Development of Recombinase Polymerase Amplification Combined with Lateral Flow Assay for Rapid Identification of *Flavobacterium covae* and *Flavobacterium oreochromis* in Tilapia and Asian Seabass

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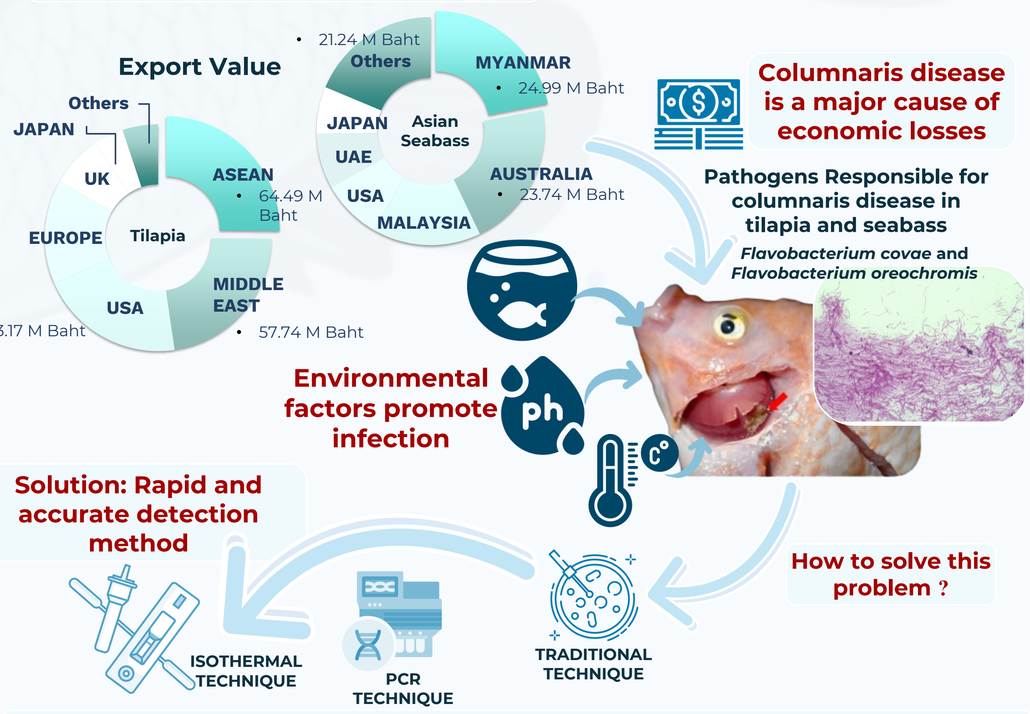
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Abstract

Columnaris disease caused by a group of *Flavobacterium* spp. can lead to significant economic losses in aquaculture industry. *Flavobacterium covae* and *F. oreochromis* are two predominant pathogens affecting tilapia and Asian seabass farming in Thailand. Although the standard PCR is effectively used for early detection of the disease, it remains limited by time consumption, high equipment costs, and the requirement of skilled users to perform the tests. Therefore, this study aims to develop a duplex recombinase polymerase amplification (RPA) technique in combination with a lateral flow assay (LFA) to accelerate the early monitoring of *F. covae* and *F. oreochromis* in tilapia and Asian seabass. The study involved the design of primers specific for *F. covae* (*aroB* gene) and *F. oreochromis* (*odE1* gene), optimization of RPA conditions, specificity, and sensitivity tests. Our results showed that the optimal condition for the duplex RPA was an incubation at 39°C for 35 min, followed by 20 min of LFA. The developed RPA-LFA was highly specific for both *F. covae* and *F. oreochromis* without cross-reactivity with other fish pathogens. The RPA-LFA was also highly sensitive with a limit of detection (LoD) as low as 0.64 pg/μL and 0.4 ng/μL for *F. covae* and *F. oreochromis*, respectively. Additionally, the developed RPA-LFA was challenged with real samples collected from fish farms with the records of the disease outbreak. From a total of 25 gill samples from infected fish, the assay detected *F. covae* in 6 samples, *F. oreochromis* in 10 samples, and both bacterial species in 2 samples. These results showed a consistency of 96% with the PCR technique. It is anticipated that the developed duplex RPA-LFA technique could become an invaluable bioanalytical alternative for controlling the outbreak of columnaris disease, especially for onsite applications and resource-limited laboratories, and subsequently supporting aquaculture industry of Thailand.

