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Quantitative analysis of myxosporean parasites (*Enteromyxum leei, E. fugu* and *Parvicapsula anisocaudata*) detected from emaciated marine fish in Korea

YOUNG-JUHN LEE

DEPARTMENT OF MARINE LIFE SCIENCES

GRADUATE SCHOOL
JEJU NATIONAL UNIVERSITY

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Young-Juhn Lee
(Supervised by professor Jun-Bum Jeong)

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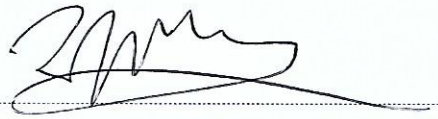
This thesis has been examined and approved

Thesis director, In-Kyu Yeo, Prof. of Department of Life Marine Science,
Jeju National University

Lyu-Jin Jun, Ph D. of Department of Life Marine Science,
Jeju National University



Joon-Bum Jeong, Prof. of Department of Life Marine Science,
Jeju National University

Date

Department of Marine Life Sciences
GRADUATE SCHOOL
JEJU NATIONAL UNIVERSITY

CONTENTS

Summary

Quantitative analysis of myxosporean (*Enteromyxum leei* and *Parvicapsula anisocaudata*) were performed using real-time PCR on the internal organs (head kidney, body kidney, intestine, spleen, brain, liver, heart, muscle, blood, and eye) of emaciated olive flounder, *Paralichthys olivaceus* from farm-A. The highest DNA copy number of *E. leei* was shown in the intestine (1.3×10^8 copies/mg tissue) of emaciatied *P. olivaceus* and DNA copy number in the other internal organs (1.3×10^3 - 4.6×10^5 copies/mg tissue) showed lower than intestine. From the result of real-time PCR for *P. anisocaudata,* it was considered mildly infected, due to the low DNA copy numbers of the head kidney (1.3×10^3 copies/mg tissue) and body kidney (9.1×10^3 copies/mg tissue). In order to investigate whether myxosporean parasites can be detected in a non-lethal methods, quantitative analysis of *E. leei* and *P. anisocaudata* isolated from rearing water of three farms were performed by real-time PCR. The DNA copy number of *E. leei* from rearing water of farm-A and farm-B were 8×10^4 and 5×10^5 copies/L. However, it was not detected in farm-C, for *P. anisocaudata* from rearing water from farm-B and farm-C were detected (2.0×10^6 and 5.1×10^6 copies/L). These results indicated that the two species of myxosprean can be diagnosed using rearing water, and it was considered that it could be used as a non-lethal diagnostic method through rearing water. Result of multiplex PCR using EM-F/R primer set (Kim et al., 2015) and EL-F/R primer set (Kang et al., 2020), It was confirmed that two clear bands were detected targeting *P. anisocaudata* and *E. leei*. As a result of performing multiplex-PCR on 9 tissues of olive flounder, one clear band of *P. anisocaudata* in kidney tissue and one clear band of *E. leei* in intestine tissue were detected. These results showed the multiplex PCR method used in this study can detect two types of myxosporeans through one PCR, and it was expected to be used as an

economical, fast and accurate diagnostic method to diagnosis myxosporeans causing emaciation disease.

As a result of monitoring *E. leei* and *P. anisocaudata* from 2021 to 2022, . *E. leei* was detected starting from June when the water temperature increased until november, and it was confirmed that *P. anisocaudata* was detected throughout the year. Among 117 olive flounders suspected of emaciation disease, 73 olive flounders (62.4%) were confirmed that was single infection by *P. anisocaudata* and 4 olive flounders (3.4%) were confirmed that was single infection by *E. leei.* 28 olive flounders (23.9%) were confirmed that was co-infected by *P. anisocaudata* and *E. leei* and 12 olive flounder (10.3%) was confirmed that was non-infected, In the case of olive flounder single infected by *P. anisocaudata*, it was confirmed that almost all of them showed emaciation symptoms, the average relative condition factor, rCF values of individuals single-infected by *P. anisocaudata* was 84.6%, and it was confirmed that symptoms appeared from mild to severe. In the case of *E. leei*, it was confirmed that only 4 out of 22 farms were detected and the average rCF values of individuals single-infected by *E. leei* was 80.6%, it was confirmed them showed emaciation symptoms similarly *P. anisocaudata,* it was in contrast to the previously reported result that *P. anisocaudata* has a low correlation with emaciation disease (Shin et al., 2018).

Myxosporean parasite *E. fugu* was occurred for the first time in Korea. Result of sequencing, it was confirmed that *E. leei* and *E. fugu* were co-infected to tiger puffer, *Takifugu rubripes*. To early diagnosis *E. fugu* and *E. leei*, we developed 1-step PCR, 2-step PCR, real-time PCR and multiplex PCR for detection of *E. fugu*, *E. leei.* Myxosporeans detection was tested using 1-step PCR, 2-step PCR, real-time PCR, multiplex PCR. These results showed that real-time PCR and 2-step PCR were higher sensitive compared to PCR results and real-time PCR was higher sensitivity than 2-step PCR. Multiplex PCR has similar sensitivity to 1-step PCR. The real-time PCR

method developed in this study has advantage that it is less time consuming than 1-step PCR technique because real time PCR does not require endpoint detection. 2-step PCR was consuming more time than 1-step PCR while, it has higher sensitive and exclude cross-reaction with other myxosporeans. In the case of multiplex PCR, has a similar sensitivity to 1-step PCR so it is considered helpful to less consuming time and resource. The diagnostic methods conducted in this study are expected to identify emaciation diseases in advance and reduce economic losses through rapid control.

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1. Development and optimization of diagnostic methods for diagnosing and monitoring emaciation disease

1.1. Introduction

An emaciation disease is occurring in farmed olive flounder in Jeju, which shows symptoms such as abdominal distension, eye depression and skull protrusion, and loss of fish weight, finally leading to death. From 2016 to 2017, the mortality rate due to emaciation disease in Jeju olive flounder, *Paralichthys olivaceus* was 10.3%, indicating a high damage (Shim et al., 2019). Although various studies have been conducted on the causing emaciation disease in olive flounder, a clear relationship to the causative agent has not yet been elucidated (Kim et al., 2011; Choi et al., 2012; Sekiya et al., 2016; Kim et al., 2015a; Kim et al., 2015b; Kim and Jeong, 2018; Shin et al., 2018). So, detailed study and preparation of countermeasures are needed.

The occurrence of emaciation disease in seawater fish farming was first reported in tiger puffer, *Takifugu rubripes* (Tun et al., 2000), and has since been reported to olive flounder, red sea bream, *Pagrus major*, and black snapper, *Oplegnathus punctatus*. The occurrence of emaciation disease has been reported in various fish species (Yasuda et al., 2005; Yanagida et al., 2008). In Korea, the incidence of emaciation disease has been continuously occurring mainly in Jeju since 2007. Myxosporean has been suggested causing emaciation disease, and in Japan, *Enteromyxum leei* was reported causing emaciation disease in olive flounder and *E. fugu* and *Leptotheca fugu* have been reported causing emaciation disease in tiger puffer. (Yasuda et al., 2005;

Yanagida et al., 2004; Ogawa and Yokoyama, 2001). In Korea, it has been continuously reported that *E. leei* and *P. anisocaudata* were detected when farmed olive flounder showing emaciation symptoms (Shin et al., 2018; Sekiya et al., 2016; Kim et al., 2018). In this study, multiplex PCR method was developed for the rapid diagnosis of pathogens of causing emaciation disease. In addition, for development non-lethal diagnostic method for emaciation disease, the pathogens of causing emaciation disease was isolated by filtering the rearing water of the tanks infected with *E. leei* or *P anisocaudata,* the pathogens copies was quantified using the real-time PCR method.

1.2. Material and methods

1.2.1. Experimental fish

Olive flounder from farm suspected emaciation disease in Jeju area and the rearing water from the tank were collected and transported to the laboratory, and through PCR test, it was confirmed that they were infected with *E. leei* and *P. anisocaudata*. Eleven types of tissues (head kidney, body kidney, intestine, muscle, gills, heart, blood, brain, liver, spleen and eyes) were isolated from one infected olive flounder, the isolated tissue was used in an experiment to analyze the degree of infection by *E. leei* and *P. anisocaudata*.

1.2.2. Experimental water

The rearing water sample from farm-A, B, C was collected 2 L from the tanks and transported to labotory. Each of water sample was passed through a 5 μm membrane filter (Whatman, Germany) using a vacuum filtration system. After that, the membrane filter was put into a 50 mL conical tubes, and 6 mL of a sterile phosphate buffered saline, PBS solution was added and vortexed strongly to drop-off the parasites attached to the filter. After the drop-off process was completed, the solution was divided into microtubes and centrifuged at 1,800 × g for 10 minutes to collect the pellets into one microtube (Lee et al., 2021). The pellet. The collected pellet was used for DNA extraction.

1.2.3. DNA extraction

Eleven types of tissues (head kidney. body kideny, intestine, spleen, liver, gills, brain, muscle, heart, blood, and eye) were isolated from one olive flounder from farm-A. All tissues were quantified to 50 mg and proceeded to the experiment and using DNeasy® Blood & Tissue Kit (Qiagen Hilden,

Germany) total DNA was isolated from each tissue. 180 μL of ATL buffer and 20 μL of proteinase K were added to each tissue quantified at 50 mg, and reacted at 56 ℃ until the tissue was dissolved after then, reaction was completed, 200 μL of AL buffer was added and reacted for 10 min after the reaction, 200 μL of 100% ethanol was added, transferred to a spin column, and centrifuged at 6,000 × g for 1 min. After the column was transferred to a new tube, 500 μL of AW1 buffer was added, and centrifuged at 6,000 × g for 1 min, and 500 μL of AW2 buffer was added and centrifuged at 20,000 × g for 3 min, followed by washing. After that, the column was transferred to a microtube, and DNA was finally isolated using 50 μL of AE buffer. The isolated DNA was stored at -80 ℃ until the experiment.

1.2.4. PCR analysis

The PCR analysis was performed for olive flounder infected by *E. leei* and *P. anisocaudata*. PCR was performed using EL-F/EL-R primer set specific to *E. leei* and EM-F/EM-R primer set specific to *P. anisocaudata.* PCR condition was added 1 μM each primer, 2.5 mM each dNTP, 10 × IP-Taq Buffer, 2.5 U IP-Taq DNA polymerase (LaboPassTM IP-Taq 500 unit 2.5U/μL, COSMO genetech, USA). Add total DNA extracted from olive flounder tissue as template DNA in a microtube, after then, final volume of the PCR mixture was made to 20 μL to add distilled water. For PCR condition, pre-denaturation at 95 ℃ for 3 min under PCR conditions, the reaction was repeated 35 times by denaturation at 95 ℃ for 30 s, annealing at 60 ℃ for 30 s, and extension at 72 ℃ for 30 s. Then, it was post-extension at 72 ℃ for 7 min. After PCR is done, the amplified product was electrophoresed on 1% agarose gel with SYBR® Safe gel stain 10,000X in DMSO (Invitrogen) added using 1 × TAE buffer as a buffer for electrophoresis and then electrophoresis product was confirmed with a UV detector.

1.2.5. Multiplex PCR analysis

Multiplex PCR was performed by combining the EL-F/EL-R primer set (1,468 bp) for the detection of *E. leei* and the EM-F/EM-R primer set (812 bp) for the detection of *P. anisocaudata*. PCR condition was added 1 μM each primer, 2.5 mM each dNTP, 10 × IP-Taq Buffer, 2.5 U IP-Taq DNA polymerase (LaboPassTM IP-Taq 500 unit 2.5U/μL, COSMO genetech, USA). Add total DNA extracted from olive flounder as template DNA in a microtube, after then, final volume of the PCR mixture was made to 20 μL to add distilled water. For PCR condition, pre-denaturation at 95 °C for 3 min under PCR conditions, the reaction was repeated 35 times by denaturation at 95 °C for 30 s, annealing at 60 °C for 30 s, and extension at 72 °C for 30 s. Then, it was post-extension at 72 ℃ for 7 min. After PCR is done, the amplified product was electrophoresed on 1% agarose gel with SYBR® Safe gel stain 10,000X in DMSO (Invitrogen) added using 1 × TAE buffer as a buffer for electrophoresis and then electrophoresis product was confirmed with a UV detector.

