DEVELOPMENT OF A MULTIPLEX PCR FOR DETECTION OF PATHOGENIC FLAVOBACTERIA CAUSING COLUMNARIS DISEASE IN TILAPIA AND ASIAN SEABASS

Puttipong Parangkul[1,*], Patcharapong Thangsunan[1], Pattanapong Thangsunan[1]

[1]Division of Biochemistry and Biochemical Innovation, Department of Chemistry, Faculty of science, Chiangmai university, Chiang Mai, Thailand

*Author's email address: puttipong_pa@cmu.ac.th

FACULTY OF SCIENCE
CHIANG MAI UNIVERSITY

Abstract

Tilapia and Asian seabass have been considered as two important fish species for the economy of Thailand. However, the aquaculture of these species is frequently threatened by an infection of *Flavobacterium* spp. which are primary causative pathogens of columnaris disease. The disease outbreak can reduce production yields and cause significant economic losses. Therefore, accurate and rapid diagnostic techniques for early identification of bacterial infection is of great importance. This study aimed to develop a multiplex PCR technique for simultaneous identification of four bacterial species causing columnaris disease, including *Flavobacterium covae, F. davisii, F. oreochromis,* and *F. columnare* in tilapia and Asian seabass. Four primer pairs were used in PCR experiments. The *FC-aroB, FD-hemW,* and *FO-E1* primer pairs were specifically designed for *F. covae, F. davisii,* and *F. oreochromis* respectively, whereas the *FcoI-cslA* primers were obtained from the previous work and used to identify all four species that had previously been classified into *F. columnare* group. Using singleplex PCR, the optimal PCR condition included an annealing temperature at 54 °C, and primer concentrations for *FC-aroB, FD-hemW, FO-E1,* and *FcoI-cslA* primers at 0.3, 0.2, 0.2, and 0.2 µM, respectively. However, the annealing temperature for the multiplex PCR assay was optimized at 55 °C with the same primer concentrations as used in the singleplex PCR. The developed multiplex PCR exhibited high specificity for the target DNA of all four flavobacteria, without cross-reactivity observed with other bacterial species tested. The multiplex PCR was highly sensitive with a detection limit of 2 ng/µL for *F. covae*, 400 pg/µL for *F. oreochromis*, 80 pg/µL for *F. davisii*, and 16 pg/µL for *F. columnare*. After challenged with a total of 25 bacterial DNA samples collected from local farms with the history of the disease outbreak, the multiplex PCR revealed 12 positive samples for *F. oreochromis*, 7 positive samples for *F. columnare*, and 6 positive samples for *F. covae*. However, no samples tested positive for *F. davisii*. These obtained data aligned well with the results from *16S rRNA* gene sequencing analysis. We anticipate that this multiplex PCR technique could be useful as an effective tool for controlling the outbreak of columnaris disease in tilapia and Asian seabass farming, which perfectly supports aquaculture industry of Thailand.

Introduction

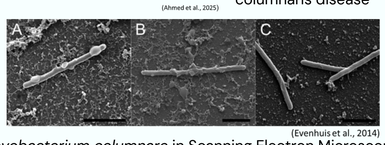
Tilapia and Asian sea bass are vital to Thailand's aquaculture, but their production yields have heavily been impacted by Columnaris disease, now known to be caused by four distinct Flavobacterium species (*F. covae, F. davisii, F. oreochromis,* and *F. columnare*).

To overcome the limitations of Singleplex PCR methods, this study developed a highly sensitive Multiplex PCR for simultaneous detection of four flavobacterial species. This tool enables faster diagnostics and more effective disease control for the Thai aquaculture industry.



(Ahmed et al., 2025)

Fig.1 Tilapia with the symptoms of columnaris disease



(Evenhuis et al., 2014)

Fig.2 *Flavobacterium columnare* in Scanning Electron Microscope (SEM)

Table 1 Comparison between singleplex PCR and multiplex PCR techniques.

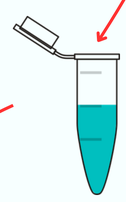
Comparison Aspect	Singleplex PCR	Multiplex PCR
Number of Primer Pairs	One primer pair per reaction	Multiple primer pairs in a single reaction
Applications	Detection of a single target gene or pathogen per reaction	detection of multiple target genes or pathogens in one reaction
Time and Cost	More time-consuming and costly when analyzing multiple targets	More time-efficient and cost-effective for multiple targets

Methodology

Table 2 Primers used in this experiment.

