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Research Note

A Modified Approach for Isolation of *Vibrio vulnificus* from Aquaculture Settings

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Abstract

The isolation of *Vibrio vulnificus* is crucial for understanding its prevalence and pathogenicity. The isolation procedure to enhance the detection of *V. vulnificus* in aquaculture samples was optimized using a double plate differentiation method. The plates used were Thiosulphate Citrate Bile Salt Sucrose Agar (TCBS) and Hichrome Agar Vibrio. Two independent studies were conducted: one involving the addition of fish samples with physiological saline as diluent, and the other with Alkaline Peptone Water (APW) enrichment. Our findings revealed that direct plating without enrichment, prior to the selection of presumptive colonies on TCBS agar, significantly increased the likelihood of isolating *V. vulnificus.* The molecular identity of this pathogen was confirmed using PCR targeting the *vvh* gene. Additionally, significant variations in the abundance of *V. vulnificus* were observed in different aquatic samples, emphasizing the importance of sample type in isolation efficiency. The enrichment procedure yielded a 2.9% prevalence, whereas the direct plating resulted in a 10.2% prevalence of the bacteria. Furthermore, this study underscores the necessity of direct plating to optimize the isolation process of *V. vulnificus* from diverse aquatic sources. The developed procedure for isolation of *V. vulnificus* is less time-consuming, less expensive (<92.4%), and statistically significant compared to conventional isolation procedures. These results contribute to a better understanding of *V. vulnificus* ecology and its potential implications for public health.

Keywords: Aquaculture, protocol optimization, *Vibrio vulnificus*, *vvh* locus, double plate differentiation

Introduction

Vibrio vulnificus, a zoonotic pathogen, has the capacity to induce diseases in humans and aquatic animals, occasionally culminating in sepsis and mortality. Among the crucial foodborne pathogens, *V. vulnificus* displays the highest fatality rate (> 50%), with males and older individuals with compromised immunity being at high risk of infection (Oliver, 2015). Based on their associated biochemical profiles linked to virulence, the species are further categorized into three biotypes: biotype 1 (associated with human infections), biotype 2 (known as an eel pathogen but occasionally infects humans), and biotype 3 (causing wound infections in fish handlers) (Lydon, Kinsey, Le, Gulig, & Jones, 2021). Among the three, biotype 1 strains have been documented as etiological agents of a spectrum of diseases, encompassing primary sepsis, and oftencited as to having fatality rates exceeding 50% (Heng et al., 2017). In addition to human infections, *V. vulnificus* is frequently associated with vibriosis in aquatic animals, particularly shellfish. Its natural

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habitat comprises warm coastal environments (preferably a water temperature of more than 18°C) characterized by low salinity or brackish conditions (Velez, Leighton, Decho, Pinckney, & Norman, 2023). Despite its preference for warm temperatures, occurrences of this species have been documented in various regions across the globe, spanning different climatic zones, including India (Heng et al., 2017).

In India, *V. vulnificus* is recurrently isolated from seafood and marine environments, with incidence rates reported as 13-16% in marine fishes, 38.5% in molluscan shellfish, and 43-75% in oysters (D'Souza et al., 2020). Significantly, vibriosis caused by these species not only amplifies the disease burden but also poses challenges to the growth of the aquaculture industry, with a projected annual economic loss, estimated at USD 9 billion (Sem, Gani, Chong, Natrah, & Shamsi, 2023). Therefore, understanding the occurrence and traits of this hazardous pathogen is paramount for devising targeted mitigation strategies. Consistent screening for *V. vulnificus* and other pathogens in aquaculture serves as a proactive measure to protect the safety of seafood products, uphold public health standards, and sustain the industry's trajectory and reputation. This approach not only promotes responsible aquaculture practices but also aligns with the overarching objective of delivering safe and nutritious seafood to consumers.