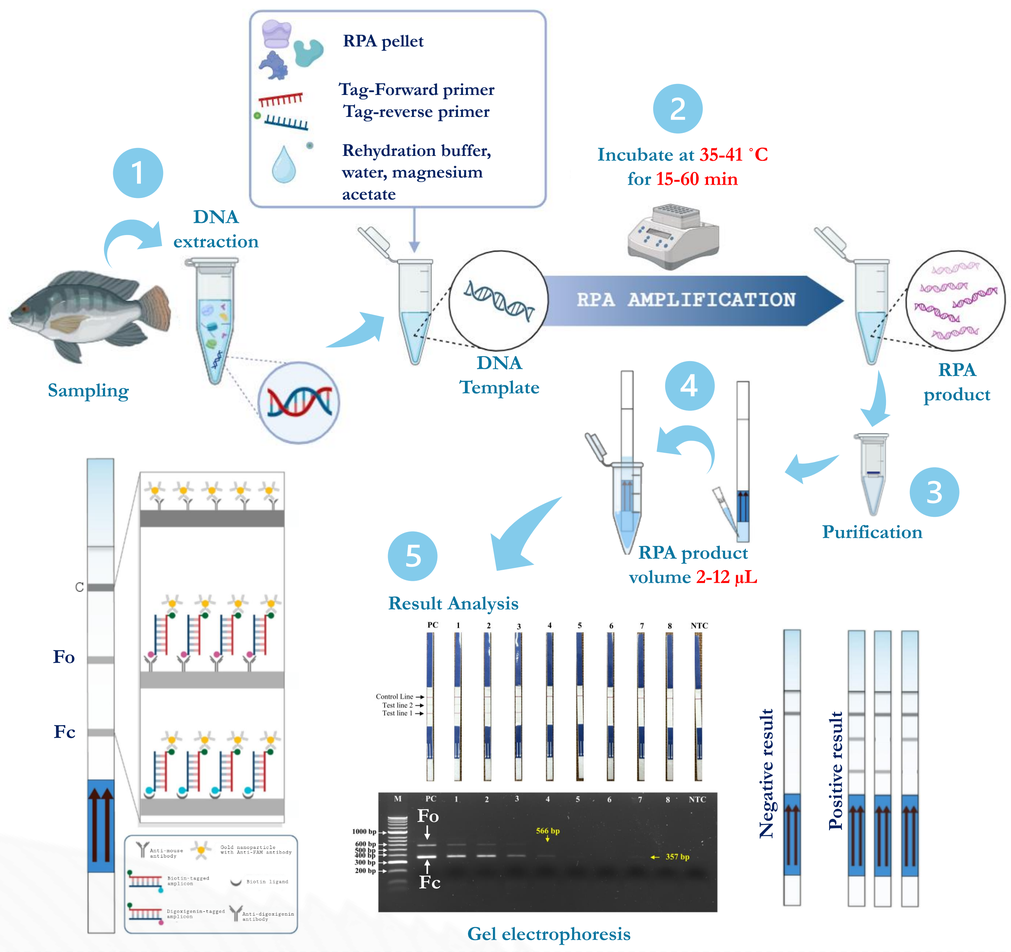
Introduction

Why are these fish species important ?



Our research aims to develop a simple point-of-care diagnostic test by combining isothermal RPA with lateral flow assay to detect *F. covae* and *F. oreochromis* infection.

Methodology



Conclusion

- **Isothermal Efficiency** — Operation at a constant temperature (**37–39°C**)
- **Rapid Results** reducing the process from 2 h to only **30–60 min**
- **Field Validation** **96% agreement rate** compared to gold-standard PCR.
- **Extreme Sensitivity** — **Low detection limits**
- **High Accuracy** — **High specificity** for *F. covae* and *F.oreochromis*.