Flavobacterium species	Primer	Gene	Nucleotide sequence (5'–3')	Reference
Flavobacterium covae	*FC – aroB* F	*aroB*	CCAAAATTAGAAA CTGAACTATCT	This study
	FC – aroB R	*aroB*	TGATAAAGATTGTA AGTAATAGGAA	This study
Flavobacterium davisii	*FD – hemW* F	*hemW*	AAGCATTAGTTAA AGGGAAATCGTCA	This study
	FD – hemW R	*hemW*	AGCATAACTGGAA ATATGAGGAATA	This study
Flavobacterium oreochromis	*FO – E1* F	*odE1*	CAAATGGAGTAAT TGAAGCAATCA	This study
	FO – E1 R	*odE1*	TTGTTCTATCTCCC AATTGAGCG	This study
Flavobacterium columnare	*FcoI-cslA* F	*cslA*	AATGACTTCAACTA AGAACAGTAGGGTG CTGA	Mabrok et al. (2020)
	FcoI-cslA R	*cslA*	CTTGTTTATCATCTT CTTGATGTCCTCC	Mabrok et al. (2020)

- 10X PCR Buffer
- $MgCl_2$
- dNTP Mixture
- Forward primers
- Reverse primers
- DNA template
- Taq DNA polymerase



PCR reaction mixture

Optimization of Multiplex PCR

↓ Annealing Temperature at 48.6 - 56.5 °C
Concentration of primers at 0.1 - 0.5 µM

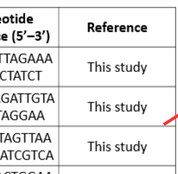
Specificity test

Sensitivity test

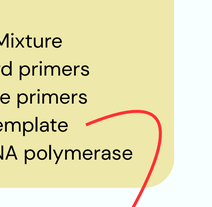
Test with real samples collected from Infected fish



thermal cycler



PCR result



gel electrophoresis

Results and Discussion

Optimization of PCR Technique

Optimization of Annealing Temperature in Singleplex PCR

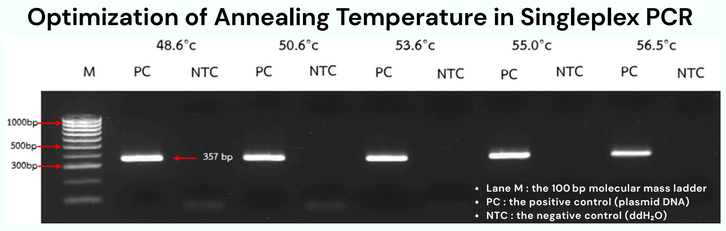


Fig.3 Optimization of annealing temperature for *FC – aroB* primers in singleplex PCR

Optimization of Primer Concentration in Singleplex PCR

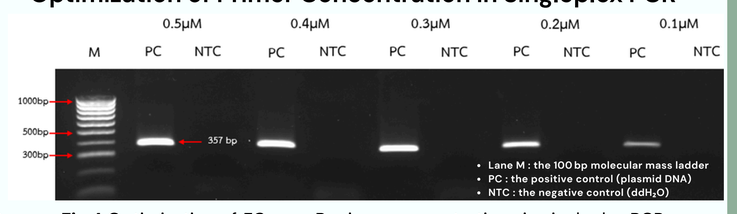


Fig.4 Optimization of *FC – aroB* primer concentrations in singleplex PCR

Optimization of Annealing Temperature in Multiplex PCR

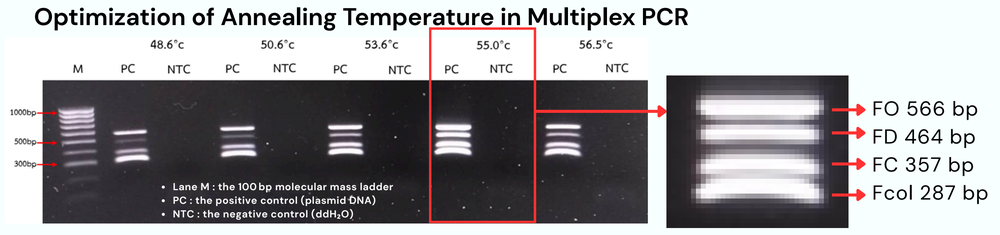


Fig.5 Optimization of annealing temperature in multiplex PCR

- The optimal temperature for primer binding is 55°C.
- The optimal primer concentrations for each species :
  - *Flavobacterium covae*: 0.3 µM
  - *Flavobacterium davisii*: 0.2 µM
  - *Flavobacterium oreochromis*: 0.2 µM
  - *Flavobacterium columnare*: 0.2 µM