For the isolation of *V. vulnificus*, both enrichment and direct plating methods are commonly employed, with the choice between them contingent upon various factors, including the initial bacterial load, the presence of competing bacteria, and the desired sensitivity of detection (Muthulakshmi & Mothadaka, 2023). Enrichment entails pre-incubating the sample in a selective medium, such as alkaline peptone water (APW), to foster the growth of *Vibrio* spp. and facilitate their enrichment (Lesmana, Rockhill, Sutanti, & Sutomo, 1985). APW provides an optimal environment for the proliferation of *V. vulnificus* along with other *Vibrio* spp., enabling it to reach detectable levels. This method proves particularly beneficial when the initial bacterial load is low or when *V. vulnificus* coexists within a complex microbial community. Following enrichment, a portion of the culture may be streaked onto selective agar, such as thiosulfate-citrate-bile salts-sucrose (TCBS) agar, for the selection of presumptive colonies.

Conversely, direct plating involves inoculating the sample directly onto a selective agar medium, such as TCBS agar, without prior enrichment. TCBS agar selectively inhibits the growth of many non-*Vibrio* bacteria while promoting the growth of *Vibrio* species (McCormack et al., 1974). This approach is often preferred when the initial bacterial load is relatively high. Moreover, although TCBS agar facilitates the differentiation of sucrose-fermenting *V. cholerae* from other sucrose non-fermenting species, it is not a suitable method for specifically isolating *V. vulnificus*, as it is one of several sucrose non-fermenting species. In aquatic environments, *V. parahaemolyticus* is a major competitor to *V. vulnificus*. Both species are sucrose non-fermenters, rendering them indistinguishable using TCBS agar. Such circumstances underscore the necessity for a modified isolation approach that enhances the likelihood of isolating *V. vulnificus* over other species, particularly *V. parahaemolyticus*. This objective forms a primary focus of the current study.

Materials and Methods

The *V. vulnificus* VV02 strain, obtained from ICAR-CMFRI reference culture collection in Kochi, India, was employed as the positive strain throughout the study. Alkaline Peptone Water (Himedia) served as the enrichment medium, whereas TCBS (BD Difco) was used for the selection of presumptive colonies. Cultures were maintained at -80°C as glycerol stocks (20%). To maintain viability, cells were regularly revived in Trypticase Soy broth (TSB, BD Difco) supplemented with 2% (w/v) sodium chloride.

Etroplus sp. samples, collected from aquaculture farms in Kochi, India, were spiked with *V. vulnificus* VV02 strain at a concentration of 10^7 CFU/mL and subsequently incubated at room temperature for four hours. Prior to inoculation, the collected fish samples were confirmed to be free of *V. vulnificus* through PCR analysis targeting the *vvh* gene using primers (forward 5'-TGTTTATGGTGTTTGCTCG-3'; reverse 5'-TCCTACCCAACAGCCAATG-3') with an expected amplicon length of 519 bp (Hill et al, 1991). The cyclic conditions used for the detection of the *vvh* gene were as follows: Initial denaturation at 94°C for 3 minutes, denaturation at 94°C for 30 seconds, annealing at 60°C for 30 seconds, extension at 72°C for 1 minute, 30-35 cycles, and final extension at 72°C for 5-10 minutes. To recover the bacteria, 25 g of fish sample were added to 225 mL of sterile physiological saline (0.85%). In a separate

batch, 25 grams of the sample were transferred to 225 ml of sterile APW (pH 9) enrichment broth. Both batches underwent serial dilution with saline and enriched samples in alkaline peptone water, respectively. The serially diluted samples were then spread plated onto TCBS plates, and the bacterial count on the TCBS plates was determined and incubated at 30°C for 24h (Uchiyama, 2000).

To validate the effectiveness of the isolation procedure, a total of 68 samples, including fish (n= 22), sediment (n =11), and water (n = 35) were collected from aquatic farms in Kochi, Kerala, India. The modified protocol involved transferring 25 g of fish samples (including both the edible meat portion and the gut portion), 25 g homogenized sediment sample, and 25 mL of pond water separately into 225 mL of physiological saline, followed by plating onto TCBS agar.