1.2.6. Real-time PCR analysis

Using the genomic DNA of *E. leei* as a template DNA, PCR was performed using the EL-F/EL-R primer set, and the PCR product was cloned into pGEM-T Easy vector (Promega, USA) and competent cell DH5α. After transformation into the strain, plasmid DNA was isolated. In the same way, PCR was performed using the EM-F/EM-R primer set using the genomic DNA of *P. anisocaudata* as a template, and the PCR product was cloned into pGEM-T Easy vector (Promega, USA), and competent cells. After transformation into the DH5α strain, plasmid DNA was isolated. The absolute standard concentration of the isolated plasmid DNA was calculated. After then, Plasmid DNA at concentrations of 3.85×10^{10} copies/g and 3.88×10^{10} copies/g, respectively, was obtained from the isolated clones of *E. leei* and *E.*

fugu genes. The obtained plasmid DNA was serially diluted 10-fold and used to obtain a standard calibration curve for real-time PCR analysis. For real-time PCR analysis, previously reported ELNMF/MyNMR primer set specific to *E. leei* and RTEM-F/RTEM-R specific to *P. anisocaudata* were used (Table 2), and SensiMixPlus SYBR (Roche, USA) in Microtube, 10 μL, 1 μM of each forward primer and reverse primer was added. Plasmid DNA or total DNA extracted from olive flounder was used as template DNA, and distilled water was added to make the final volume 20 μL. Under the conditions of real-time PCR, post extension was performed at 95 °C for 10 min, followed by 45 cycles of at 95 °C for 20 s, 60 °C for 20 s , and at 72 °C for 20 s as 1 cycle. Quantitative analysis was performed using LightCycler® Nano SW1.0 (Roche, USA). Real-time PCR analysis was performed in triplicate and the values were expressed as average values.

Table 1. Primer sets used in this study

Target	Primer	Oligonucleotide sequences (5' to 3')	Expected size	Function	Reference
E. leei	EL-F	GATGAAACTGCGAAGCGCTC	1,468 bp	PCR	Kang et al (2020)
	EL-R	CACAAGTTGATGACTTGCGC			
P. anisocaudata	EM-F	CAACCGCAATGTGTTTACTC	812 bp	PCR	Kim et al (2015)
	EM-R	CCAAACAACCTGCCACAATG			
E. leei	ELNMF	CGGTGACGCCAATCCGTG	198 bp	Real-time PCR	Shin et al (2018)
	MyNMR	GACGGTATCTGATCGTCTTCGA			
P. anisocaudata	EMRT-F	CGATACATGTTGGTCGAC	147 bp	Real-time PCR	Kim & Jeong (2018)
	EMRT-R	CGAATCGCATTAATTATC			

1.3. Results

1.3.1. PCR anaylsis results

As a result PCR analysis of olive flounder samples from farm-A suspected of emaciation symptoms in Jeju, it was comfirmed that was co-infected by two types of myxosporeans (Table 2&3). Among the 11 tissues of the infected fish, *E. leei* was confirmed PCR analysis in the intestine and muscle, and *P. anisocaudata* was confirmed in the head and body tissues, respectively negative in the other tissues. As a result of PCR analysis on the rearing water of farm-A, *E. leei* was confrimed and *P. anisocaudata* was negative (Table 2&3).

1.3.2. Multiplex PCR anaylsis results

As a result of multiplex-PCR analysis, it was confirmed that specific bands clearly appeared (Fig. 1). A specific band was detected at 1,468 bp in the DNA sample was single infected by *E. leei* and a specific band was detected at 812 bp in the DNA sample was single infected by *P. anisocaudata.* It was confirmed that two specific bands were detected at the 1,468 bp and 812 bp positions in DNA samples was co-infected by *E. leei* and *P. anisocaudata* (Fig. 1). In addition, it was confirmed that the sensitivity was not lowered compared to single PCR, and that it was suitable for the diagnosis of emaciation disease. In addition, as a result of applying the mutiplex-PCR method to spleen, kidney, liver, gill, muscle, intestine, brain and heart tissues collected from olive flounder in suspected emaciation disease farm, it was confirmed that specific bands appeared clearly (Fig. 2).

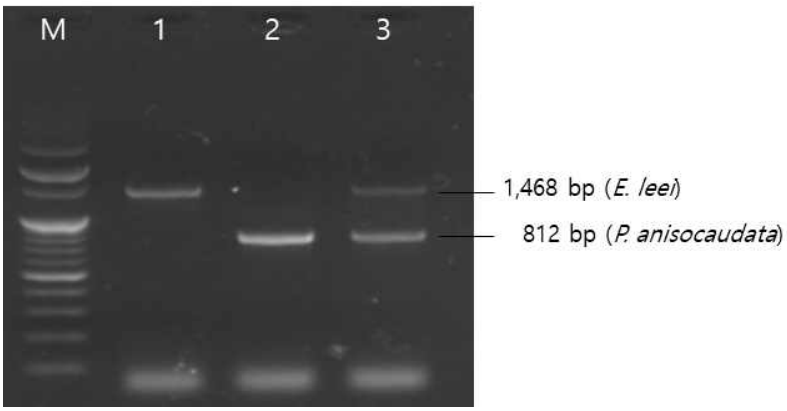

Fig. 1. Multiplex-PCR amplification. lane 1, *E. leei* positive sample; lane 2, *P. anisocaudata* positive sample; lane 3, DNA sample co-infected by *E. leei* and *P. anisocaudata*; M, 100 bp DNA ladder.

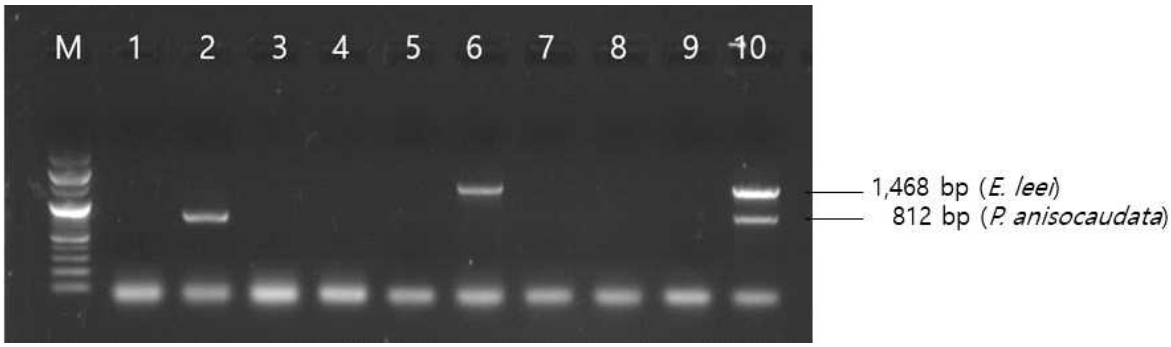

Fig. 2. Multiplex-PCR amplification. lane 1, spleen DNA; lane 2, kidney DNA; lane 3, liver DNA; lane 4, gill DNA; lane 5, muscle DNA; lane 6, intestine DNA; lane 7, brain DNA; lane 8, heart DNA; lane 9, blood DNA; lane 10, positive control (*E. leei* and *P. anisocaudata*); M, 100 bp DNA ladder.

1.3.3. Real-time PCR results

As a result of real-time PCR analysis of *E. leei* on 11 types of tissues of farm-A olive flounder showing emaciation symptoms, the intestinal tissue confirmed that was highest DNA copy value at 1.3×10^8 copies/mg (Table 2). The rest of the tissues were confirmed values of 1.3×10^3 ~ 4.6×10^5 copies/mg, confirming that *E. leei* was detected in all types of tissues examined. In addition, result of real-time PCR analysis of rearing water from farm-A, values of rearing water sample was confrimed 8×10^4 copies/L. As a result of real-time PCR analysis of *P. anisocaudata,* values of 1.3×10^3 ~ 9.1×10^3 copies/mg were confirmed in the head kidney and body kidney, and were not detected in other tissues including rearing water (Table 3). It was confirmed two types of myxosporeans, *E. leei* (5×10^5 copies/L) and *P. anisocaudata* (2.0×10^6 copies/L) were detected in farm-B, and only one type of myxosporean, *P. anisocaudata* (5.1×10^6 copies/L) was detected in farm-C (Table 2&3). Therefore, it was confirmed that the sensitivity of the real-time PCR method was higher than conventional PCR method in diagnosing emaciation disease.

Table 2. The DNA copy numbers of *E. leei* in each internal organ of emaciated olive flounder from farm-A and rearing water from farm-A, farm-B, farm-C

Aquafarm	Organ	Result		
		Real-time PCR (copies/mg tissue)	PCR	Ct
Farm-A	H.K	1.3×10^3	-	29.6
	B.K	1.4×10^3	-	29.6
	Intestine	1.3×10^8	+	13
	Muscle	4.6×10^5	+	21
	Brain	1.1×10^4	-	26.6
	Blood	1.0×10^4	-	26.7
	Gill	1.8×10^4	-	25.9
	Spleen	9.3×10^3	-	26.9
	Heart	7.5×10^4	-	23.9
	Liver	4.2×10^4	-	24.7
	Eye	1.1×10^5	-	23.2
	Rearing water	8×10^4 (copies/L)	-	28.5
Farm-B	Rearing water	5×10^5 (copies/L)	+	25.6
Farm-C	Rearing water	-	-	-

+, positive; -, negative

Table 3. The DNA copy numbers of *P. anisocaudata* in each internal organ of emaciated olive flounder from farm-A and rearing water from farm-A, farm-B, farm-C

Aquafarm	organ	Result		
		Real-time PCR (copies/mg tissue)	PCR	Ct
Farm-A	H.K	1.3×10^3	+	37.5
	B.K	9.1×10^3	+	34
	Intestine	-	-	-
	Muscle	-	-	-
	Brain	-	-	-
	Blood	-	-	-
	Gill	-	-	-
	Spleen	-	-	-
	Heart	-	-	-
	Liver	-	-	-
	Eye	-	-	-
	Rearing water	-	-	-
Farm-B	Rearing water	2.0×10^6(copies/L)	+	32
Farm-C	Rearing water	5.1×10^6(copies/L)	+	30

+, positive; -, negative

1.4. Discussion

As the damage caused by fish parasites spreads around the world, efforts are being made to reduce infection from the seed production stage, but it is known that it is currently difficult to completely block it. Microscopy, immunoassay, and western blotting test methods for detecting parasites can be difficult in diagnosing early infection and incubation period parasitic diseases, and detection using PCR is generally used as one of the methods to compensate for these shortcomings (Yasuda et al., 2005). Since the 1-step PCR method requires a band confirmation process through electrophoresis on an agarose gel after the PCR reaction, and it is difficult to quantitatively investigate the degree of infection, real-time PCR method that can detect pathogens quickly and quantitatively has recently been developed. In this study, 1-step PCR and real-time PCR were performed by isolating 11 types of tissues (head body, body, intestine, muscle, gill, heart, blood, brain, liver, spleen and eye) from olive flounder (Farm-A) showing emaciation symptoms. In this way, the degree of infection by *E. leei* and *P. anisocaudata* was analyzed. As reported by Yasuda et al., 2005, intestinal tissue (1.3×10^8 copies/mg) was analyzed to be the main target site of infection by *E. leei*, followed by high levels in muscle and eyes, it was confirmed that *E. leei* was also detected in all other tissue parts to be analyzed. In addition, it was confirmed that *E. leei* was detected in both methods, PCR and real-time PCR (8×10^4 copies/L) in the rearing water of olive flounder farm. These results suggested it could be possible to detect emaciation disease using olive flounder sample as well as rearing water sample, applicability of Non-lethal diagnosis for olive flounder infected by emaciation disease.In olive flounder from farm-A, *P. anisocaudata* was detected at a low concentration only in kidney tissue, and it was confirmed that it was not detected in other tissues

including rearing water. *Parvicapsula* sp. was detected at a very high concentration of 3.2×10^5 ~ 1.7×10^7 copies/mg from tissues such as kidney, intestine, spleen, brain and liver of olive flounder with severe emaciation symptoms (Kim and Jeong, 2018). In comparison with these results, farm-A olive flounder was judged to be mildly infected by *P. anisocaudata* and severely infected by *E. leei*. Cases of co-infection by *E. leei* and *P. anisocaudata* are frequently seen in olive flounder farms with emaciation symptoms in Jeju. It is considered necessary to accumulate and compare detailed data on emaciation, cumulative mortality, feed reduction rate, duration of infection and extent of damage in farms infected with myxosporean and those infected with co-infected. As a result of real-time PCR anaylsis of suspected emaciation olive flounder in farm-A, *E. leei* was detected in eleven types of olive flounder tissues in cluding rearing water sample from farm-A. In the case of 1-step PCR analysis of same samples, *E. leei* was detected in only two types of tissue, intestine and muscle. Therefore, the sensitivity of the real-time PCR method in diagnosing emaciation disease was higher than 1-step PCR method, and it is possible to determine the degree of infection by quantitative analysis of myxosporeans thus, it was considered be more useful diagnosis method.