Acknowledgements

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Results and Discussion

Optimization for Duplex RPA-LFA

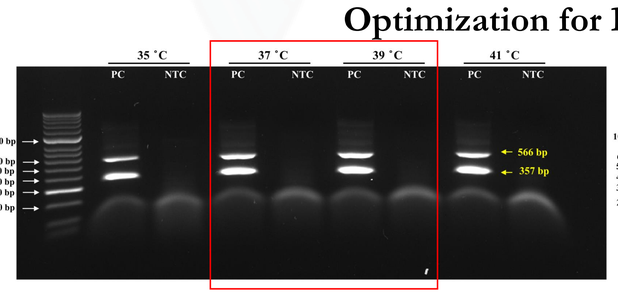


Figure 1: Optimization of Incubation Temperature for Duplex RPA

- **Optimal Temperature Range**: The system was optimized to a thermal window of **37–39°C**.
- **Diagnostic Precision**: Operating the assay within this window successfully minimized the **non-specific DNA amplification.**

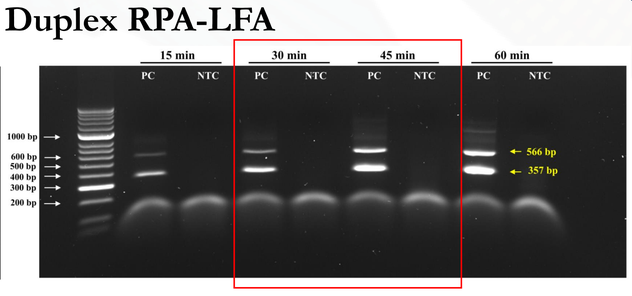


Figure 2: Optimization of Incubation Time for Duplex RPA

- **Optimal Time Window:** An incubation period of **30–45 min.** was identified as the ideal duration for the reaction.
- **Artifact Suppression:** Operating the assay within this timeframe successfully minimized the formation of **non-specific products** that tended to appear at longer durations.

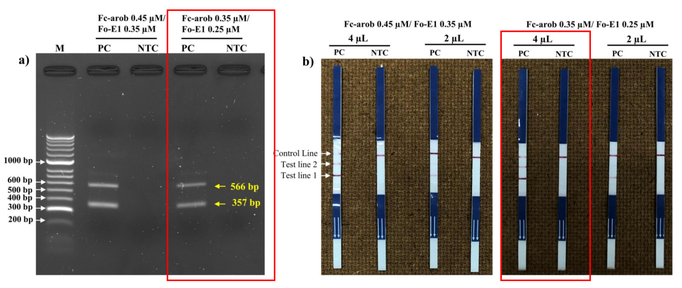
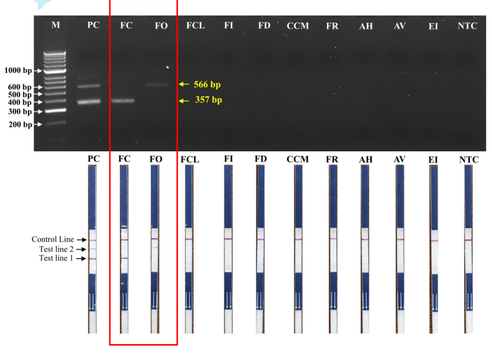


Figure 3: Optimization of Condition for LFA

- **Primer Optimization**: Reducing primer concentrations to **0.35 µM (*Fc*)** and **0.25 µM (*Fo*)** successfully eliminated non-specific bands.
- **Loading Volume**: Evaluation of various loading volumes showed that **4 µL** provided superior band intensity on the LFA compared to 2 µL.

Specificity Test of RPA-LFA



Bacterial Species	
Flavobacterium covae	FC
Flavobacterium oreochromis	FO
Flavobacterium columnare	FCL
Flavobacterium indicum	FI
Flavobacterium davisii	FD
Candidatus Chryseobacterium massiliae	CCM
Flectobacillus roseus	FR
Aeromonas hydrophila	AH
Aeromonas veronii	AV
Edwardsiella ictaluri	EI

Figure 4: Specificity of the Duplex RPA-LFA Against Common Aquatic Pathogens.

- **Target-Specific Amplification**: The assay successfully amplified RPA products in the reactions containing only the targeted ***F. covae*** and ***F. oreochromis*** DNA.
- **Methodological Consistency**: Both **gel electrophoresis** and **LFA results** showed **100% consistency**, with no false positives observed for non-target species.