The green colonies from TCBS were transferred to Hichrome Vibrio agar, and the mauve colored colonies were selected for further study. The isolates were stored in Marine agar slants. The molecular identity of the isolates was then confirmed by detecting the *vvh gene*, as previously described.

The Kruskal-Wallis ANOVA was performed to compare the *Vibrio* counts in samples treated with the two methods (Kruskal and Wallis,1952). This nonparametric method is used to compare the relative abundance of vibrios.

Results and Discussion

The *Vibrio* count from the saline dilution, namely the green (non-sucrose fermenting) and yellow (sucrose fermenting) colonies, was recorded. *V. vulnificus* belongs to the non-sucrose fermenting category. Therefore, the main competitors are *V. parahaemolyticus* and *V. mimicus*. The colonies of *V. mimicus* can tolerate up to 0-3% salt, while *V. parahaemolyticus* and *V. vulnificus* have almost the same salt tolerance strengths, that is 1-8% for *V. parahaemolyticus* and 1-6% for *V. vulnificus*.

In the enrichment-streaked plate, the colony count reflects the presence of yellow and green colonies but does not provide an accurate representation of CFU per gram, as seen in the direct plating method. Similarly, the total *Vibrio* count from fish samples was recorded and presented in Table 1. The result showed that APW enrichment yielded 2.95% prevalence of *V. vulnificus*, while the direct plating gave four times higher prevalence for the same samples, that is 10.2%.

The present study revealed a notably higher likelihood of isolating *V. vulnificus* from samples treated with physiological saline. Additionally, the abundance of other species, particularly the primary competitor *V. parahaemolyticus*, was significantly lower in samples treated with physiological saline compared to those enriched in APW cultures. The discrepancy in the initial count was amplified during the enrichment process. Thus, the chance of isolating *V. vulnificus* using an inoculation loop is minimal compared to the more abundant *V. parahaemolyticus*. PCR detection of the *vvh* gene confirmed the molecular identity of *V. vulnificus*.

The present study has standardised a modified isolation approach, leading to an enhanced isolation rate of *V. vulnificus*, overcoming the limitations of conventional isolation procedures, such as enrichment with alkaline peptone water followed by streaking on TCBS agar. The competition between *V. vulnificus* and other vibrios in marine and estuarine environments, where these bacteria coexist, reveals competitive interactions for resources

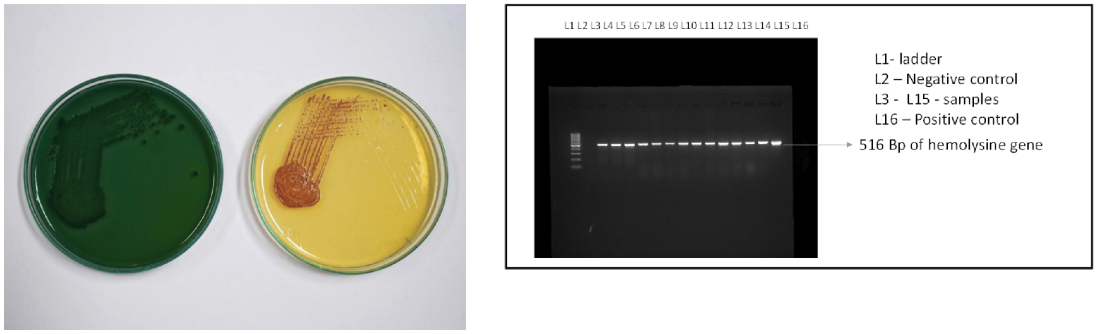

Fig. 1. Identification of *Vibrio vulnificus* by using TCBS agar, Hichrome Vibrio agar and PCR amplification.