2. Monitoring of emaciation disease in 2021-2022

2.1. Introduction

Since 2007 occurence of emaciation disease in Korea especially in Jeju. In Korea, emaciation disease in olive flounder has been occurring continuously since 2007, mainly in the Jeju. Myxosporean have been suggested as the cause of emaciation disease in Korea, and in Japan, *E. leei* of olive flounder, *E. fugu* of tiger puffer, and *Leptotheca fugu* are representatively reported (Yasuda et al., 2005; Yanagida et al., 2004; Ogawa and Yokoyama, 2001). The causative pathogens was investigated for farmed olive flounder suspected emaciation symptoms in Korea, it has been consistently reported that *E. leei* and *P. anisocaudata* were detected (Shin et al., 2018; Sekiya et al., 2016; Kim and Jeong, 2018). In relation to the occurrence of emaciation disease in Korea, study has been conducted on *Parvicapsula.* sp including *E. leei,* and a correlation analysis between emaciation olive flounder and the detection rate of *Parvicapsula.* sp has been reported through emaciation disease monitoring from 2010 to 2013 (Kim et al., 2015b). In a recent study, *P. anisocaudata* and *E. leei* were identified as the causative pathogens of emaciation disease in farmed olive flounder in Korea and it was reported that the association of *E. leei* was much higher than *P. anisocaudata* (Shin et al., 2018). However, according to Kim et al., 2018, it was reported that *P. anisocaudata* was distributed in high concentrations in the gastric submucosa and that the association with emaciation disease could not be ruled out. So, in this study, *E. leei* and *P. anisocaudata* are monitored for olive flounder farms with suspected emaciation symptoms to analyze the correlation with emaciation disease and two types of myxosporeans.

2.2. Material and methods

2.2.1. Experimental fish

From June 2021 to May 2022, 138 olive flounder were collected and transported to the laboratory from 22 farms suspected of emaciation disease in Jeju (Fig. 3). After measuring the weight and length to compare the emaciation degree, the intestine, the main target organ of *E. leei*, and the kidney, the target organ of *P. anisocaudata*, were isolated from olive flounders. The isolated tissue was used in an experiment to analyze the infection by *E. leei* and *P. anisocaudata.*

2.2.2. DNA extractiom

Two types of tissues (kidney, intestine) were isolated from all olive flounder from 23 farms. All tissues were quantified to 50 mg and proceeded to the experiment and using DNeasy® Blood & Tissue Kit (Qiagen Hilden, Germany) total DNA was isolated from each tissue. 180 µL of ATL buffer and 20 µL of proteinase K were added to each tissue quantified at 50 mg, and reacted at 56 °C until the tissue was dissolved after then, reaction was completed, 200 µL of AL buffer was added and reacted for 10 min after the reaction, 200 µL of 100% ethanol was added, transferred to a spin column, and centrifuged at 6,000 × g for 1 min. After the column was transferred to a new tube, 500 µL of AW1 buffer was added, and centrifuged at 6,000 × g for 1 min, and 500 µL of AW2 buffer was added and centrifuged at 20,000 × g for 3 min, followed by washing. After that, the column was transferred to a microtube, and DNA was finally isolated using 50 µL of AE buffer. The isolated DNA was stored at -80 °C until the experiment.

2.2.3. Multiplex PCR analysis

Multiplex PCR was performed by combining the EL-F/EL-R primer set (1,468 bp) for the detection of *E. leei* and the EM-F/EM-R primer set (812 bp) for the detection of *P. anisocaudata*. PCR condition was added 1 μM each primer, 2.5 mM each dNTP, 10 × IP-Taq Buffer, 2.5 U IP-Taq DNA polymerase (LaboPassTM IP-Taq 500 unit 2.5U/μL, COSMO genetech, USA). Add total DNA extracted from olive flounder tissue as template DNA in a microtube, after then, final volume of the PCR mixture was made to 20 μL to add distilled water. For PCR condition, pre-denaturation at 95 °C for 3 min under PCR conditions, the reaction was repeated 35 times by denaturation at 95 °C for 30 s, annealing at 60 °C for 30 s, and extension at 72 °C for 30 s. Then, it was post-extension at 72 ℃ for 7 min. After PCR is done, the amplified product was electrophoresed on 1% agarose gel with SYBR® Safe gel stain 10,000X in DMSO (Invitrogen) added using 1 × TAE buffer as a buffer for electrophoresis and then electrophoresis product was confirmed with a UV detector.

2.2.4. Comparison of condition factor (CF)

To confrim the emaciation degree, condition factor was calculated by following the formular, body weight × 10^2/body length^3. Relative condition factor was calculated based on standard condition factor (National Institute of Fisheries Science 2016) of cultured olive flounder (rCF = CF/standard CF × 100).

2.2.5. Statistical analysis

Statistical analyses were performed using SPSS 29.0 software (SPSS Inc., USA). Kruskal wallis test and Mann-Whitney U test were used to investigate a relation between relative condition factor and individual of infected by myxosporean olive flounder. Differences were considered

significant at $P < 0.05$.

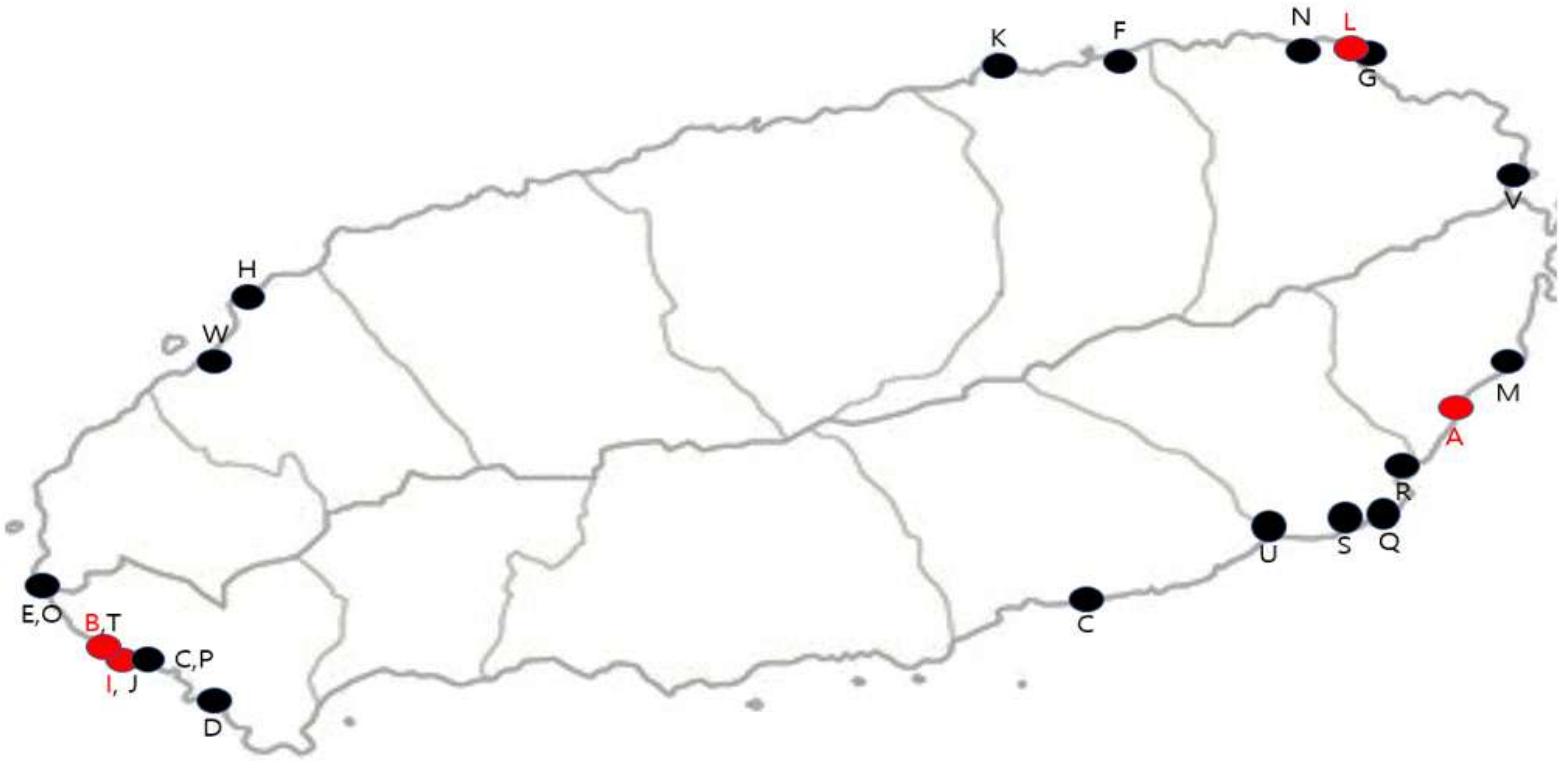

Fig. 3. Location of the Jeju olive flounder sampling station. Red dot, *E. leei* and *P. anisocaudata* positive farm; Black dot, *P. ansiocaudata* positve farm.

Table. 4 Primer sets used in this study

Target	Primer	Oligonucleotide sequences (5' to 3')	Expected size	Function	Reference
E. leei	EL-F	GATGAAACTGCGAAGCGCTC	1,468 bp	PCR	Kang et al (2020)
	EL-R	CACAAGTTGATGACTTGCGC			
P. anisocaudata	EM-F	CAACCGCAATGTGTTTACTC	812 bp	PCR	Kim et al (2015)
	EM-R	CCAAACAACCTGCCACAATG			

2.3. Results

2.3.1. Multiplex PCR anaylsis results

From June 2021 to May 2022, as a results multiplex PCR analysis of olive flounder samples from suspected emaciation symptoms olive flounder farms in Jeju, *E. leei* and *P. anisocaudata* which are known to cause emaciation disease, were detected in 21 out of 22 farms (95.5%). *E. leei* was detected starting from June when the water temperature increased until november, and it was confirmed that *P. anisocaudata* was detected throughout the year. Among 117 olive flounders suspected of emaciation disease, 73 olive flounders (62.4%) were confirmed that was single infection by *P. anisocaudata* and 4 olive flounders (3.4%) were confirmed that was single infection by *E. leei.* 28 olive flounders (23.9%) were confirmed that was co-infected by *P. anisocaudata* and *E. leei* and 12 olive flounder (10.3%) was confirmed that was non-infected (table 5).

Table 5. Detection frequency of myxosporean parasites in olive flounder

Year	Months	Infection rate (%) (positive no. / total no.)			
		P. anisocaudata	*E. leei*	Co-infection	Non infection
2021	6	10/17 (58.8%)	1/17 (5.9%)	1/17 (5.9%)	5/17 (29.4%)
	7	9/20 (45.0%)	1/20 (5%)	10/20 (50%)	0/20 (0%)
	8	20/27 (74.1%)	2/27 (7.4%)	3/27 (11.1%)	2/27(7.4%)
	9	7/12 (58.3%)	0/12 (0%)	4/12 (33.3%)	1/12 (8.3%)
	10	0/5 (0%)	0/5 (0%)	5/5 (100%)	0/5 (0%)
	11	2/7 (28.6%)	0/7 (0%)	5/7 (71.4%)	0/7 (0%)
	12	3/3 (100%)	0/3 (0%)	0/3 (0%)	0/3 (0%)
2022	1	3/3 (100%)	0/3 (0%)	0/3 (0%)	0/3 (0%)
	2	4/4 (100%)	0/4 (0%)	0/4 (0%)	0/4 (0%)
	4	13/13 (100%)	0/13 (0%)	0/13 (0%)	0/13 (0%)
	5	2/6 (33.3%)	0/6 (0%)	0/6 (0%)	4/6 (66.7%)
Total		73/117 (62.4%)	4/117 (3.4%)	28/117 (23.4%)	12/117 (10.3%)