Sensitivity Test of LFA

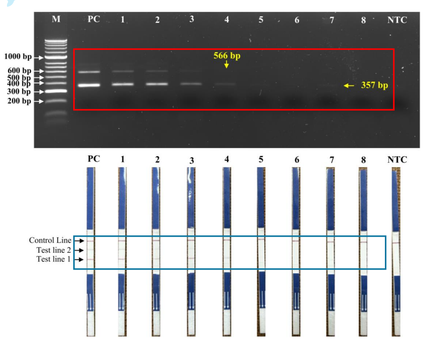


Figure 5: Determination of Limit of Detection (LOD) for the RPA-LFA

- **Fc Sensitivity:** Detected as low as **0.64 pg/µL**.
- **Fo Sensitivity:** Detected as low as **0.4 ng/µL**.
- **Method Correlation:** 100% agreement between Gel and LFA results, validating the LFA for field-use without loss of sensitivity.

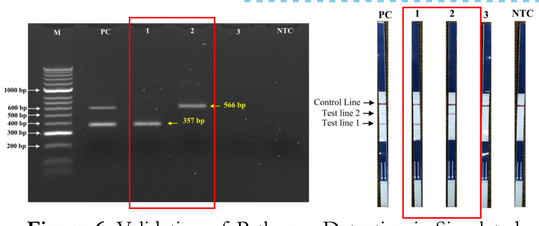
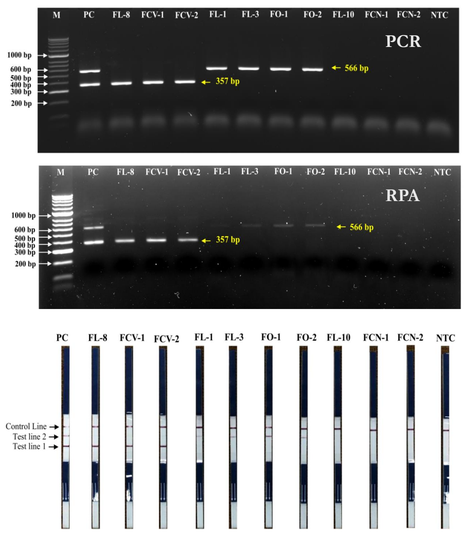


Figure 6: Validation of Pathogen Detection in Simulated Natural Infection

Testing RPA-LFA with Challenged Samples

- **Natural Infection Simulation:** The system successfully identified infections in Nile Tilapia under conditions designed to mimic natural environments.
- **Tissue Detection:** Analysis confirmed the infection of both *F. covae* and *F. oreochromis* in the challenged fish samples.

Testing RPA-LFA with Real Sample



- **High Correlation**: The results demonstrated a high degree of correlation, achieving a **96% similarity rate** between the RPA-LFA and traditional PCR results.

Sample NO.	Sample	Host	RPA with LFA		PCR	
			F. covae	*F. oreochromis*	*F. covae*	*F. oreochromis*
1	FL-8	Asian seabass	+	-	+	-
2	FCV-1	Asian seabass	+	-	+	-
3	FCV-2	Asian seabass	+	-	+	-
4	FL-1	Red Tilapia	-	+	-	+
5	FL-3	Red Tilapia	-	+	-	+
6	FO-1	Black Tilapia	-	+	-	+
7	FO-2	Red Tilapia	-	+	-	+
8	FL-10	Koi carp	-	-	-	-
9	FCN-1	Fancy carp	-	-	-	-
10	FCN-2	Fancy carp	-	-	-	-
11	FL-17	Arowana	+	-	+	-
12	FL-18	Giant catfish	+	-	+	-
13	FL-20	Walking catfish	+	-	+	-
14	C7	Asian seabass	-	+	-	+
15	FL-2	Red Tilapia	-	+	-	+
16	FL-4	Red Tilapia	-	+	-	+
17	FL-5	Asian sea bass	+	+	-	+
18	FL-9	Asian sea bass	+	+	+	+
19	FL-19	Siamese pangasius	-	+	-	+
20	FL-23	Asian sea bass	-	+	-	+
21	FL-24	Asian sea bass	-	+	-	+
22	FL-11	Koi carp	-	-	-	-
23	FL-14	Koi carp	-	-	-	-
24	FL-12	Koi carp	-	-	-	-
25	FL-13	Koi carp	-	-	-	-

The RPA-LFA was tested against 25 biological samples collected directly from fish farms across Thailand.

Figure 7: Field Validation Using Real-World Samples

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