Specificity Test of Multiplex PCR



Fig.6 Specificity test of multiplex PCR

The results demonstrate that this multiplex PCR assay possesses high specificity, effectively differentiating each species based on distinct DNA band sizes without cross-reactivity with other common pathogenic bacteria in tilapia and Asian seabass. Furthermore, the assay exhibits high sensitivity, with detection limits as follows:

- *Flavobacterium covae*: 2 ng/µL
- *Flavobacterium davisii*: 400 pg/µL
- *Flavobacterium oreochromis*: 80 pg/µL
- *Flavobacterium columnare*: 16 pg/µL

Sensitivity Test of Multiplex PCR

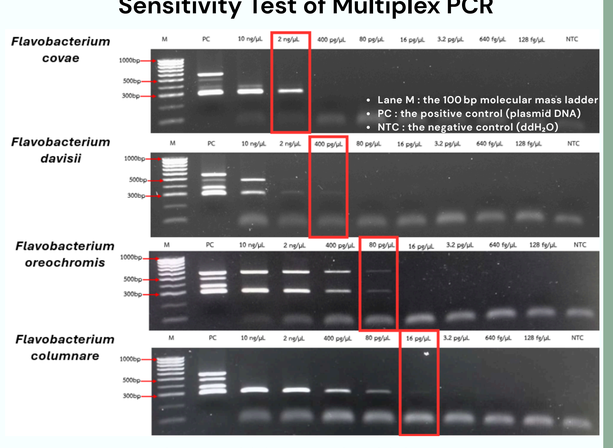


Fig.7 Sensitivity test of multiplex PCR

Testing Multiplex PCR with Real Samples

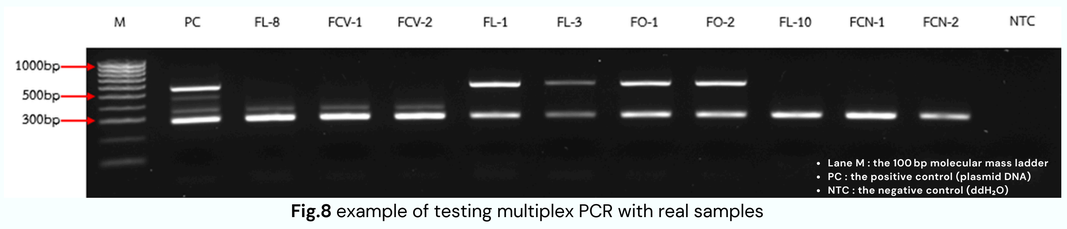


Fig.8 example of testing multiplex PCR with real samples

Table 3 Detection results of pathogenic flavobacteria in infected fish using multiplex PCR and *16S rRNA* seqencing analysis.