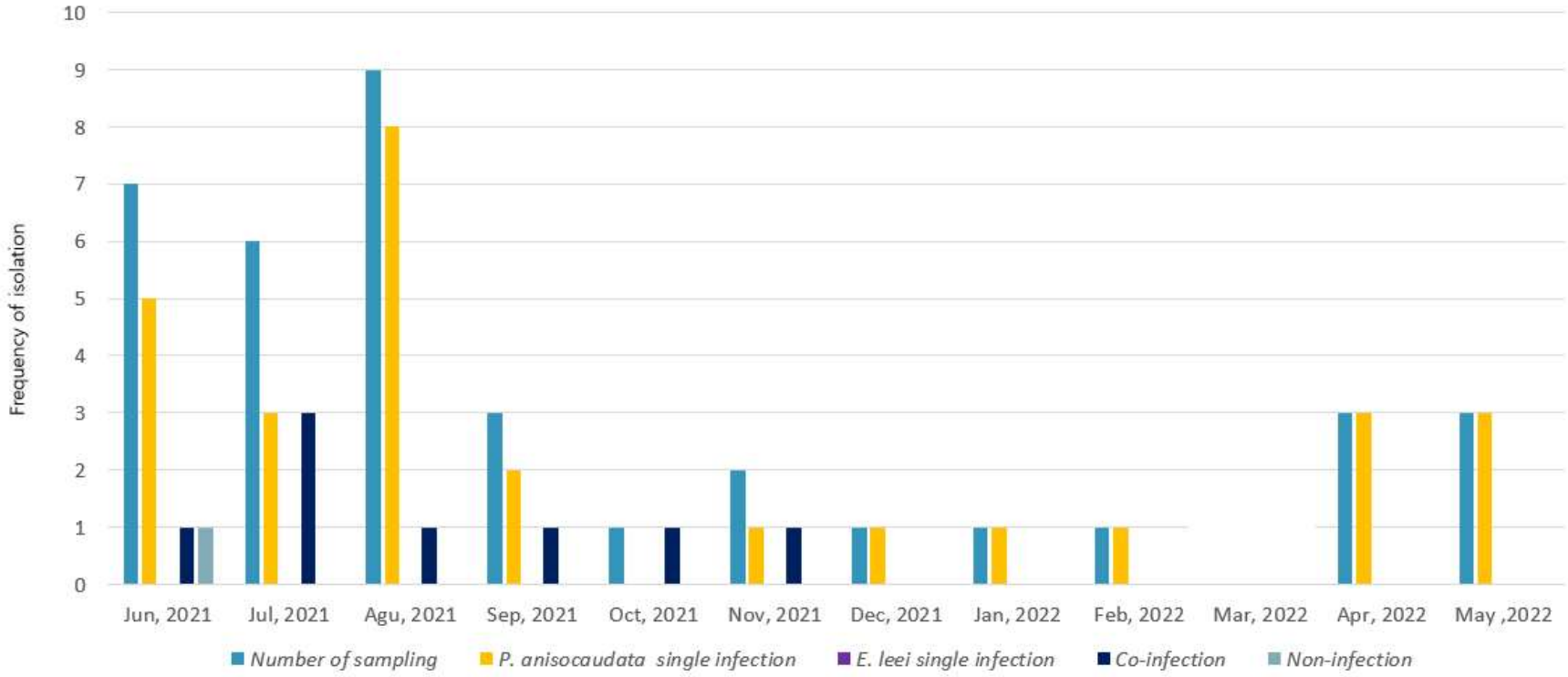

Fig. 4. Frequency of isolation myxosporeanparasite, *E. leei* and *P. anisocaudata* by monthly sampling

2.3.2. Comparison of condition factor

It was confirmed that the CF values of 12 olive flounders that were not infected with emaciation disease was at a higher or similar level compared to the standard CF presented by NIFS, respectively Table 6. In the case of four olive flounders that were infected by *E. leei* was at a lower or similar level compared to the standard CF, respectively Table 7. It was confirmed that the CF values of 28 olive flounders that were co-infected by *E. leei* and *P. anisocaudata* was at a lower level compared to the standard CF, respectively Table 8. It was confirmed that the CF values of 73 olive flounders that were infected by *P. anisocaudata* was at a lower level compared to the standard CF (table 9)(fig. 4).

2.3.3. Comparison of relative condition factor (rCF)

The average rCF value of olive flounder of single-infected by *E. leei* was confirmed that was 80.6%, single-infected by *P. anisocaudata* was confirmed that was 84.6%. In the case of co-infected by *E. leei* and *P. anisocaudata,* it was confirmed that was 74.8%, in the case of non-infection, it was confirmed that was 105.4%.

Table 6. The rCF value of normal olive flounder

Date	Length (cm)	Weight (g)	CF	Standard CF	rCF	Detection of myxosporean parasites (*E. leei* and *P. anisocaudata*)	
						E. leei	*P. anisocaudata*
21.06.08	34	400	1.01	1.19	84.87	-	-
	46	1070	1.1	1.27	86.61	-	-
	34	410	1.04	1.19	87.39	-	-
	34	450	1.14	1.19	95.80	-	-
21.06.22	31	375	1.25	1.17	106.84	-	-
21.08.13	29	320	1.31	1.16	112.93	-	-
21.08.30	16	50	1.22	1.03	118.45	-	-
21.09.03	44	1000	1.17	1.25	93.60	-	-
22.05.17	31	445	1.49	1.17	127.67	-	-
22.05.24	29	305	1.25	1.16	107.81	-	-
	28	350	1.59	1.15	138.64	-	-
	33	450	1.25	1.2	104.35	-	-

Table 7. The rCFvalue of olive flounder infected by *E. leei*

Date	Length (cm)	Weight (g)	CF	Standard CF	rCF	Detection of myxosporean parasites (*E. leei* and *P. anisocaudata*)	
						E. leei	*P. anisocaudata*
21.06.08	33	400	1.16	1.19	97.48	++ (Intestine)	-
21.07.22	33	390	1.1	1.19	92.44	++ (Intestine)	-
21.08.30	39	450	0.75	1.22	61.48	++ (Intestine)	-
	39	500	0.84	1.22	68.85	++ (Intestine)	-

Table 8. The rCF value of olive flounder co-infected by *E. leei* and *P. anisocaudata*

Date	Length (cm)	Weight (g)	CF	Standard CF	rCF	Detection of myxosporean parasites (*E. leei* and *P. anisocaudata*)	
						E. leei	*P. anisocaudata*
21.08.13	26	170	0.96	1.13	84.96	-	++ (Kidney)
	28	250	1.14	1.15	99.13	-	++ (Kidney)
	27	190	0.96	1.14	84.21	-	++ (Kidney)
21.08.19	28	200	0.91	1.15	79.13	-	+ (Kidney)
	29	210	0.86	1.16	74.14	-	++ (Kidney)
21.08.24	28	200	0.91	1.15	79.22	-	++ (Kidney)
	27	180	0.91	1.14	80.22	-	++ (Kidney)
	26	170	0.97	1.13	85.60	-	++ (Kidney)
21.08.30	34	440	1.11	1.19	93.28	-	++ (Kidney)
	37	550	1.08	1.21	89.26	-	++ (Kidney)
	24	120	0.87	1.11	78.20	-	++ (Kidney)
	23	110	0.90	1.1	82.19	-	++ (Kidney)
	28	210	0.96	1.15	83.48	-	++ (Kidney)
	27	210	1.06	1.14	92.98	-	++ (Kidney)
	27	230	1.04	1.15	90.43	-	++ (Kidney)
21.09.06	21	80	0.86	1.08	79.63	-	++(Kidney)
	21	70	0.76	1.08	70.37	-	++(Kidney)
	21	75	0.8	1.08	74.07	-	++(Kidney)
	21	80	0.86	1.08	79.63	-	++(Kidney)
	23	100	0.82	1.1	74.55	-	++(Kidney)

21.09.30	35	360	0.84	1.2	69.97	-	++(Kidney)
	35	360	0.84	1.2	69.97	-	++(Kidney)
21.11.03	28	250	1.14	1.15	99.03	-	++(Kidney)
21.11.23	38	500	0.91	1.22	74.69	-	++(Kidney)

Table 9. The rCF value of olive flounder infected by *P. anisocaudata*

Date	Length (cm)	Weight (g)	CF	Standard CF	rCF	Detection of myxosporean parasites (*E. leei* and *P. anisocaudata*)	
						E. leei	*P. anisocaudata*
21.06.03	22	110	1.02	1.09	93.58	-	++ (Kidney)
	23	120	0.98	1.1	89.09	-	++ (Kidney)
21.06.08	35	400	0.93	1.2	77.50	-	++ (Kidney)
	48	1230	1.11	1.28	86.72	-	+ (Kidney)
21.06.28	21	85	0.92	1.08	84.98	-	++ (Kidney)
	17	50	1.02	1.04	97.86	-	++ (Kidney)
	18	55	0.94	1.05	89.82	-	++ (Kidney)
	21	85	0.91	1.08	84.26	-	++ (Kidney)
	17	50	1.01	1.04	97.12	-	++ (Kidney)
	18	55	0.94	1.05	89.52	-	++ (Kidney)
21.07.07	24	110	0.79	1.11	71.17	-	++ (Kidney)
	23	100	0.82	1.1	74.55	-	++ (Kidney)
21.07.13	32	350	1.06	1.19	89.08	-	+ (Kidney)
21.07.19	40	650	1.01	1.23	82.11	-	++ (Kidney)
	36	450	0.96	1.2	80.00	-	++ (Kidney)
	34	440	1.11	1.19	93.28	-	+ (Kidney)
	39	660	1.11	1.22	90.98	-	++ (Kidney)
21.07.22	36	480	1.02	1.2	85.00	-	++ (Kidney)
21.07.29	35	440	1.02	1.2	85.00	-	++ (Kidney)
21.08.03	26	190	1.08	1.13	95.58	-	++ (Kidney)

21.08.06	22	100	0.93	1.09	85.32	-	+ (Kidney)
	26	170	0.96	1.13	84.96	-	++ (Kidney)
	23	120	0.98	1.1	89.09	-	++ (Kidney)
	26	200	1.13	1.13	100.00	-	+ (Kidney)
21.08.13	26	170	0.96	1.13	84.96	-	++ (Kidney)
	28	250	1.14	1.15	99.13	-	++ (Kidney)
	27	190	0.96	1.14	84.21	-	++ (Kidney)
21.08.19	28	200	0.91	1.15	79.13	-	+ (Kidney)
	29	210	0.86	1.16	74.14	-	++ (Kidney)
21.08.24	28	200	0.91	1.15	79.22	-	++ (Kidney)
	27	180	0.91	1.14	80.22	-	++ (Kidney)
	26	170	0.97	1.13	85.60	-	++ (Kidney)
21.08.30	34	440	1.11	1.19	93.28	-	++ (Kidney)
	37	550	1.08	1.21	89.26	-	++ (Kidney)
	24	120	0.87	1.11	78.20	-	++ (Kidney)
	23	110	0.90	1.1	82.19	-	++ (Kidney)
	28	210	0.96	1.15	83.48	-	++ (Kidney)
	27	210	1.06	1.14	92.98	-	++ (Kidney)
	27	230	1.04	1.15	90.43	-	++ (Kidney)
21.09.06	21	80	0.86	1.08	79.63	-	++(Kidney)
	21	70	0.76	1.08	70.37	-	++(Kidney)
	21	75	0.8	1.08	74.07	-	++(Kidney)
	21	80	0.86	1.08	79.63	-	++(Kidney)
	23	100	0.82	1.1	74.55	-	++(Kidney)

21.09.30	35	360	0.84	1.2	69.97	-	++(Kidney)
	35	360	0.84	1.2	69.97	-	++(Kidney)
21.11.03	28	250	1.14	1.15	99.03	-	++(Kidney)
21.11.23	38	500	0.91	1.22	74.69	-	++(Kidney)
21.12.09	32	380	1.16	1.18	98.28	-	++(Kidney)
	29	270	1.11	1.16	95.44	-	++(Kidney)
	29	260	1.07	1.16	91.90	-	++(Kidney)
22.01.24	34	380	0.97	1.19	81.25	-	++(Kidney)
	31	270	0.91	1.17	77.46	-	++(Kidney)
	30	260	0.96	1.16	83.01	-	++(Kidney)
22.02.15	27	190	0.97	1.14	84.68	-	++(Kidney)
	26	180	1.02	1.13	90.63	-	++(Kidney)
	28	190	0.87	1.15	75.26	-	++(Kidney)
	28	200	0.91	1.15	79.22	-	++(Kidney)
22.04.12	28	180	0.82	1.15	71.30	-	++(Kidney)
	26	170	0.97	1.13	85.60	-	++(Kidney)
	26	160	0.91	1.13	80.56	-	++(Kidney)
22.04.13	34	390	0.99	1.19	83.38	-	++(Kidney)
	35	400	0.93	1.2	77.75	-	++(Kidney)
	33	380	1.06	1.19	88.86	-	++(Kidney)
	36	410	0.88	1.2	73.23	-	++(Kidney)
	33	360	1.00	1.19	84.18	-	++(Kidney)
22.04.26	23	100	0.82	1.1	74.72	-	++(Kidney)
	24	140	1.01	1.11	91.24	-	++(Kidney)

22.04.26	23	100	0.82	1.1	74.72	-	++(Kidney)
22.04.26	21	100	1.08	1.08	99.98	-	++(Kidney)
22.04.26	23	100	0.82	1.1	74.72	-	++(Kidney)
22.05.09	28	250	1.14	1.15	99.03	-	++(Kidney)
22.05.09	24	150	1.09	1.11	97.75	-	++(Kidney)

2.3.4. Statistical analysis

The significance vaules was statistically significant at 0.000 for association of rCF of co-infection and rCF of non-infection and the significance vaules was statistically significant at 0.004 for association of single-infected by *P. anisocaudata* rCF and rCF of non-infection. In the case of infection of *E. leei*, the significance vaules was statistically non-significant at 0.060 for association of rCF of single infection of *E. leei* and rCF of non-infection (table 10). For post-hoc analysis with Mann-Whitney U test was performed (table 11).