Table 1. List of samples and details

Sl.No	Sample	Type of sample	TPC CFU/mL	TVC in saline CFU/mL	Saline green colonies	Saline yellow colonies	APW green colonies	APW yellow colonies	APW positive for *Vibrio vulnificus*	Saline positive for *Vibrio vulnificus*
1	14A	Animal	740000	11400	3400	8000	20400	100	0	0
2	15A	Animal	256000	2800	0	2800	0	0	0	1
3	16A	Animal	750000	14200	2600	11600	96000	0	0	0
4	17A	Animal	190000	7000	0	7000	14000	0	0	0
5	18A	Animal	2000000	50000	0	50000	166000	0	0	0
6	14S	Sediment	24000	80000	70000	10000	2000	0	0	0
7	15S	Sediment	101000	11000	7000	4000	2000	0	0	0
8	16S	Sediment	100000	190000	190000	0	88000	88000	0	0
9	17S	Sediment	29000	16000	14000	2000	30000	16000	0	0
10	18S	Sediment	1980000	300	100	200	0	0	0	0
11	14I	Water	10000	3300	3200	100	300	0	0	0
12	14O	Water	25000	0	0	0	100	0	0	0
13	14C	Water	30000	0	0	0	0	0	0	0
14	15I	Water	51000	300	300	0	300	100	0	0
15	15O	Water	41000	1000	0	1000	600	0	0	0
16	15C	Water	27500	500	200	300	100	100	0	0
17	16O	Water	55000	100	0	0	100	0	0	1
18	16I	Water	33000	300	0	300	100	100	0	0
19	16C	Water	112000	400	100	300	0	0	0	0
20	17O	Water	17000	20	0	20	50	10	0	0
21	17I	Water	115000	160	0	160	10	0	0	0
22	17C	Water	26000	230	230	0	80	0	0	0
23	18O	Water	162000	300	100	200	0	0	0	0
24	18I	Water	43000	70	0	70	0	0	0	0
25	18C	Water	90000	10	10	0	10	0	0	0
26	River 2	Water	18000	700	300	400	100	0	0	0
27	River 3	Water	4000	500	300	200	200	0	0	0
28	*Epinephelus diacanthus*	Animal	900000	100	0	100	0	0	0	0
29	*Epinephelus malabaricus*	Animal	350000	0	0	0	0	0	0	0
30	*Lutjanus malabaricus*	Animal	58000	100	0	0	0	0	0	0
31	*Sphyraena jello*	Animal	750000	0	0	0	0	0	0	0
32	*Arius felis*	Animal	310000	400	0	400	100	0	0	0

33	*Megalospis sp*	Animal	2100000	200	200	0	100	100	0	0
34	*Platycephalus indicus*	Animal	430000	1000	1000	0	0	0	0	0
35	*Lethrinus sp*	Animal	2080000	600	0	600	400	300	0	0
36	*Scarus sp*	Animal	880000	800	300	500	300	0	0	0
37	*Cephalopholis sp*	Animal	3920000	100	0	100	100	0	0	0
38	19A	Animal	3900000	2000	0	2000	13000	0	0	0
39	20A	Animal	5000000	127000	127000	0	20000	20000	0	0
40	21A	Animal	8000000	7000	7000	0	5000	5000	0	0
41	22A	Animal	950000	22000	22000	0	3000	3000	0	0
42	23A	Animal	750000	84000	50000	34000	107000	106000	1	1
43	24A	Animal	1890000	77000	74000	3000	8000	8000	0	0
44	19S	Sediment	31000	0	0	0	0	0	0	0
45	20S	Sediment	50000	9000	0	9000	0	0	0	1
46	21S	Sediment	60000	0	0	0	100	100	0	0
47	22S	Sediment	30000	100	100	0	0	0	0	0
48	23S	Sediment	60000	100	100	0	0	0	0	0
49	24S	Sediment	120000	19000	19000	0	0	0	0	0
50	19C	Water	13800	0	0	0	100	100	0	0
51	20C	Water	7000	0	0	0	400	400	0	1
52	21C	Water	178000	0	0	0	500	300	0	0
53	22C	Water	17200	400	200	200	200	200	1	1
54	23C	Water	35000	100	0	100	0	0	0	0
55	24C	Water	10500	100	0	100	200	0	0	0
56	19I	Water	11000	200	200	0	0	0	0	0
57	20I	Water	272000	0	0	0	0	0	0	0
58	21I	Water	4400	100	100	0	0		0	0
59	22I	Water	2500	0	0	0	0	0	0	0
60	23I	Water	11300	0	0	0	0	0	0	0
61	24I	Water	4400	0	0	0	0	0	0	0
62	19O	Water	3100	0	0	0	0	0	0	0
63	20O	Water	25100	0	0	0	0		0	0
64	21O	Water	20500	0	0	0	0	0	0	0
65	22O	Water	17100	0	0	0	0	0	0	0
66	23O	Water	23700	3400	3400	0	0	0	0	1
67	24O	Water	2000	0					0	0
68	River 4	Water	17600	1100		200	200	200	0	0
Total									2	7

