



Infection of *Enteromyxum leei* in cultured starry flounder *Platichthys stellatus*

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Abstract

Enteromyxum leei has been identified as the causative agent of emaciation disease in a wide range of marine fish hosts. In this study, we aimed to determine the effect of the parasitic infection of *Enteromyxum* species on starry flounder that were cultured in aquaculture farms of Jeju island in Korea. As the mortality of cultured olive flounder *Paralichthys olivaceus* because of *E. leei* infection increased, some fish farms on Jeju island attempted to culture the starry flounder *Platichthys stellatus*, as an alternative. Myxosporeans with a developmental stage similar to *E. leei* were found in the intestines of cultured starry flounders. The partial 18S rDNA of myxosporeans showed 100% similarity with *E. leei*. To reveal the effect of *E. leei* infection on starry flounder, the intensity of *E. leei* infection measured using quantitative polymerase chain reaction, and the condition factor (CF) of fish were measured and analyzed statistically. The results showed that high-intensity *E. leei* infection significantly decreased the CF of the starry flounder. However, the pathogenicity of *E. leei* to starry flounder is low, considering its mortality and clinical signs.

Keywords: *Enteromyxum leei*, *Platichthys stellatus*, Emaciation disease, Starry flounder

Introduction

Myxozoans are microscopic endoparasites that belong to the phylum Cnidaria; they complete their life cycles in vertebrate (intermediate hosts; mainly fish) and invertebrate (definitive hosts; annelids or bryozoans) hosts (Chang et al., 2015; Lom & Dyková, 2006). Myxozoans comprises more than 2,000 described species, several of which can cause diseases in economically important fish (Lom & Dyková, 2006). Notably, emaciation disease caused by myxosporean infection (*Enteromyxum leei*, *Enteromyxum scophthalmi*, and *Sphaerospora fugu*) causes mass mortality in important aquaculture fish worldwide (such as olive flounder

Paralichthys olivaceus, gilt-head sea bream *Sparus aurata*, tiger puffer *Takifugu rubripes*, and turbot *Scophthalmus maximus*) (Diamant, 1992; Ogawa & Yokoyama, 2001; Palenzuela et al., 2002; Sekiya et al., 2016; Tun et al., 2002).

The starry flounder *Platichthys stellatus* of the family Pleuronectidae inhabits the coastal waters of the Pacific and Arctic oceans, and the East Sea of Korea (Kang et al., 2012; Orcutt, 1950). Since the inception of aquaculture of starry flounder began in the early 2000s, it has become a candidate marine flatfish for aquaculture in Korea as it can feed at low temperatures and is disease-resistant (Han et al., 2019; Kim et al., 2019; NIFS, 2019). Moreover, some fish farms in Jeju have attempted to culture starry

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flounder instead of olive flounder as the mortality of cultured olive flounder by emaciation disease has increased recently (Shim et al., 2019). The Marine Science Research Institute of Jeju National University monitored the parasitic infections of olive flounder and starry flounder that are cultured on Jeju island and found *Enteromyxum* species in starry flounder intestines. The present study aimed to identify *Enteromyxum* species and determine the effect of the parasitic infection of these species in cultured starry flounder.

Materials and Methods

Fish samples and parasitological examination

Ten starry flounder individuals ($n = 20$, total length: 22.9 ± 2.0 cm; body weight: 183.8 ± 79.4 g) were obtained from a starry flounder farm on September 16 and November 10, 2022, respectively. Upon arrival to Marine Science Research Institute, the 20 individuals were administered at lethal over dose of Tricaine Methanesulfonate (Syndel, Ferndale, WA, USA), and wet smears of fresh gill, skin, and intestinal scrapings were examined using a light microscope. The kidney and liver tissues were used to isolate the bacteria, which were then incubated on a brain-heart infusion agar plate (BHIA) supplemented with 1% NaCl at 25 °C for 48 h.