Table 10. Kruskal wallis test results of analysis items by rCF of non-infection

Statistical analysis method	Infection type	Test Statistic	Std. Error	Std. Test Statistic	*p**
Kruskal wallis test	rCF of Co-infection by *E. leei* and *P. anisocaudata*	-68.898	11.967	-5.757	.000
	rCF of *E. leei* single-infection	-50.523	19.635	-2.573	.060
	rCF of *P. anisocaudata* single-infect.ion	37.184	10.877	3.419	.004

**p: Asymptotic significance probability*

Table 11. Mann-Whitney test results of analysis items by rCF of non-infection

Statistical analysis method	Infection type	Mann Whitney U	Wilcoxon W	Z	*p**
Mann-Whitney U test	rCF of Co-infection by *E. leei* and *P. anisocaudata*	11	417	-4.447	<.001
	rCF of *E. leei* single-infection	7	17	-1.958	0.056
	rCF of *P. anisocaudata* single-infection	100	2801	-3.998	<.001

**p: Asymptotic significance probability*

2.4. Discussion

In the case of fish infected with *E. leei,* it is known that the osmotic pressure control system of the intestinal tissue was broken, resulting in emaciation symptoms due to dehydration (Kim et al., 2011). And it was also known to have an osmotic pressure-regulating function in the kidneys. Olive flounders infected with *P. anisocaudata* was observed a high rate of infection in the kidneys. One of the major roles of fish kidneys is the regulation of osmotic pressure, especially in marine fish, it was known to reabsorb water from the renal capillaries. It was reported that *P. anisocaudata* penetrates deeply into the tubule lumen of olive flounder, and that if severely infected, it would be negatively affected tubule function (Cho et al., 2005), olive flounder heavily infected by *P. anisocaudata* was presumed to be considered to have low nutrient and water reabsorption rates due to renal tubule dysfunction. It is known that typical marine fish produce very little urine to regulate osmotic pressure. In contrast, in this study, in the case of flounder infected with *P. anisocaudata* through monitoring, the majority of bladder distension was observed, which is considered to be an abnormality in osmotic pressure regulation. Therefore, it is considered that further studies on osmotic pressure regulation due to renal tubule dysfunction caused by *P. anisocaudata* are needed.

Multiplex PCR was performed for two types of myxosporeans, *E. leei* and *P. anisocaudata* in olive flounder farm suspected emaciation disease, a high infection rate of 95.5% was confirmed that was infected by *P. anisocaudata* in 21 out of 22 farms. In the case of olive flounder single infected by *P. anisocaudata*, it was confirmed that almost all of them showed emaciation symptoms, the average rCF values of individuals single-infected by *P. anisocaudata* was 84.6%, and emaciation symptoms appeared from mild to

severe. The significance vaules was statistically significant at 0.004 for association of single-infected by *P. anisocaudata* rCF and rCF of non-infection (Table 10). In the case of *E. leei*, it was confirmed that only 4 out of 22 farms were detected and the average rCF values of individuals single-infected by *E. leei* was 80.6%, it was confirmed them showed emaciation symptoms similarly *P. anisocaudata*. However the significance vaules was statistically non-significant at 0.060 for association of rCF of single infection of *E. leei* and rCF of non-infection (Table 10). It was in contrast to the previously reported result that *P. anisocaudata* has a low correlation with emaciation disease (Shin et al., 2018). Also, it was confirmed that the rCF values of 28 olive flounders that were co-infected by *E. leei* and *P. anisocaudata* was 74.8%. The significance vaules was statistically significant at 0.000 for association of rCF of co-infection and rCF of non-infection. These results showed emaciation degree was high in the case of co-infected by *E. leei* and *P. anisocaudata* than, it was considered that further research was needed.

3. Occurrence of the myxosporean emaciation disease caused by *Enteromyxum fugu* in cultured tiger puffer, *Takifugu rubripes* in Korea

3.1. Introduction

Myxosporean parasite *E. fugu* was occurred for the first time in Korea. Result of sequencing, it was confirmed that *E. leei* and *E. fugu* were co-infected. There are four species in *Enteromyxum* genus: *E. leei, E. scophthalmi, E. fugu* and *E. caesio* and these are a pathogenic myxosporean parasite is known causative pathogens of enteromyxosis and emaciation disease in fishes: *Pagrus major, Oplegnathus punctatus, Paralichthys olivaceus* (Freeman et al., 2020; Palenzuela et al., 2002; Yanagida et al., 2004; Sohn et al 2021). Especially in Japan, suggested that emaciation disease tiger puffer, *Takifugu rubripes* was infected by *E. leei* and *E. fugu* (Yasuda et al., 2005; Ishimatsu et al., 2007; Yanagida et al., 2008) and myxosporean emaciation disease has been a serious problem, infected fish has a severe emaciation, typically sunken eyes, bony ridges on the head, weight loss and tapering body, intestinal inflammation, causing low food conversion rates, and eventually death (Ogawa & Yokoyama., 2001, Sitjà-Bobadilla & Palenzuela., 2012).

Diagnosis of the enteric myxosporean in tiger puffer relies on light microscopic observation of histological and PCR, Polymerase chain reaction method. Histological method is accurate diagnosis method. However, myxosoprean parasites have highly variable developmental stages in

morphology (Tin Tun et al., 2000) and also confirmatory diagnosis could be confusing in the absence of species-defining spores (Yanagida et al., 2004, Shin *et al.*, 2018). For these reasons, histological diagnosis has a disadvantage in that it takes a long time and require technical expertise (Yanagida et al., 2004, Shin et al., 2018). Therefore, we used the PCR method for the diagnosis of enteric myxosporean in tiger puffer, but the specific primer reported in Korea for *E. leei* (Kang et al., 2020) can combine *E. fugu* sequence. Also *E. leei* and *E. fugu* PCR products produced by EL-F/R primer set has similar base pair, so when using this primer, it is impossible to accurately diagnosis emaciation fish infected between *E. leei* and *E. fugu*. To solve this problem, we developed new specific primer sets for 1-step PCR to detection *E. leei* and *E. fugu*. Also, there are no treatments or available vaccines for myxosporean infections in fish (Yokoyama et al., 2012) and infection fish has reduced appetite, limits drug administration through feeding, for these reasons myxosporean infection control strategies are mostly preventions for instances, a good management technique, reducing fish densities and improving water quality (Fontes et al., 2015). Therefore, early diagnosis is important to controlling the disease (Sohn et al., 2021). However, there are not enough pathogens in the early stages of infection in fish, thus, in the early stage infection, the DNA of the pathogen extracted from the main target organ has low concentration so, high-sensitivity method is essential for early diagnosis.. Therefore, 1-step PCR is insufficient for early diagnosis. Especially myxosporean parasite *E. fugu* occurred for the first time in Korea, it was necessary to develop a rapid diagnostic method and a highly sensitive diagnostic method to prevent the spread of the disease. Real-time PCR has a high sensitivity than 1-step PCR (Mackay et al., 2002) and faster than 1-step PCR because they don't have to endpoint detection, so it is suitable to early diagnosis. But real-time PCR method is involved with an expensive equipment therefore, it is difficult to use in 1-step diagnosis facilities which

do not have expensive laboratory equipment. In addition, 2-step PCR has high sensitivity and high specificity (Massung et al., 1998), so it can be an accurate diagnosis but, it doesn't require another equipment. So, it helps to early diagnosis in 1-step diagnosis facilities. For these reasons, in this study, we tried to develop real-time PCR method for early diagnosis of emaciation disease and also, we tried to develop 2-step PCR method for early diagnosis of emaciation disease in 1-step diagnosis facilities without real-time PCR equipment.

3.2. Material and methods

3.2.1. Experiment fish and water sample

On September, 2021 four tiger puffers were transported to the laboratory from a farm suspected of emaciation disease. After measuring the height and weight, total of nine tissues were dissected and isolated from the tiger puffer: kidney, intestine, spleen, liver, gills, brain, muscle, heart and eye. Condition factor was calculated by the following the formula (body weight × 10^2/body length$^{3.}$)(Table 12).

To confirm if olive flounder, *Paralichthys olivaceus* could be infected by *E. fugu*, in June, 2022, from olive flounder farm located near by the tiger puffer fish farm 15 fishes 60 days old, 5 L inflow water, 5 L rearing water were transported to the laboratory. For DNA extraction, the intestine and kidney were separated from each olive flounder and then separated tissues were pooled by grouping 5 individuals. Each of grouping tissue samples was homogenized, quantification at 50 mg each of homogenized grouping tissue samples, they were used for DNA extraction. The rearing water sample was collected 5 L from the tanks and 5 L flowing into the tank were collected. Each of water sample was passed through a 5 μm membrane filter (Whatman, Germany) using a vacuum filtration system. After that, the membrane filter was put into a 50 mL conical tube, and 6 mL of a sterile phosphate buffered saline, PBS solution was added and vortexed strongly to drop-off the parasites attached to the filter. After the drop-off process was completed, the solution was divided into microtubes and centrifuged at 1,800 × g for 10 minutes to collect the pellets into one microtube (Lee et al., 2021). The pellet. The collected pellet was used for DNA extraction.

3.2.2. DNA extraction

Nine types of tissues (kidney, intestine, spleen, liver, gills, brain, muscle, heart and eye) were isolated from four tiger puffers, all tissues were quantified to 50 mg and proceeded to the experiment and using DNeasy® Blood & Tissue Kit (Qiagen Hilden, Germany) total DNA was isolated from each tissue. 180 µL of ATL buffer and 20 µL of proteinase K were added to each tissue quantified at 50 mg, and reacted at 56 ℃ until the tissue was dissolved after then, reaction was completed, 200 µL of AL buffer was added and reacted for 10 min after the reaction, 200 µL of 100% ethanol was added, transferred to a spin column, and centrifuged at 6,000 × g for 1 min. After the column was transferred to a new tube, 500 µL of AW1 buffer was added, and centrifuged at 6,000 × g for 1 min, and 500 µL of AW2 buffer was added and centrifuged at 20,000 × g for 3 min, followed by washing. After that, the column was transferred to a microtube, and DNA was finally isolated using 50 µL of AE buffer. The isolated DNA was stored at -80 ℃ until the experiment.

3.2.3. Primer design

Using the MACAW program, *E. fugu* (accession number AY520573.1) and *E. leei* (accession number DQ139796.1) sequences were compare to design some specific primer sets for *E. leei* and *E. fugu*. We designed EF-1F, EF-2R, REF-R primer sets which are specific to *E. fugu* and EL-1F/EL-1R, EL-2F/EL-2R primer sets which are specific to *E. leei*. EL-1F/EL-1R primer set was used for 1-step PCR of *E. leei*, and EL-2F/EL-2R primer set was used for 2-step PCR of *E. leei*. EF-1F and previously reported EF-R primer set (Yanagida et al., 2004) was used for 1-step PCR of *E. fugu,* and previously reported EF-F (Yanagida et al., 2004) and EF-2R primer set was used for 2-step PCR of *E. fugu*. The previously reported ELNMF/MyNMR primer set (Shin et al., 2018) was used for Real-time PCR analysis of *E. leei* and the previously reported EF-F (Yanagida et al., 2004) and REF-R primer set was used for real-time PCR analysis of *E. fugu* (Table 12).