0 represents absence of *Vibrio vulnificus.*
1 represents the presence of *Vibrio vulnificus*

and space. Such interactions can significantly influence the population dynamics and survival of individual species (Hoang, Nguyen, Do, Nguyen, & Pham, 2022).

The main competitor in isolation of *V. vulnificus* is *V. parahaemolyticus* as they share a similar biochemical profile with nearly identical salt tolerance. Intragenus inhibition through quorum sensing is a persistent phenomenon to be reckoned while targeting a particular *Vibrio* species. The enrichment media and conditions used in these conventional approaches are designed to favour the growth of certain target organisms, such as *V. parahaemolyticus* or *V. cholerae*. However, this selective pressure can lead to the suppression or exclusion of other non-target vibrios that may also be present in the sample. Direct plating on non-selective media allows for the growth of a broader range of vibrios without the selective pressure of enrichment (Colwell, 2000).

During the enrichment process, faster-growing or more competitive *Vibrio* spp. can outcompete and suppress the growth of slow-growing or less competitive species. This often results in the underrepresentation of the less competitive species, like *V. vulnificus,* in the final enriched culture (Tantillo, Fontanarosa, Di Pinto, & Musti, 2004). Additionally, *Vibrio* species such as *V. vulnificus* may enter a Viable but Non Culturable (VBNC) state in response to the stressful conditions of enrichment media, such as high salinity or selective agents. These VBNC cells may not resuscitate or grow during the enrichment process, leading to their underrepresentation or exclusion from the final culture (Fakruddin, Mannan, & Andrews, 2013). The new approach developed in the study is based on direct plating, which allows for the isolation and identification of rare or previously unknown *Vibrio* spp. that may not be targeted by selective enrichment media. This can lead to the discovery of new species or strains and provide a more comprehensive understanding of the diversity of vibrios in each environment (Thompson, Iida, & Swings, 2004).