Molecular identification of *Enteromyxum* species and detection by polymerase chain reaction (PCR)

Twenty samples of 50–100 mg of intestinal tissue were collected in microtubes and stored at -20°C . DNA was extracted directly from the samples using the QIAamp DNA Mini Kit (Qiagen, Hilden, Germany) following the manufacturer's protocol and was eluted in 100 μL AE buffer. We amplified parts of the 18S rDNA by polymerase chain reaction (PCR) using a combination of primers (Table 1) designed in previous studies (Barta et al., 1997; Hillis & Dixon, 1991; Sekiya et al., 2016). In addition, *E. leei* was detected by PCR using primers specific for *E. leei* (Sekiya et al.,

2016) (Table 1). The PCR comprised the following steps: initial denaturation at 95 °C for 5 min, followed by 35 cycles at 95 °C for 30 s, 58 °C for 30 s, and 72 °C for 30 s, and a final extension at 72 °C for 7 min. PCR products were treated with the AccuPrep Genomic PCR Purification Kit (Bioneer, Daejeon, Korea) to remove excess primers and dNTPs and directly sequenced using BigDye™ Terminator v3.1 in an ABI 3730xl Sequencer. BLASTN analysis was performed using National Center for Biotechnology Information database based on the 18S rDNA sequence of the isolate.

Condition factor (CF) and quantitative polymerase chain reaction (qPCR)

The total length and body weight of the fish were measured to calculate the condition factor ($\text{CF} = \text{body weight} \times 100 / [\text{total length}]^3$). To investigate the effect of the intensity of *E. leei* infection on the CF of starry flounders, quantitative PCR (qPCR) was carried out as the previously described (Sohn et al., 2021). The qPCR was performed in a 10 μL total reaction mixture containing 3 μL of template, 0.5 μL of each primer (Table 1) (10 pmol/ μL), 5 μL of $2 \times$ TaKaRa Ex Taq™ SYBR Premix (TaKaRa, Shiga, Japan) and 1 μL of nuclease-free water. The thermal profile was as follows: 95 °C for 30 s, followed by 45 cycles at 95 °C for 5 s and 60 °C for 30 s.

Statistical analyses

Statistical analyses were performed using the SPSS 16.0 software (version 16.0; SPSS, Chicago, IL, USA). The relationship between CF of starry flounder and the intensity of *E. leei* infection were investigated using regression analysis and one-way analysis of variance. Differences were considered statistically significant at $p < 0.05$.

Results

Detection and identification of *Enteromyxum* species

No parasitic infection was observed in the skin and gill samples;

Table 1. Oligonucleotide primers used for molecular identification and quantitative PCR in this study

Purpose	Target	Name	Sequence (5'-3')	Forward/Reverse	References
Identification	18S rRNA	18e	CTGGTTGATCCTGCCAGT	Forward	Hillis & Dixon, 1991
	18S rRNA	EL-F	GGATATTGTCAGAGTATGTT	Forward	Sekiya et al., 2016
	18S rRNA	mEL-R	AGAAGCCAACGTATATGATTA	Reverse	Sekiya et al., 2016
	18S rRNA	ERIB10	CTTCCGCAGGTTCCACCTA	Reverse	Barta et al., 1997
Quantitative PCR	28S rRNA	EL-qF	ACTCGGCTATGTGGGCAGTG	Forward	Sohn et al., 2021
	28S rRNA	EL-qR	ATTGTTAATTGCTAGTCGTAAGAGCAAG	Reverse	Sohn et al., 2021

PCR, polymerase chain reaction.

however, many primary cells (including accompanying cells) similar to the developmental stage of *E. leei* were found in the intestine samples (Fig. 1A). All the fish examined had no gross lesions, regardless of whether parasites were detected (Fig. 1B and 1C). Microscopic investigation showed that the detection rate for *Enteromyxum* species in the intestinal samples was 20% and 70% for samples obtained in September and November, respectively. Partial sequences of 18S rDNA (1,680 bp) were obtained from *Enteromyxum* species and deposited in GenBank (accession number OQ130171). The sequences showed 100% (1,680/1,680) nucleotide identity with the 18S rDNA (accession number MF161396) of *E. leei*. The PCR results (with primers specific for *E. leei*) showed 50% and 100% detection in September and November, respectively. No bacteria grew on BHIA plates from the kidney and liver specimens.