3.2.4. 1-step PCR analysis

The 1-step PCR analysis was used to test for infection four emaciation tiger puffer by *E. leei* and *E. fugu*. 1-step was performed using EF-1F/EF-R primer set specific to *E. fugu* and EL-1F/EL-1R primer set specific to *E. leei* (Table 12). PCR condition was added 1 μM each primer, 2.5 mM each dNTP, 10 × IP-Taq Buffer, 2.5 U IP-Taq DNA polymerase (LaboPassTM IP-Taq 500 unit 2.5U/μL, COSMO genetech, USA). Add total DNA extracted from tiger puffer tissue as template DNA in a microtube, after then, final volume of the PCR mixture was made to 20 μL to add distilled water. For PCR condition, pre-denaturation at 95 °C for 3 min under PCR conditions, the reaction was repeated 35 times by denaturation at 95 °C for 30 s, annealing at 60 °C for 30 s, and extension at 72 °C for 30 s. Then, it was post-extension at 72 ℃ for 7 min. After PCR is done, the amplified product was electrophoresed on 1% agarose gel with SYBR® Safe gel stain 10,000X in DMSO (Invitrogen) added using 1 × TAE buffer as a buffer for electrophoresis and then electrophoresis product was confirmed with a UV detector.

3.2.5. 2-step PCR analysis

To obtain template DNA for 2-step PCR, first step PCR analysis was performed using EL-1F/EL-1R primer set specific to *E. leei* and EF-1F/EF-R primer set specific to *E. fugu* (Table 12). PCR products are electrophoresed on 1% agarose gel with SYBR® Safe gel stain 10,000X in DMSO (Invitrogen) added with 1 × TAE buffer as a buffer for electrophoresis and then the PCR product was confirmed using a UV detector. 2-step PCR analysis was performed using the identified first step PCR product as template DNA and 2-step PCR was performed using EF-F/EF-2R primer set specific to *E. fugu* and EL-2F/EL-2R primer set specific to *E. leei* (Table 12). 2-step PCR condition was 1 μM each primer, 2.5 mM each dNTP, 10 × IP-Taq Buffer, 2.5 U IP-Taq DNA polymerase (LaboPassTM IP-Taq 500 unit 2.5U/μL, COSMO genetech, USA) and template DNA extracted from tiger puffer in microtube After, the final volume of the PCR mixture was made up to 20 μL with distilled water. After pre-denaturation at 95 °C for 3 min under PCR conditions, the reaction was repeated 35 times by denaturation at 95 °C for 30 s, annealing at 60 °C for 30 s, and extension at 72 °C for 30 s. Then, it was post-extension at 72 ℃ for 7 min. PCR amplified products are electrophoresed on 1% agarose gel with SYBR® Safe gel stain 10,000X in DMSO (Invitrogen) added with 1 × TAE buffer as a buffer for electrophoresis and confirmed.

3.2.6. Sequencing for identifying myxosporean (*E. leei* and *E. fugu*)

To identify which myxosporean infected to tiger puffers, PCR analysis was performed using primer set EL-F/EL-R specific to *E. leei* and EF-F/EF-R primer set specific to *E. fugu.* PCR products was purified using HiGeneTM Gel & PCR Purification kit (Biofact, Korea) then, using the purified PCR products sequencing was performed (Solgent, Korea).

3.2.7 Multiplex PCR analysis

Multiplex PCR was developed to identify two types of myxosporean through a single PCR. PCR was performed using 2 pairs of primers for multiplex PCR described in table 12. PCR analysis was used to test for infection four emaciation tiger puffers by *E. leei* and *E. fugu*. PCR condition was added 1 μM each primer, 2.5 mM each dNTP, 10 × IP-Taq Buffer, 2.5 U IP-Taq DNA polymerase (LaboPassTM IP-Taq 500 unit 2.5U/μL, COSMO genetech, USA). Add total DNA extracted from tiger puffer tissue as template DNA in a microtube, after then, final volume of the PCR mixture was made to 20 μL to add distilled water. For PCR condition, pre-denaturation at 95 °C for 3 min under PCR conditions, the reaction was repeated 35 times by denaturation at 95 °C for 30 s, annealing at 62 °C for 30 s, and extension at 72 °C for 30 s. Then, it was post-extension for 7 min at 72 °C. Then, it was post-extension for 7 min at 72 ℃. After PCR is done, the amplified product was electrophoresed on 1% agarose gel with SYBR® Safe gel stain 10,000X in DMSO (Invitrogen) added using 1 × TAE buffer as a buffer for electrophoresis and then electrophoresis product was confirmed with a UV detector.

3.2.8 Real-time PCR analysis

Using the genomic DNA of *E. leei* as a template DNA, PCR was performed using the NEL-F/NEL-R primer set, and the PCR product was cloned into pGEM-T Easy vector (Promega, USA) and competent cell DH5α. After transformation into the strain, plasmid DNA was isolated. In the same way, PCR was performed using the EF-1F/EF-R primer set using the genomic DNA of *E. fugu* as a template, and the PCR product was cloned into pGEM-T Easy vector (Promega, USA), and competent cells. After transformation into the DH5α strain, plasmid DNA was isolated. The absolute standard concentration of the isolated plasmid DNA was calculated. After then, Plasmid DNA at concentrations of 3.85×10^{10} copies/g and 3.88×10^{10} copies/g, respectively, was obtained from the isolated clones of *E. leei* and *E. fugu* genes. The obtained plasmid DNA was serially diluted 10-fold and used to obtain a standard calibration curve for real-time PCR analysis. For real-time PCR analysis, previously reported ELNMF/MyNMR primer set specific to *E. leei* and EF-F/REF-R specific to *E. fugu* were used (Table 12), and SensiMixPlus SYBR (Roche, USA) in Microtube, 10 μL, 1 μM of each forward primer and reverse primer was added. Plasmid DNA or total DNA extracted from tiger puffer was used as template DNA, and distilled water was added to make the final volume 20 μL. Under the conditions of real-time PCR, post extension was performed at 95 °C for 10 min, followed by 45 cycles of at 95 °C for 20 s, 60 °C for 20 s , and at 72 °C for 20 s as 1 cycle. Quantitative analysis was performed using LightCycler ® Nano SW1.0 (Roche, USA). Real-time PCR analysis was performed in triplicate and the values were expressed as average values.

Table 12. Primer sets used in this study

Target	Purpose	Name	Oligonucleotide sequences (5'-3')	Expected size (bp)	Reference
E. leei (18S rRNA)	1-step PCR	EL-1F	CTATTCGATGGTATACATAG	1,338	This study
		EL-1R	GTCTACTCTTTCGAGCAAAC		
	2-step PCR	EL-2F	ACTAGCTAGAAACCAACAAG	609	This study
		EL-2R	AACACGGATTGGCGTCACCG		
	Real-time PCR	ELNMF	CGGTGACGCCAATCCGTG	191	Shin *et al.*, (2018)
		MyNMR	GACGGTATCTGATCGTCTTCGA		
	PCR	EL-F	GATGAAACTGCGAAGCGCTC	1,468 / 1,464*	Kang *et al.*, (2020)
		EL-R	CACAAGTTGATGACTTGCGC		
E. fugu (SSU rRNA)	1-step PCR	EF-1F	TATCGTAGGCGGAAGCCTGG	1,085	This study
		EF-R	AGAACCTACAATTGGTCTGT		Yanagida et al., (2005)
	2-step PCR	EF-F	GGAGCAGTTCCAATGGGATT	585	Yanagida et al., (2005)
		EF-2R	CGGTGAGACTAACATGTATC		This study
	Real-time PCR	EF-F	GGAGCAGTTCCAATGGGATT	185	Yanagida et al., (2005)
		REF-R	ATACCACAATAGCTTCAACT		This study
E. leei	Multiplex PCR	EL-2F	ACTAGCTAGAAACCAACAAG	1,271	This study
		EL-1R	GTCTACTCTTTCGAGCAAAC		
E. fugu	Multiplex PCR	EF-1F	TATCGTAGGCGGAAGCCTGG	797	This study
		EF-2R	CGGTGAGACTAACATGTATC		

Table 13. Prevalence of infection with the three myxosporean in tiger puffer

Fish	No.	B.L. (cm)	B.W. (g)	Condition factor	*E. leei*			*E. fugu*		
					1-step PCR	2-step PCR	Real-time PCR	1-step PCR	2-step PCR	Real-time PCR
Tiger Puffer	1	22.5	150	1.31*	P	P	P	P	P	P
	2	22.0	180	1.69	P	P	P	P	P	P
	3	20.0	130	1.63	P	P	P	P	P	P
	4	21.0	140	1.51	P	P	P	P	P	P

P, positive.

3.2.9 Comparison sensitivity

To compare the sensitivity, we select the No. 1 tiger puffer which highest degree of emaciation others tiger puffer, The experiment was performed using DNA of the intestine, which is the target tissue of *E. leei* and *E. fugu*. The purity and yields of the total DNA extract obtained from No.1 tiger puffer intestine sample was high(purity = A260/A280 ratio 1.838 and yield = 60 ng/μl). DNA concentration was measured with a NanoDrop2000 spectrophotometer (Beckman Coulter DU®730, UV - Vis spectrophotometer). The quantification of nucleic acids was calculated following formula: DNA concentration = OD260 × extinction coefficient × dilution factor. To determine the detection limit, intestine DNA of No. 1 tiger puffer was diluted 10-fold from 60 ng/μl to 6 fg/μl. To compare sensitivity 1-step PCR, 2-step PCR, and real-time PCR methods were performed through each of diluted DNA.

3.3. Result

3.3.1. Sequencing for identify myxosporean (*E. leei* and *E. fugu*)

Result of sequencing alignment of *E. leei* PCR product, it was confirmed that it has 100% identities (1370/1370) to *E. leei* (accession number MH465674.1) and result of sequencing alignment of *E. fugu* PCR product, it was confirmed that it has 99.41% identities (1009/1015) to *E. fugu* (AY520573.1). These results show tiger puffers were co-infected by *E. leei* and *E. fugu.*

3.3.2. 1-step PCR analysis

To confirm the specificity of new PCR primer set, PCR analysis was performed using 1-step PCR primer set EL-1F/EL-1R, 2-step PCR primer set EL-2F/EL-2R specific to *E. leei* and 1-step PCR primer set EF-1F/EF-R, 2-step PCR primer set EF-F/EF-2R specific to *E. fugu* (Table 12). As the template DNA for PCR, the intestine tissue DNA of olive flounder infected with *E. leei* was used. PCR results for *E. leei*, *E. leei* was detected by new primer set EL-1F/EL-1R, EL-2F/EL-2R in contrast *E. fugu* was not detected by new primer set EF-1F/EF-R, EF-F/EF-2R (data not shown). On September, four tiger puffers were transported laboratory from a farm suspected of having emaciation disease. Dissecting and isolating the intestine, which is the main target tissue of *E. leei* and *E. fugu* after then, DNA isolation, PCR analysis was performed after DNA isolation. Isolated DNA was used for performing 1-step PCR primer set EL-1F/EL-1R, EF-1F/EF-R. As a result, it was confirmed that both *E. leei* and *E. fugu* were detected in all tiger puffers (Table 13). By combining the PCR results and external

symptoms, No. 1 tiger puffer was selected and DNA was isolated from 9 tissues (kidney, intestine, spleen, liver, gills, brain, muscle, heart and eyes). 1-step PCR was performed using the 1-step PCR primer set EL-1F/EL-1R specific for *E. leei*, and the 1-step PCR primer set EF-1F/EF-R specific for *E. fugu*. As a result of PCR, *E. fugu* detection was confirmed in 6 tissues other than the brain and heart (Table 14). In the case of *E. leei* detection was confirmed in 6 tissues except for the brain and heart, identical to the results of *E. fugu* (Table 14).

3.3.3. 2-step PCR analysis

3.3.3.1. 2-step PCR analysis result of tiger puffers

4 tiger puffers were isolated from 9 tissues (kidney, intestine, spleen, liver, gills, brain, muscle, heart and eyes). First, for 2-step PCR analysis, 1-step PCR was performed using 1-step PCR primer set EL-1F/EL-1R specific for *E. leei*, and the 1-step PCR primer set EF-1F/EF-R specific for *E. fugu*. The 1-step PCR product was used as a template DNA for 2-step PCR and PCR was performed using specific for *E. leei* primer set EL-2F/EL-2R, specific for *E. fugu* primer set EF-F/EF-2R. The 1-step PCR product was used as a template DNA for 2-step PCR and PCR was performed using specific for *E. leei* primer set EL-2F/EL-2R, specific for *E. fugu* primer set EF-F/EF-2R. As a result of 2-step PCR, *E. fugu* was detected in all nine tissues. In the case of *E. leei*, the detection was confirmed in all 9 tissues as in the results of *E. fugu*.

3.3.3.2 2-step PCR analysis result of olive flounder

As a 2-step PCR result, *E. fugu* was confirmed that it was detected group 2 olive flounder kidney and intestine, group 3 olive flounder kidney, inflowing water sample and rearing water sample however it was confirmed that it was not detected all sample at 1-step PCR respectively (Fig. 6).

