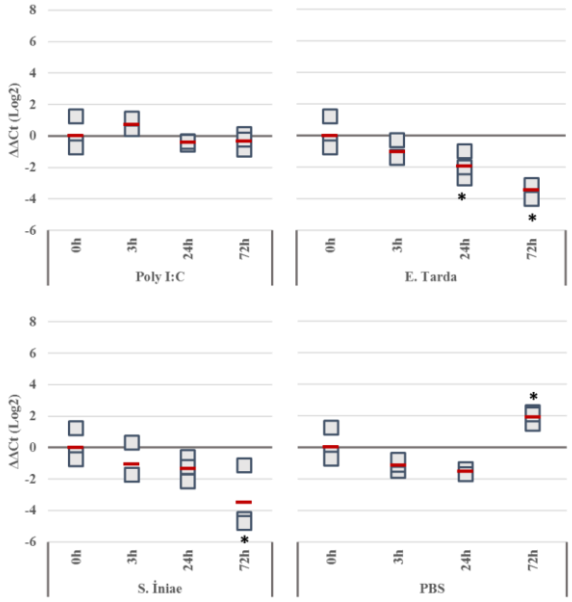

Figure 7. Quantitative RT-PCR results represent mRNA expression levels of ***JfCXCL9_Like*** under different conditions. Lines on the treatment groups represent the average values of groups. Asterisk (*) indicates statistically significant differences (p<0.05) against 0h mRNA expression levels.

CONCLUSION

In conclusion, our study successfully cloned and sequenced the cDNA of *JfCXCL9_L* and *JfCXCL10_L* genes in Japanese flounder. Furthermore, qRT-PCR experiments demonstrated that *JfCXCL10_L* expression mirrored that of *IL1-β*, with upregulation after Poly I:C and FKC_{ET} treatments and downregulation after FKC_{SI} treatment. This suggests a role for *JfCXCL10_L* in the immune response to viral and gram-negative bacterial stimuli. *JfCXCL9_L* mRNA levels were significant after FKC_{ET} and FKC_{SI} treatments, showing downregulation to some extent. Notably, at specific time points, *JfCXCL9_L* levels were lower compared to Poly I:C treatment. These findings shed light on the immune response mechanisms and potential functions of *JfCXCL9_L* and *JfCXCL10_L* in Japanese flounder.

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CONFLICT OF INTEREST

The authors declare that there is no conflict of interest.

AUTHOR CONTRIBUTION

Planning the study: HK, EB; Literature: EB; Methodology: IH, HK, EB; Performing the experiments: EB; Data analysis: EB; Writing: EB; Supervision: IH, HK. All the authors approved the final draft.

ETHICAL APPROVAL STATEMENTS

All animal experiments were conducted at the laboratories of TUMSAT (Tokyo, Japan, 2017) according to the guideline issued by the Ministry of the Environment, Japan, and the Tokyo University of Marine Science and Technology's Regulations on the Handling of Animal Experiments.

DATA AVAILABILITY STATEMENT

The data available from the corresponding author upon a reasonable request

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