Sample	Host	*F. covae*	*F. davisii*	*F. oreochromis*	*F. columnare*	sequencing results
FL-8	Asian sea bass	+	-	-	-	***Flavobacterium covae***
FCV-1	Asian sea bass	+	-	-	-	***Flavobacterium covae***
FCV-2	Asian sea bass	+	-	-	-	***Flavobacterium covae***
FL-1	Red tilapia	-	-	+	-	***Flavobacterium oreochromis***
FL-3	Red tilapia	-	-	+	-	***Flavobacterium oreochromis***
FO-1	Black tilapia	-	-	+	-	***Flavobacterium oreochromis***
FO-2	Red tilapia	-	-	+	-	***Flavobacterium oreochromis***
FL-10	Koi carp	-	-	-	+	***Flavobacterium columnare***
FCN-1	Fancy carp	-	-	-	+	***Flavobacterium columnare***
FCN-2	Fancy carp	-	-	-	+	***Flavobacterium columnare***
FL-17	Arowana	+	-	-	-	***Flavobacterium covae***
FL-18	Giant catfish	+	-	-	-	***Flavobacterium covae***
FL-20	Walking catfish	+	-	-	-	***Flavobacterium covae***
C7	Asian sea bass	-	-	+	-	***Flavobacterium oreochromis***
FL-2	Red tilapia	-	-	+	-	***Flavobacterium oreochromis***
FL-4	Red tilapia	-	-	+	-	***Flavobacterium oreochromis***
FL-5	Red tilapia	-	-	+	-	***Flavobacterium oreochromis***
FL-9	Asian sea bass	-	-	+	-	***Flavobacterium oreochromis***
FL-19	Siamese pangasius	-	-	+	-	***Flavobacterium oreochromis***
FL-23	Asian sea bass	-	-	+	-	***Flavobacterium oreochromis***
FL-24	Asian sea bass	-	-	+	-	***Flavobacterium oreochromis***
FL-11	Koi carp	-	-	-	+	***Flavobacterium columnare***
FL-14	Koi carp	-	-	-	+	***Flavobacterium columnare***
FL-12	Koi carp	-	-	-	+	***Flavobacterium columnare***
FL-13	Koi carp	-	-	-	+	***Flavobacterium columnare***
total	-	6	0	12	7	-

$$\text{Percentage Similarity} = \frac{\text{Number of consistent results}}{\text{Total number of identified sequences}} \times 100$$

$$\text{Percentage Similarity} = \frac{25}{25} \times 100$$

$$\text{Percentage Similarity} = 100\%$$

From the 25 samples, *Flavobacterium covae* was detected in 6 samples, *F. oreochromis* in 12 samples, and *F. columnare* in 7 samples. Interestingly, *F. davisii* was not detected in any of the samples tested. This study focuses on infections in tilapia and Asian seabass, which are primarily associated with *F. oreochromis* and *F. covae*, respectively. The experimental results were consistent with the sequencing results, demonstrating that this multiplex PCR is a highly effective tool for accurate detection and species classification within a single reaction.

Acknowledgements

This research would like to express sincere gratitude to Asst. Prof. Dr. Pattanapong Thangsunan for his invaluable guidance and support. Appreciation is also extended to the Division of Biochemistry and Biochemical Innovation, Department of Chemistry, Chiang Mai University, for providing facilities and support. Finally, sincere thanks are given to all laboratory members for their assistance throughout the study.

References

- Mabrok, M., Chokmangmeepisarn, P., LaFrentz, B. R., Kayansamruaj, P., Dong, H. T., & Rodkhum, C. (2020). Development of a species-specific polymerase chain reaction for highly sensitive detection of Flavobacterium columnare targeting chondroitin AC lyase gene. Aquaculture, 521, 734597. https://doi.org/10.1016/j.aquaculture.2019.734597
- LaFrentz, B. R., Králová, S., Burbick, C. R., Alexander, T. L., Phillips, C. W., Griffin, M. J., Waldbieser, G. C., García, J. C., de Alexandre Sebastião, F., Soto, E., Loch, T. P., Liles, M. R., & Snekvik, K. R. (2022). The fish pathogen Flavobacterium columnare represents four distinct species … and emended description of Flavobacterium columnare. Systematic and Applied Microbiology, 45(2), 126293. https://doi.org/10.1016/j.syapm.2021.126293
- Nguyen, D. H. M., Chokmangmeepisarn, P., Khianchaikhan, K., Morishita, M., Uchuwittayakul, A., LaFrentz, B. R., Rodkhum, C., et al. (2025). Comparative genomic analysis of Flavobacterium species causing columnaris disease of freshwater fish in Thailand: insights into virulence and resistance mechanisms. BMC Veterinary Research, 21, 357. https://doi.org/10.1186/s12917-025-04488-3

Conclusion

- Optimal PCR condition was established at an annealing temperature of 55°C and primer concentrations between 0.2 - 0.3 µM.
- Multiplex PCR is highly specific for four flavobacterial species without cross-reactivity with other common aquatic bacteria.
- Multiplex PCR exhibited high sensitivity with low detection limits to pg levels.
- Multiplex PCR was successfully implemented with real-world situation.
- The developed Multiplex PCR is accurate, rapid, and highly effective for diagnosing flavobacterial infections in the Thai aquaculture industry.