Relationship between intensity of *Enteromyxum leei* and condition factor (CF) of starry flounder

The CF of starry flounders investigated ranged from 1.20 to 1.82 and the cycle of quantification (Cq) value showed 12.2 to 34.1. As shown in Fig. 2 and Table 2, the CF was positively associated with Cq value (Fig. 2 and Table 2). Based on the Cq value, infection intensity was divided into three parts (high, moderate, low or

negative). The mean CF in high infection intensity of parasites (Cq value: less than 23; more than 4.5×10^5 copies/100 mg) group is 1.36. The mean CF in moderate infection intensity of parasites (Cq value: 23–27; 4.5×10^5 – 2.1×10^4 copies/100 mg) group is 1.47, whereas the mean CF in low or negative (Cq value: more than 27; less than 2.1×10^4 copies/100 mg) group is 1.63 (Table 3).

Discussion

Recently, there has been a change in the aquaculture industry of Jeju island, Republic of Korea. The production of olive flounder decreased from 26,093 tons in 2016 to 21,463 tons in 2021, whereas the production of starry flounder increased from 446 to 2,610 tons between this period. The proportion of starry flounder production in 2021 accounted for approximately 10% of the total aquaculture production on Jeju island (KOSTAT, 2017; KOSTAT, 2022). This change can likely be attributed to the mass mortality of cultured olive flounder due to diseases (in particular, emaciation disease). Unfortunately, to date, there are no effective chemotherapeutics or vaccines against emaciation disease.

The mortality associated with emaciation disease of cultured olive flounder caused by *E. leei* shows seasonal changes in Korea. The mortality begins in September, and reaches its highest mortality

