한국어병학회지 제30권 제1호 (2017)
J. Fish Pathol., 30(1) : 67~70

www.ksfp.org
pISSN 1226-0819, eISSN 2233-5412
http://dx.doi.org/10.7847/jfp.2017.30.1.067

Note

PCR-based identification of *Pseudomonas fluorescens* in diseased olive flounder, *Paralichthys olivaceus*, in Jeju Island, South Korea

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Pseudomonas is currently causing increasing mortality in farmed olive flounder in Jeju Island. It was previously reported that *P. anguilliseptica* is the pathogen causing the mortality. It is not known whether other sub-species are involved or not. In this study, *P. fluorescens* was identified from diseased olive flounder by a PCR-based diagnosis. Based on genomic sequencing and BLAST analysis, 5 out of 6 samples were closer with *P. fluorescens* than *P. anguilliseptica*. Our finding suggests that *P. fluorescens* may be the dominant species causing the disease in farmed olive flounder in Jeju Island, South Korea.

Key words: *Pseudomonas fluorescens*, Dominant species, Olive flounder, 16s rRNA, PCR

Pseudomonas fluorescens is a gram-negative, rod-shaped bacterium that secretes a soluble fluorescein particularly under low iron availability. It grows well in mineral salts media with carbon sources (Kreig et al., 1984). The bacterium was identified and characterized as a pathogen to olive flounder, *Paralichthys olivaceus*, in China. It causes the 'red skin disease' all year round upon inappropriate handling and leads to mortality (Zang et al., 2009). However, to our knowledge, *P. fluorescens* has not been reported in farmed olive flounder from Jeju Island, South Korea. Jeju is the main production area for the olive flounder in South Korea.

In farmed olive flounder from South Korea, *Streptococcus iniae*, *Streptococcus parauberis*, *Edwardsiella tarda,* and *Vibrio sp.* were known as the major bacterial pathogens. *S. iniae* (β-haemolysis) and *S. parauberis* (α-haemolysis) are gram-positive and catalase-negative bacteria that cause streptococcosis in the olive flounder (Nho et al., 2009). *E. tarda* is a gram-negative bacterium that cause edwardsiellosis in many fish (Park et al., 2012). Moreover, *P. anguilliseptica* has been recently focused on as a pathogen causing increasing mortality in farmed olive flounder in Jeju Island and this bacterium could cause up to 90 cumulative percent mortality with an infection dose of 3×10^8 CFU in fingering of olive flounder (Kang et al., 2015 and Jang et al., 2014b).

To investigate the prevalence of bacterial pathogens in farmed olive flounder, we randomly sampled the bacterial pathogens from diseased olive flounder such as from those showing dark body colour, haemorrhages, reddening of the body, exophthalmos, loss of appetite, and swollen abdomen, every week during the period from November 2015 to March 2016. The liver was the target organ for screening the bacterial pathogens in diseased olive flounder. Bacteria were

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Table 1. Gene specific primers used in this study

Pathogen	Gene specific primers (5' to 3')		Reference
Streptococcus parauberis	SP718-Forward SP718-Reverse	TTTCGTCTGAGGCAATGTTG GCTTCATATATCGCTATACT	(Lan et al., 2008)
Streptococcus iniae	SI300-Forward SI300-Reverse	CTAGAGTACACATGTAGCTAAG GGATTTTCCACTCCCATTAC	(Zlotkin et al., 1998)
Edwardsiella tarda	ET415-Forward ET415-Reverse	GCATGGAGACCTTCAGCAAT GCGGAGATTTTGCTCTTCTT	(Mata et al., 2004)
Yersinia ruckeri	YR-forward YR-reverse	TCCAGCACCAAATACGAAGG ACATGGCAGAACGCAGATC	(Keeling et al., 2012)
Aeromonas hydrophila	AH703-forward AH703-reverse	CCCCCTGGACAAAGACTGAC ACTTCTGGTGCAACCCACTC	GenBank: AY827493.1
Pseudomonas sp.	Psan-F Psan-3	TTGGGAGGAAGGGCAGTAACC TGCGCCACTAAAATCTCAAG	(Romalde et al., 2004)

Table 2. The bacterial pathogens detected from two fish farms in Jeju Island, South Korea. *Edwardsiella tarda* is 'T', *Streptococcus iniae* is 'I', *Streptococcus parauberis* is 'P', and an unknown bacterium is '+' in the bacterial screening from disease olive flounder. The unknown bacterium showed pink-coloured colonies on SS agar.