3.3.4. Multiplex PCR analysis

To confirm the sensitivity of multiplex PCR, No. 1 tiger puffer intestine DNA was serially diluted 10-fold from 60 ng/μl to 6 fg/μl. The DNA copies values corresponding to each of the diluted DNA were shown in Table 15. As a result of multiplex PCR using diluted DNA, it was confirmed that neither *E. leei* nor *E. fugu* was detected in 6 pg/μl. In the case of *E. leei* was detected up to 60 pg/μl (5.5×10^5 copies/mg tissue), *E. fugu* was detected up to 60 pg/μl (3.65×10^4 copies/mg tissue) respectively (Fig. 5).

3.3.5. Standard curve efficiency

The real-time PCR assay to *E. fugu*, when performed in duplicate, was able to reliably detect the target plasmid DNA over a 9-log (from 3.88×10^3 to 3.88×10^9 copies/mg tissue) and a linear relationship with a correlation of determination (R^2) of 0.996 and slope of −3.33 and real-time PCR efficiency was 99.3%(Fig. 6a). The real-time PCR assay to *E. leei*, when performed in duplicate, was able to reliably detect the target plasmid DNA over a 9-log (from 3.85×10^3 to 3.85×10^9 copies/mg tissue) and a linear relationship with a correlation of determination (R^2) of 0.999 and slope of −3.519 and real-time PCR efficiency was 92.4% (Figure 6b). The qPCR efficiency was calculated using the formula: real-time PCR efficiency = $(10^{-1/\text{slope}} - 1) \times 100$.

3.3.6. Real-time PCR analysis

As a result of quantitative analysis of *E. fugu* in 9 tissues of No. 1 tiger puffer from a farm showing emaciation symptoms, intestinal tissue showed the highest DNA copy value of 4.39 $\times 10^6$ copies/mg tissue (Table 14). The rest of the tissues showed a value of 2.09 $\times$ 10^2 - 3.31 $\times$ 10^4 copies/mg tissue, confirming that *E. fugu* DNA was detected in all types of tissues tested. As a result of the analysis of *E. leei*, it showed the highest DNA copy value of 2.52 $\times$ 10^8 copies/mg tissue in intestinal tissue (Table 14). The rest of the tissue showed a value of 8.69 $\times$ 10^3 - 8.13 $\times$ 10^5 copies/mg tissue, confirming that *E. fugu* DNA was detected in all tissues tested. As a result of the 1-step PCR analysis, it was confirmed that *E. fugu* and *E. leei* were not detected in the brain and heart, whereas the real-time PCR method had higher sensitivity than the 1-step PCR method (Table 14).

3.3.7. Comparison of sensitivity

As a result of 1-step PCR, 2-step PCR and real-time PCR analysis of *E. fugu* and *E. leei* using diluted No. 1 tiger puffer intestine DNA, in the case of *E. fugu*, the 1-step PCR result showed up to 60 pg/μl (3.65 $\times 10^4$ copies/mg tissue) detection was confirmed, and as a result of 2-step PCR, detection was confirmed up to 600 fg/μl (1.07 $\times 10^2$ copies/mg tissue), and as a result of real-time PCR, detection was confirmed up to 600 fg/μl (1.07 $\times 10^2$ copies/mg tissue). As a result of the 1-step PCR analysis of *E. leei*, detection was confirmed up to 60 pg/μl (5.50 $\times 10^5$ copies/mg tissue) in the PCR result, 2-step PCR analysis showed that detection was confirmed same as real-time PCR analysis showed that detection was confirmed up to 6 fg/μl (1.11 $\times 10^2$ copies/mg tissue). as a real-time PCR result. When checking the results of the analysis of *E. leei* and *E. fugu*, 2-step PCR was confirmed to

have higher sensitivity than PCR (Table 15). In the results of *E. fugu*, 2-step PCR was confirmed to have lower sensitivity than real-time PCR (Table 15), and it was confirmed that the same concentration was detected in the results of *E. leei* (Table 15).

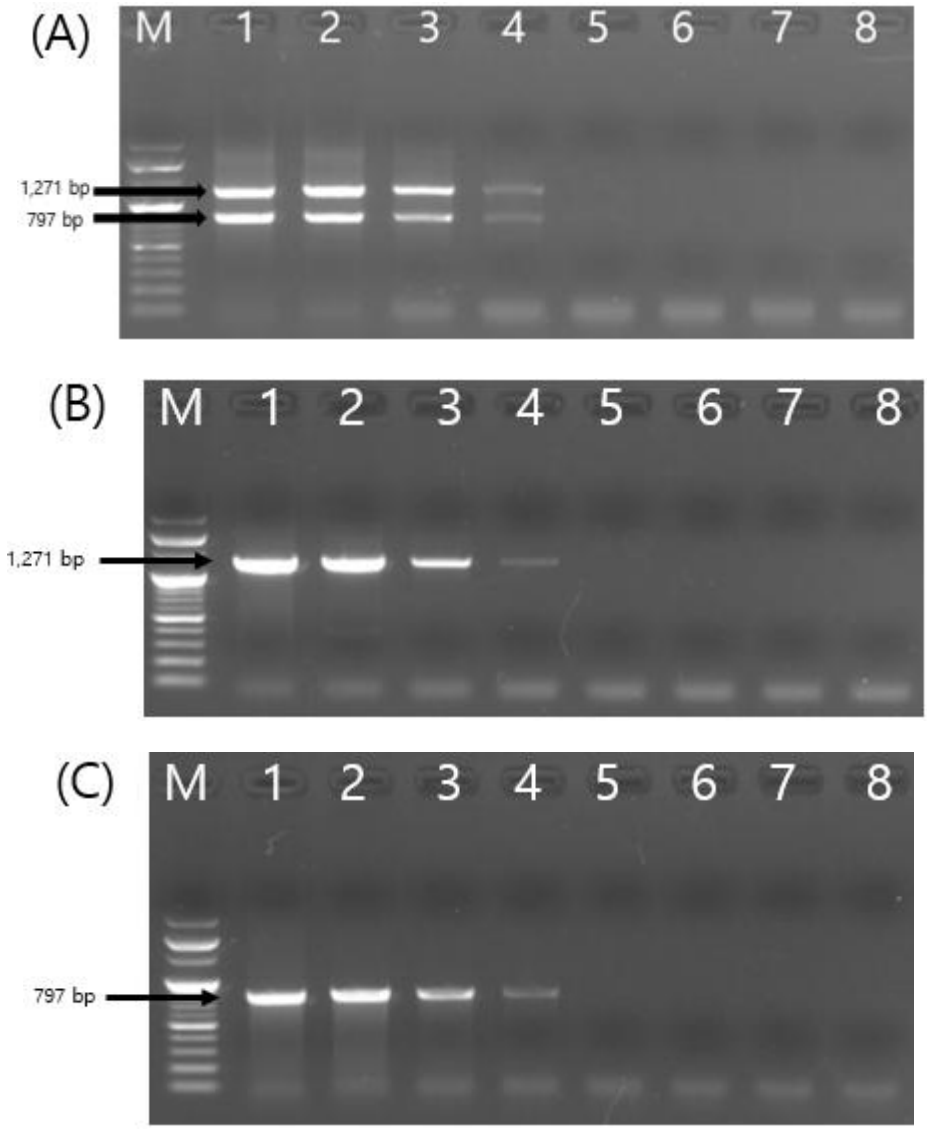

Fig. 5. (A) multiplex PCR amplification result using 10-fold diluted DNA from tiger puffer intestine DNA with the EL-2F/EL-1R primer set and EF-1F/EF-2R primer set. (B) 1-step PCR amplification result using the EL-2F/EL-1R primer set, (C) 1-step PCR amplification result using the EF-1F/EF-2R primer set. lane 1, intestine DNA (60 ng/μL); lane 2, intestine DNA (6 ng/μL); lane 3, intestine DNA (600 pg/μL); lane 4, intestine DNA (60 pg/μL); lane 5, intestine DNA (6 pg/μL); lane 6, intestine DNA (600 fg/μL); lane 7, intestine DNA (60 fg/μL); lane 8, intestine DNA (6 fg/μL); M, 100 bp DNA ladder.

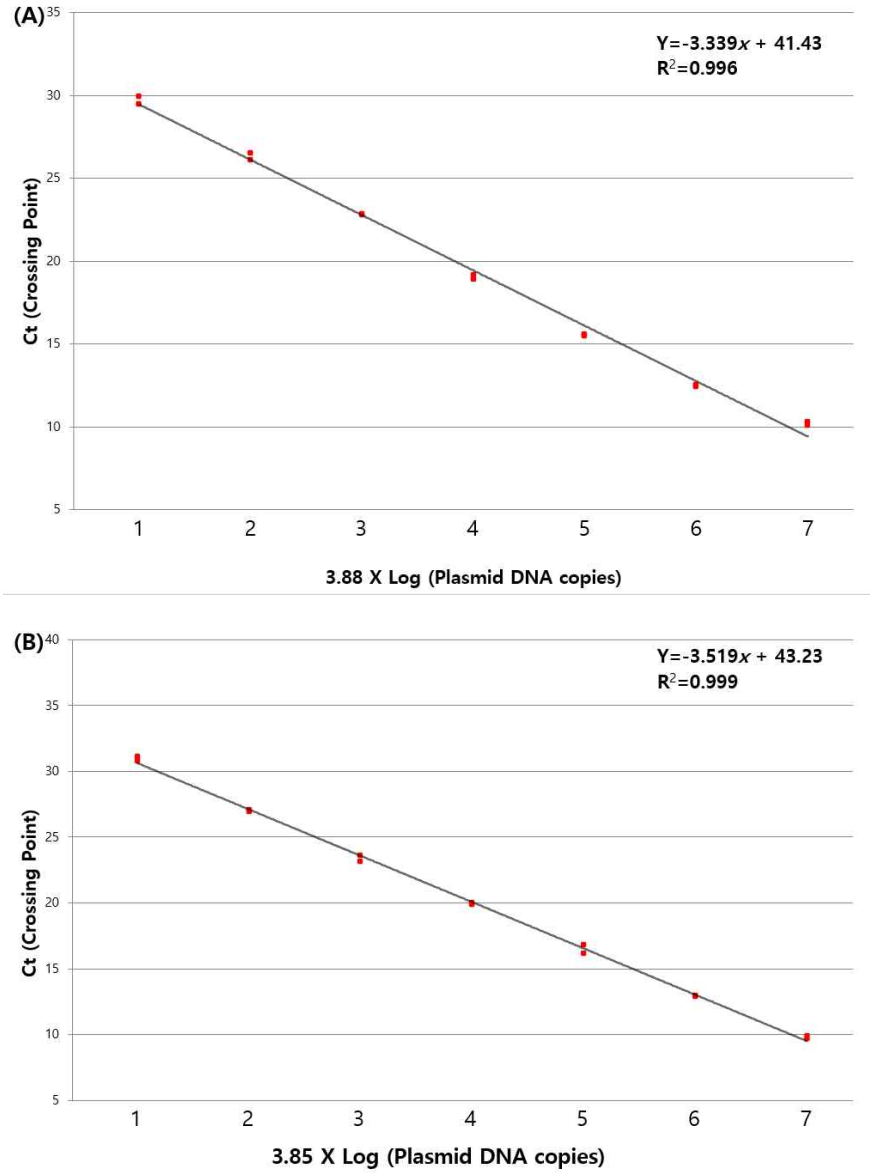

Fig. 6. Standard curve derived from 10-fold serially diluted plasmid DNA containing a partial of SSU rDNA sequence from *E. fugu* (A), standard curve derived from 10-fold serially diluted plasmid DNA containing a partial of 18S rDNA sequence from *E. leei* (B).

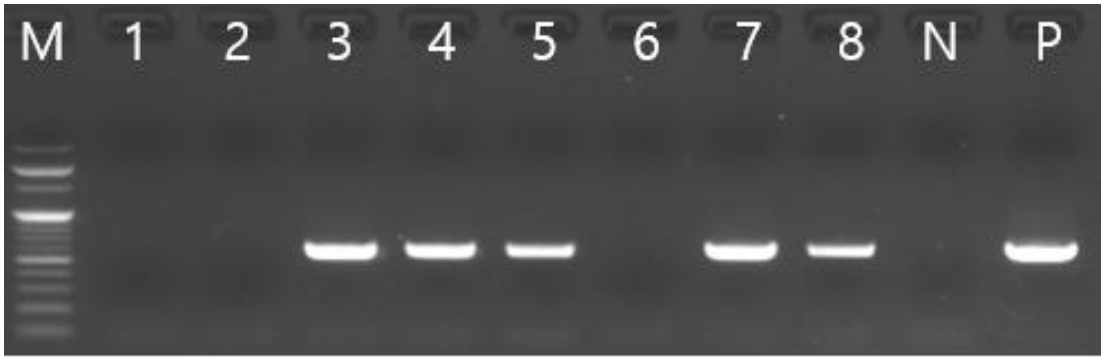

Fig. 7. 2-step PCR amplification result. Lane 1, group 1 kidneys DNA; lane 2, group 1 intestines DNA; lane 3, group 2 kidneys DNA; lane 4, group 2 intestines DNA; lane 5, group 3 kidneys DNA; lane 6, group 3 intestines DNA; lane 7, inflowing water sample DNA; lane 8, rearing water sample DNA; N, negative control; P, positive control; M, 100 bp DNA ladder.