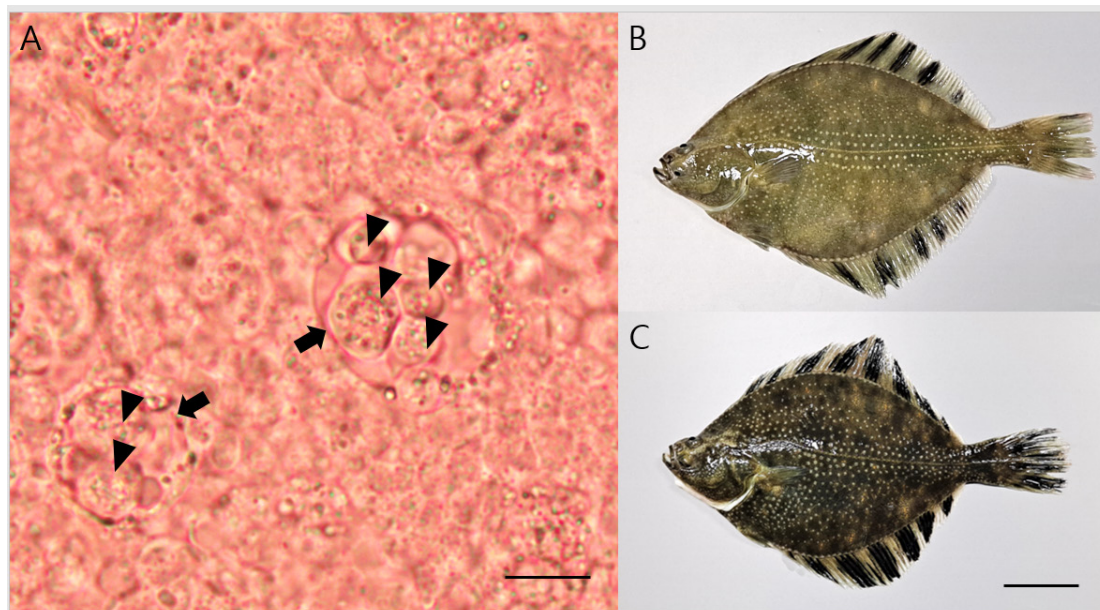


Fig. 1. Developmental stage of myxosporeans found in cultured starry flounder *Platichthys stellatus*. A: Primary (arrows) and accompanying (arrowheads) cells in intestinal scraping (scale bar = 10 μm). B: Not infected starry flounder. C: Infected starry flounder (scale bar = 5 cm).

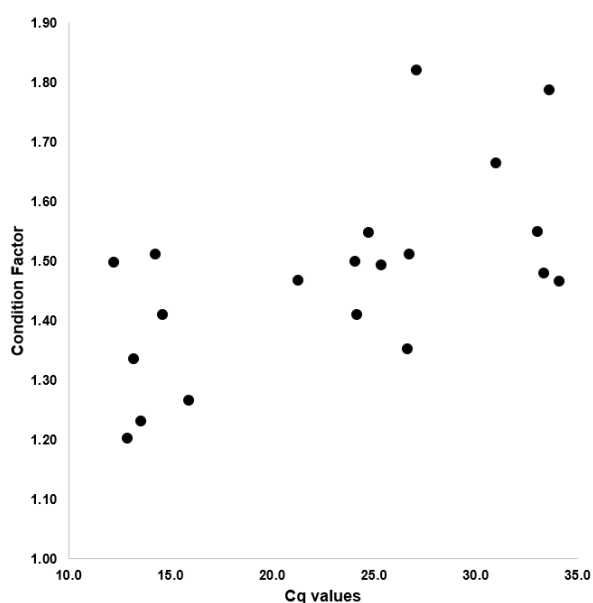


Fig. 2. Difference in condition factor of cultured starry flounder depends on Cq values. Cq, cycle of quantification.

rate from October to November, and decreases December onwards (Shin et al., 2018). The intensity of *E. leei* infection and the decrease in the relative CF in the olive flounder corresponded to these fluctuation (Sohn et al., 2021). In the present study, the detection rate of *E. leei* in cultured starry flounder also shows that of the similar fluctuation. Thus, we speculate that the onset of *E. leei* infection occurred at approximately the same time in cultured starry flounder and cultured olive flounder. However, *Enteromyxum* species require approximately two months to reach the developmental stage in the intestines after the initial onset of a natural infection (Sohn et al., 2021); hence, the infection of *E. leei* in cultured starry flounder may have likely begun in July.

Based on culturing experience, the members of fish farms in Jeju Island believed that the starry flounder are more resistant to emaciation disease than olive flounder. Although *E. leei* has been reported in more than 50 species of fish (Yanagida, 2017), there are no reports on starry flounder yet. Based on the results of the present study, the prevalence and intensity of *E. leei* in starry flounder were not lower than those in olive flounder that were infected naturally or experimentally. However, there was a clear

Table 2. Regression analysis summary for intensity of *Enteromyxum leei* infection (Cq value) affect condition factor of starry flounder

Variable	Unstandardized coefficients		Standardized coefficients	t-value	F-value	R^2
	B	SE	β	(p-value)	(p-value)	
(Constant)	1.185	0.092		12.809 (0)*	10.889 (0.004)*	0.377
Cq value	0.013	0.004	0.614	3.300 (0.004)*		

* $p < 0.05$.

Cq, cycle of quantification.