A. J fish farm (East of Jeju)

Date	Temp (°C)	Fish size	Bacteria			
			T	I	P	+
11. 2015	20	20cm			○	○
	18	29cm				○
12. 2015	18	33cm	○			○
	18	40cm	○	○		○
	18	42cm	○			○
	18	43cm	○			○
	18	50cm	○		○	○
	18	55cm			○	○
1. 2016	18	45cm				○
	18	37cm				○
	17	45cm	○		○	○
	17	47cm				○
2. 2016	16	38cm	○			○
	16	50cm				○
	16	58cm	○			
3. 2016	15	38cm	○			
	15	50cm	○			
	15	54cm	○			○
	16.8	33cm	○		○	○
	16.8	38cm				○
	16.8	45cm				○

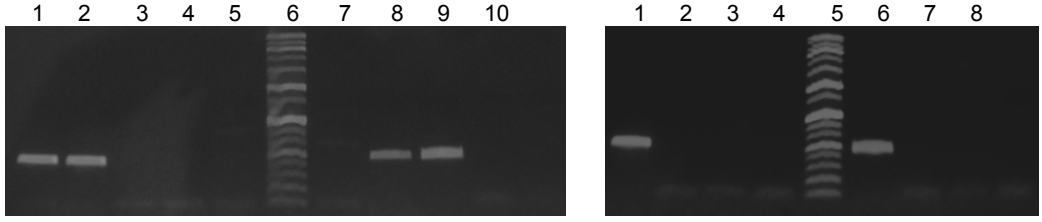
B. Y fish farm (West of Jeju)

Date	Temp (°C)	Fish size	Bacteria			
			T	I	P	+
11. 2015	20	10cm				○
	18	14cm	○	○		○
	18	24cm				○
12. 2015	18	17cm				○
	18	29cm	○			○
	18	30cm	○		○	
	18	32cm	○			
	18	35cm	○			○
	18	38cm	○			○
	17	12cm			○	○
1. 2016	17	38cm				○
	17	45cm			○	○
	14	37cm				
2. 2016	14	20cm	○			
	14	22cm				○
	14	23cm				○
	14	35cm		○	○	○
	14	44cm	○			○
3. 2016	14	20cm	○		○	○
	14	21cm	○			
	14	21cm				○
	14	26cm				○
	14	26cm				
	14	27cm	○			○
	14	33cm	○	○		○
	14	35cm	○			○

isolated using BHI, TCBS, SS, and blood agar plates. Selective agar plates (TCBS, SS agar) and the catalase test were used for the identification of bacteria, and blood agar was used to determine the haemolysis type of the bacteria. Finally, a direct colony PCR method (Sebastiao et al., 2015) with gene specific primers (GSPs) (Table 1) were used to identify the bacteria. In this study, among 47 samples, *E. tarda, S parauberis*, and *S. iniae* were detected 25, 10, and 4 times, respectively (Table 2). During screening of bacterial pathogens in diseased olive flounder, we incidentally found that the most prevalent bacterial colony was a pink one on the SS agar (38 times from 47 samples). Out of 38 samples, 18 pink-coloured colonies were sub-cultured with LB broth and stored at -80°C with 80 % sterile glycerol for further examination. To identify the unknown bacteria, three GSPs against *Yersinia ruckeri*, *Aeromonas hydrophila*, and *Pseudomonas sp.* (Table 1) were used for direct colony PCR method (Sebastiao et al., 2015). GPSs, Psan-F (21-mer) and Psan-3 (20-mer), were specifically designed to detect *P. anguilliseptica* in a previous study (Romalde et al., 2004). However, Psan-F (forward primer) was only 1 bp mismatched and Psan-3 (reverse primer) was 2 bp mismatched with *P. fluorescens* 16S rRNA genes (GenBank KT767960.1 and KT767959.1). Therefore, in current study, the GSPs, Psan-F and Psan-3, were used to detect both *P. anguilliseptica* and *P. fluorescens*.

A specific PCR band (418 bp) was detected in the direct colony PCR (denaturation at 95°C for 1 min, annealing at 52°C for 1 min, extension at 72°C for 1 min) with GSPs against *Pseudomonas sp.*. Furthermore, DNA samples from 18 isolates were extracted for conventional PCR (denaturation at 95°C for 1 min, annealing at 55°C for 1 min, extension at 72°C for 1 min). Of the 18 unknown bacterial samples, 6 were positive with GSPs against *Pseudomonas sp.* (Fig. 1A). All 6 PCR products were sequenced (SolGent Co., Ltd.). The 6 genomic sequences (377 bp, excepted 41 bp of primer parts) were analysed with the Basic Local Alignment Search Tool (BLAST) at NCBI and 97-100% similarities with the *P. fluorescens* 16S rRNA genes (GenBank KT767960.1 and

A.



J fish farm (10 samples) Y fish farm (8 samples)

B.