Table 14. 1-step PCR, 2-step PCR and real-time PCR results for *E. leei* and *E. fugu*

Tiger puffer	Tissue	*E. leei*			*E. fugu*		
		Real-time PCR (copies/mg tissue)	1-step PCR	2-step PCR	Real-time PCR (copies/mg tissue)	1-step PCR	2-step PCR
No. 1	Intestine	2.52×10^{8}	P	P	4.39×10^{6}	P	P
	Liver	2.79×10^{5}	P	P	3.31×10^{4}	P	P
	Eye	3.03×10^{5}	P	P	9.94×10^{3}	P	P
	Gill	8.13×10^{5}	P	P	5.87×10^{3}	P	P
	Kidney	7.90×10^{4}	P	P	7.00×10^{3}	P	P
	Muscle	9.50×10^{4}	P	P	2.97×10^{3}	P	P
	Spleen	1.42×10^{4}	P	P	1.02×10^{3}	P	P
	Heart	8.69×10^{3}	-	P	2.09×10^{2}	-	P
	Brain	8.85×10^{3}	-	P	1.88×10^{2}	-	P
No. 2	Intestine	2.39×10^{9}	P	P	8.79×10^{6}	P	P
	Liver	8.25×10^{2}	-	P	1.72×10^{2}	P	P
	Eye	5.50×10^{3}	P	P	5.53×10^{2}	-	P
	Gill	1.53×10^{4}	-	P	1.95×10^{2}	-	P
	Kidney	4.87×104	P	P	6.50×10^{2}	P	P
	Muscle	1.06×10^{5}	P	P	2.13×10^{3}	P	P
	Spleen	5.69×10^{2}	-	P	1.67×10^{2}	-	P
	Heart	3.11×10^{3}	P	P	3.11×10^{1}	P	-
	Brain	2.07×10^{3}	-	P	3.60×10^{2}	-	P
No. 3	Intestine	2.50×10^{7}	P	P	2.64×10^{6}	P	
	Liver	1.24×10^{4}	-	P	1.73×10^{3}	-	
	Eye	1.20×10^{3}	-	P	9.56	P	
	Gill	9.88×10^{2}	-	P	8.88×10	-	P
	Kidney	4.45×10^{4}	P	P	1.04×10^{2}	P	P
	Muscle	8.79×10^{4}	P	P	1.56×10^{3}	-	P
	Spleen	3.15×10^{3}	-	P	7.37×10^{2}	-	P
	Heart	2.11×10^{4}	-	P	6.46×10^{2}	-	P
	Brain	1.20×10^{3}	-	P	5.24×10^{2}	-	P
No. 4	Intestine	5.10×10^{5}	P	P	2.06×10^{4}	P	P
	Liver	1.45×10^{3}	-	P	2.75×10^{2}	-	P
	Eye	1.63×10^{3}	-	P	1.04×10^{2}	-	P
	Gill	2.38×10^{3}	-	P	9.10×10	-	P
	Kidney	1.41×10^{4}	-	P	9.52	-	P
	Muscle	3.39×10^{4}	P	P	2.26×10^{2}	-	P
	Spleen	2.04×10^{3}	-	P	5.63×10	-	P
	Heart	8.93×10^{3}	-	P	5.26×10	-	P
	Brain	1.22×10^{3}	-	P	2.34×10	-	-

P, positive; -, negative.

Table 15. 1-step PCR, 2-step PCR and real-time PCR results for *E. leei* and *E. fugu*

Tiger puffer	Total DNA	*E. leei*			*E. fugu*		
		Real-time PCR (copies/mg tissue)	1-step PCR	2-step PCR	Real-time PCR (copies/mg tissue)	1-step PCR	2-step PCR
No. 1	6 ng/μl	4.86×10^7	P	P	4.40×10^6	P	P
	600 pg/μl	5.65×10^6	P	P	3.97×10^5	P	P
	60 pg/μl	5.50×10^5	P	P	3.65×10^4	P	P
	6 pg/μl	5.84×10^4	-	P	2.60×10^3	-	P
	600 fg/μl	5.55×10^3	-	P	1.07×10^2	-	P
	60 fg/μl	8.65×10^2	-	P	2.46×10	-	-
	6 fg/μl	1.11×10^2	-	P	-	-	-
	600 ag/μl	-	-	-	-	-	-

P, positive; -, negative.

3.4. Discussion

In this study, the causative factors of emaciation disease in cultured tiger puffer were investigated through molecular diagnostic tools such as PCR, 2-step PCR, and real-time PCR. We did not consider bacteria to be the causative pathogen of emaciation diseases cause, it was confirmed that previous studies showed severe emaciation disease was caused by myxosporean infection and bacteria were not directly related to disease (Choi et al., 2012, Yanagida 2017). On September, 2021, the tiger puffer suspected of emaciation disease was transported to the laboratory from Jeju farm in Korea. After then, a total of nine tissues were dissected and isolated from the tiger puffer: kidney, intestine, spleen, liver, gills, brain, muscle, heart, and eye. 1-step PCR analysis was performed to confirm infection caused by emaciation disease. In the case of Japan, it was confirmed that *E. leei* and *E. fugu* were detected (Yasuda et al., 2005, Ishimatsu et al., 2007, Yanagida et al., 2008). Result of sequencing alignment of *E. leei* PCR product, it was confirmed that it has 100% identities (1370/1370) to *E. leei* (accession number MH465674.1) and result of sequencing alignment of *E. fugu* PCR product, it was confirmed that it has 99.41% identities (1009/1015) to *E. fugu* (AY520573.1). These results show tiger puffers were co-infected by *E. leei* and *E. fugu.* it has been confirmed that the primer set EL-F/EL-R specific to *E. leei* reported in Korea (Kang et al., 2020), can bind not only to *E. leei* but also to the sequence of *E. fugu*. So we developed new 1-step PCR primer set specific to *E. leei* and *E. fugu*. To confirm the specificity of the newly prepared primers, 1-step PCR was performed that olive flounder infected by *E. leei* using the EF-1F/EF-R primer set specific to *E. fugu* and EL-1F/EL-1R primer set specific to *E. leei*. It was confirmed that *E. fugu* was not detected when

using the primer set EF-1F/EF-R specific to *E. fugu* and it was confirmed that *E. leei* was not detected when using the primer set EL-1F/EL-1R specific to *E. leei.* So, it was confirmed that the *E. fugu* primer did not specifically bind to *E. leei* sequence (data not shown). Moreover, previous studies showed, myxosporeans were isolated from the aquaculture water by filtration, it was confirmed that the detection was possible (Lee et al., 2021, Shin et al., 2018) and It was reported that non-lethal method could be possible (Lee et al., 2021). Through this method, it will be able to determine which pathogens are in the farm, without diagnosis of lethal method. But this method has low concentration DNA of pathogens than lethal method, therefore it is considered that a high sensitivity diagnosis method will be helpful for this method. In addition, since *E. fugu* was first report in Korea, rapid and high sensitivity diagnosis method would be needed to detect of emaciation disease by *E. fugu* and prevent disease transmission in advance. For these reasons, in this study, we developed real-time PCR method for early diagnosis of emaciation disease and we developed 2-step PCR method for early diagnosis of emaciation disease in 1-step diagnosis facilities without real-time PCR equipment. Additionally, we developed a multiplex PCR that is less time and resource intensive than traditional PCR through just one time PCR analysis identify two pathogens. In this study, as a result of comparing the sensitivity of multiplex PCR with 1-step PCR, multiplex PCR result of *E. leei*, it was confirmed that up to 6pg/μl (5.8×10^4 copies/mg tissue) was detected and in the case of *E. fugu,* it was confirmed that up to 60 pg/μl (3.65×10^4 copies/mg) were detected. As a result of comparing with 1-step PCR, in the case of *E. leei* 1-step PCR result, it was confirmed that up to 60 pg/μl (5.50×10^5 copies/mg tissue) was detected, *E. fugu* 1-step PCR result, it was confirmed that up to to 60 pg/μl (3.65×10^4 copies/mg tissue) were detected. These results show multiplex PCR has similar sensitivity to 1-step PCR and it is considered less consuming time and resource in

diagnosis. For comparison of sensitivity, 1-step PCR, 2-step PCR, and real-time PCR were performed using the serially 10-fold diluted DNA of No. 1 tiger puffer intestine DNA. in the case of 1-step PCR targeting *E. leei*, detection was confirmed up to 60 pg/μl (5.50 $\times 10^5$ copies/mg tissue), and in the case of 2-step PCR and real-time PCR, detection was confirmed up to 6 fg/μl (1.11 $\times 10^2$ copies/mg tissue). 1-step PCR, 2-step PCR, and real-time PCR methods results for *E. fugu*, in the case of 1-step PCR, detection was confirmed up to 60 pg/μl (3.65 $\times 10^4$ copies/mg tissue), and in the case of 2-step PCR result was confirmed up to 600 fg/μl (1.07 $\times 10^2$ copies/mg tissue) and real-time PCR, detection was confirmed up to 60 fg/μl (2.46 $\times 10$ copies/mg tissue). In conclusion, the results confirmed that real-time PCR has higher sensitivity than 1-step PCR as reported by Mackay et al., 2002 and as reported by Massung et al., 1998, 2-step PCR was confirmed to have high sensitivity and high specificity than 1-step PCR. In addition, according to real-time PCR analysis of tiger puffer 9 tissues samples, the DNA of *E. leei* and *E. fugu* were highest value in the intestine. These results suggested that these two myxosporean parasites could be pathogens causing emaciation disease. Presently, myxosporean infections in fish, there are no alternative for treatments and vaccines (Yokoyama et al., 2012). Myxosporean infection control strategies are mostly preventions for instances, a good management technique, reducing farm densities and improving water quality (Fontes et al., 2015) because several studies show that myxosporean infection relies on temperature, breeding density, time of exposure and parasite load (Picard et al., 2020). However, in the case of Korea, which is surrounded by the sea on three sides and possess abundant water resources, the running water culture method using seawater has been favorably used in terms of cost and efficiency. Currently, in Korea, most olive flounder farms in Jeju, use running water culture use seawater as it is without filtration or sterilization so, it can be vulnerable to disease transmission from other farms (Park, K.S. 2002).

Olive flounder, a representative breed of the domestic aquaculture industry accounts for about 50% of the total annual production of aquaculture fish in 2021. annual production of olive flounder was 41,776 tons of which the production of farmed olive flounder in Jeju was 21,463 tons (51.4%). It accounts for more than half of the total production and grows as a major aquaculture fish species. Therefore, emaciation disease will be considered to be a great threat to the aquaculture industry in Jeju. Emaciation disease was first recognized in the late 2000s in Korea and both pathogens *E. leei* and *P. anisocaudata* were reported to be emaciation disease to olive flounder (Kim et al., 2015; Sekiya et al., 2016). *E. fugu* was occurred for the first time in Korea in this study caused emaciation disease in tiger puffer and infection of olive flounder by *E. fugu* has not been reported. If olive flounder can be infected by *E. fugu*, it will be considered to be a major threat to Korean aquaculture industry. Result on this study, *E. fugu* was confirmed that it was detected olive flounder tissue sample, inflowing water sample and rearing water sample at olive flounder farm located near the tiger puffer farm (Fig. 7). *E. fugu*, which is transmitted to olive flounder farms, is considered be transmitted from a nearby tiger puffer farm. Since the intensity of infection by *E. fugu* was low, it was impossible to determine whether it was pathogenicity. So, further study must be required to explain how to *E. fugu* enters and spreads in the intestine of fish additionally the research is needed to consider the relation *E. fugu* infects olive flounder. For these studies, challenge test must perform of intentionally infecting olive flounder with *E. fugu.* In this study, the developed new primer sets for *E. fugu* and *E. leei* can help to prevent disease transmission of *E. leei* and *E. fugu* through early diagnosis and it will be a great advantage in reducing damage by establishing a rapid control strategies.

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