Table 3. Relationship between mean condition factor and intensity of *Enteromyxum leei* infection in starry flounder

Intensity of <i>E. leei</i> infection (qPCR result)	Condition factor					
	n	Mean	SD	p-value	F-value	Scheffe
High (Cq value: less than 23) ^a	8	1.36	0.12	a:ab = 0.106	8.045	b > a
Moderate (Cq value: 23–27) ^{ab}	6	1.47	0.07	ab:b = 0.310		
Low or negative (Cq value: more than 27) ^b	6	1.63	0.15	b:a = 0.003*		

* $p < 0.05$.

qPCR, quantitative polymerase chain reaction; Cq, cycle of quantification.

difference in pathogenicity. The intensity of *E. leei* infection had a significant relationship with the CF of starry flounder, and the group with high infection intensity (Cq value: less than 23) of parasites had a significantly low CF compared to the group with low or negative (Cq value: more than 27). However, the difference in CF by the infection intensity of *E. leei* was a mean of more than 20%–30% in olive flounder (Shin et al., 2019, Shin et al., 2022; Sohn et al., 2021), whereas the mean was 16.6% in starry flounder. In addition, clinical signs of *E. leei* infection such as sunken eyes, fragile and semi-transparent intestine filled with mucous liquid, liver atrophy, and redness were not observed (data not shown). The mortality of cultured starry flounder (by *E. leei*) was also very low in the fish farm, and the feed intake did not decrease.

We suggest three possible explanations for the difference in pathogenicity 1) The difference of pathogenicity of *E. leei* depends on the host fish species. Previous studies have reported high pathogenicity in olive flounder, tiger puffer, sharpnose sea bream *Diplodus puntazzo*, and low pathogenicity in gilt-head sea bream, red sea bream *Pagrus major*, and European sea bass *Dicentrarchus labrax* (Sitjà-Bobadilla & Palenzuela, 2012; Yanagida, 2017; Yanagida et al., 2008). The exact reason for the difference in pathogenicity depending on fish species has not been clarified; however, the difference in the development of *E. leei* depends on fish species or the genetic resistance of fish species (and strain) against myxosporean infection have been reported (Baerwald et al., 2011; Bartholomew, 1998; Sitjà-Bobadilla et al., 2007; Yanagida, 2017; Yanagida et al., 2004; Yasuda et al., 2005). 2) Co-infection with pathogenic bacteria and *E. leei*: some pathogenic bacterial infections (Genera *Edwardsiella*, *Vibrio*, and *Streptococcus*) from emaciated olive flounders have been reported (Choi et al., 2012; Kim et al., 2015; Yasuda et al., 2005), and the co-infection with *Edwardsiella piscicida* affected the pathogenicity of *E. leei* (Shin et al., 2022). No bacteria were isolated from the kidney and liver of starry flounder; however, previous studies have reported pathogenic bacteria (*Photobacterium damsela* subsp. *piscicida* and *Streptococcus parauberis*) in cultured starry flounder in Korea (Cho et al., 2008; Cho et al., 2013). Thus, further studies needed to reveal how pathogenic bacterial infections affect the pathogenicity of *E. leei* in starry flounders. 3) Acquired immunity: the starry flounders investigated in the present study were introduced to the fish farm in May 2021. Thus, the fish may have already been infected in the summer-autumn season of 2021 and acquired immunity. Fish that acquired immunity against *E. leei* infection were resistant to emaciation disease by *E. leei* (Picard-Sánchez et al., 2019; Yanagida et al., 2008).

Conclusion

The infection of *Enteromyxum* species was detected in cultured starry flounder and was identified as *E. leei*. Based on the statistical analyses, we revealed that high-intensity *E. leei* infection significantly decreased the CF of starry flounder. As the production of starry flounder and mortality due to emaciation disease in Jeju island increase, further studies (such as the factors that affect the pathogenicity of *E. leei* on starry flounder, host immunity, the vaccines, and chemotherapeutics against *E. leei* infection) are needed.

Competing interests

No potential conflict of interest relevant to this article was reported.

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Availability of data and materials

Upon reasonable request, the datasets of this study can be available from the corresponding author.

Ethics approval and consent to participate

This article does not require IRB/IACUC approval because there are no human and animal participants.

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