Sample	*P. fluorescens* 16S rRNA gene		*P. anguilliseptica* 16S rRNA gene	
	KT767960.1	KT767959.1	X99540	X99541
J fish farm 1	100 %	100 %	96 %	96 %
J fish farm 2	98 %	98 %	96 %	96 %
J fish farm 7	99 %	99 %	96 %	96 %
J fish farm 8	98 %	98 %	96 %	96 %
Y fish farm 1	100 %	100 %	96 %	96 %
Y fish farm 5	97 %	97 %	97 %	97 %

Fig. 1. PCR-based identification of the unknown pink-coloured colonies on SS agar. (A) PCR results (418 bp) from the unknown bacterial isolates with GSPs against *Pseudomonas sp.*. (B) 377 bp of genomic sequence results were compared with reference sequences, *P. fluorescens* 16S rRNA (GenBank KT767960.1, KT767959.1) and *P. anguilliseptica* 16S rRNA (GenBank X99540, X99541).

KT767959.1) (Fig. 1B).

A previous study using Psan-F and Psan-3 GSPs showed that all 11 isolates of *Pseudomonas* from olive flounder in Jeju were 99-100 % similarities with *P. anguilliseptica* 16S rRNA genes (GenBank X99540 and X99541) (Jang et al., 2014a). However, in this current study, 6 positive isolates of *Pseudomonas* from olive flounder in Jeju were only 96-97% similarities with *P. anguilliseptica* 16S rRNA genes (GenBank X99540 and X99541) (Fig. 1B) but 97-100% similarities with *P. fluorescens*.

P. anguilliseptica was previously reported as a deadly fish pathogen causing serious problems for olive flounder farming in Jeju Island (Kang et al., 2015). However, our finding suggests that *P. fluorescens* could be more dominant than *P. anguilliseptica* causing mortalities in farmed olive flounder in Jeju Island, South Korea.

References

Jan.Y.H., Identification, characterization, rapid detection technique development and vaccine production of major fish pathogen, Pseudomonas anguilliseptica isolated from farmed olive flounder, Paralichthys olivaceus. In: *Marine Life Sciences*. Jeju national university., 2014a.

Jan.Y.H., S.,D., Heo.M.S., Efficacy of formalin-killed Pseudomonas anguilliseptica vaccine on immune gene expression and protection in farmed olive flounder, Paralichthys olivaceus. *Vaccine.,* 32: 1808-1813, 2014b.

Kan.B.J, D.S., Jang.Y.H., Won.S.H, Heo.M.S.: Detection of Pseudomonas anguilliseptica form Olive flounder paralichthys olivaceus using Real-time PCR with a taqman fluorescent probe. *Fish Pathology.,* 50: 7, 2015.

Keeling.S.E., J.C., Wallis.R., Brosnahan.C.L., Gudkovs. N., Mcdonald.W.L., Development and validation of real-time PCR for the detection of Yersinia ruckeri. *J Fish Dis.,* 35: 119-125, 2012.

Kreig.N.R., H.,J.G. :*Pseudomonadaceae,* Baltimore: Williams and Wilkins Co., :, 1984.

Lan.J., Z.,X.H., Wang.Y., Chen.J., Han.Y.: Isolation of an unusual strain of Edwardsiella tarda from turbot and establish a PCR detection technique with the gyrB gene. *J Appl Microbiol.,* 105: 644-651, 2008.

Mata.A.I., G.A., Casamayor.A., Blanco.M.M., Dominguez. L., Fernandez-Garayzabal.J.F.: Multiplex PCR assay for detection of bacterial pathogens associated with warm-water Streptococcosis in fish. *Appl Environ Microbiol.,* 70: 3183-3187, 2004.

Nho.S.W., S.,G.W., Park.S.B., Jang.H.B., Cha.I.S., Han. M.A., Kim.Y.R., Park.Y.K., Dalvi.R.S., Kang.B.J., Joh.S.J., Jung.T.S., Phenotypic characteristics of Streptococcus iniae and Streptococcus parauberis isolated from olive flounder (Paralichthys olivaceus). *Fems Microbiology Letters.,* 293: 20-27, 2009.

Park.S.B., A.T., Jung.T.S.: Pathogenesis of and strategies for preventing Edwardsiella tarda infection in fish. *Veterinary Research.,* 43: 2012.

Romalde.J.L.,L.-R.S.L., Ravelo.C., Magarinos.B., Toranzo. A.E.: Development and validation of a PCR-based protocol for the detection of Pseudomonas anguiliseptica. *Fish Pathol.,* 39: 9, 2004.

Sebastiao.F.A., F.L.R., Hashimoto.D.T., Pilarski.F.: Identification of bacterial fish pathogens in brazil by direct colony PCR and 16s rRNA gene sequencing. *Advances in Microbiology.,* 5: 16, 2015.

Zang.W.W., H.,Y.H., Wang.H.L, Sun.L., Identification and characterization of a virulence-associated protease from a pathogenic Pseudomonas fluorescens strain. *Veterinary Microbiology.,* 139: 183-188, 2009.

Zlotkin.A., E.A., Ghittino.C., Bercovier.H.: Identification of Lactococcus garvieae by PCR. *J Clin Microbiol.,* 36: 983-985, 1998.

Manuscript Received : Mar 31, 2017
Revised : Apr 14, 2017
Accepted : Apr 14, 2017