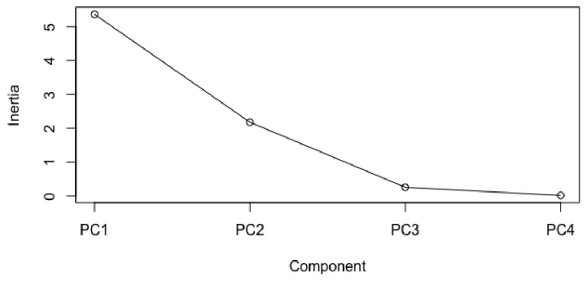

Fig. 2. PCA Screen plot

Deepanjali, Kumar, Karunasagar, and Karunasagar (2005) reported that in shrimp farm waters in India, *V. parahaemolyticus* was the predominant species, accounting for 60-70% of the total *Vibrio* population, while *V. vulnificus* constituted only 1-2%. In a study by Arias, Macian, Aznar, Garay, and Pujalte (1999), it was reported that in shrimp ponds, *V. parahaemolyticus* was the most abundant V*ibrio* species, with a ratio of approximately 10:1 compared to *V. vulnificus.* Blackstone et al. (2003) investigated the abundance of *Vibrio* species in oyster grounds and found that *V. parahaemolyticus* outnumbered *V. vulnificus* by a ratio of approximately 100:1. In oyster harvesting areas in the Gulf of Mexico, the ratio of *V. parahaemolyticus* to *V. vulnificus* ranged from 10:1 to 100:1, depending on the specific location and environmental conditions (Jones et al., 2012). Hence, instead of enrichment, the method involving direct plating yields better diversity and enhanced recovery of *V. vulnificus*.

The results of Kruskal-Wallis ANOVA performed to compare the *Vibrio* count in samples treated with the two methods indicated that the *Vibrio* count was significantly different ($p<0.05$). When the *Vibrio*

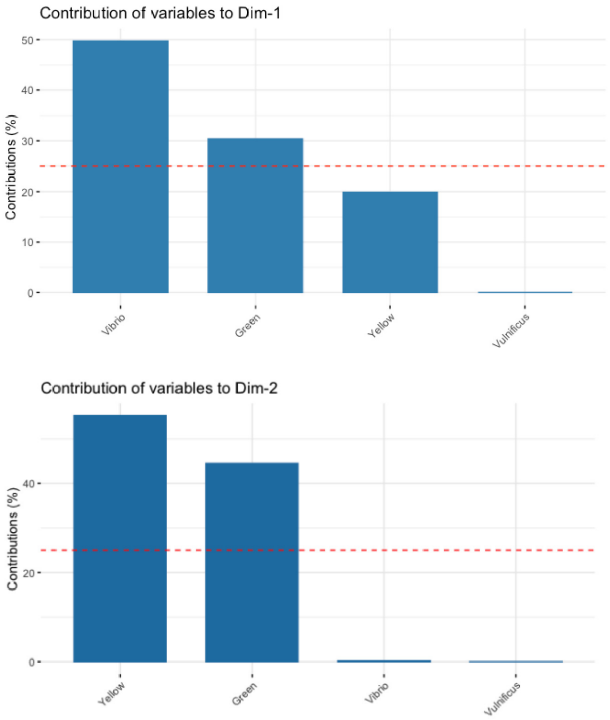

Fig. 3. Contribution of variables

count data were analyzed to assess the differences in occurrence across various type of samples-namely aquatic animals, water, and sediment, it was observed that the Kruskal-Wallis test rejected the null hypothesis at 1% significance level. Therefore, it can be concluded that the *Vibrio* count varies according to the type of sample (i.e., animal, water, or sediment). Further analysis revealed that the non-sucrose fermenting *Vibrio* species varied significantly ($p<0.05$) across different diluents, whereas the intensity of sucrose fermenters did not vary significantly with respect to the diluent. Additionally, the formation of both sucrose and non-sucrose fermenters varied significantly ($p<0.05$) across the different types of samples.

The *Vibrio* count data, after suitable transformation, were subjected to Principal Component Analysis (PCA). The first two principal components explained more than 96% of the variation in the data (Fig. 2). The contributing factors to the two principal components were examined using a contribution plot. The variable "Green" contributed to PC1, while both "Green" and "Yellow" contributed more than 25% to PC2.

The PCA biplot visually depicts the samples pertaining to two variables: APW enrichment and saline (Fig. 3). The green and yellow colonies, represented by the variables "Green" and "Yellow", are plotted in opposite directions, indicating their complimentary nature. Fifty percent of the saline diluent samples were separated from the APW samples, although a few samples are seen to be plotted synchronously in the plot. The *Vibrio* count varied significantly across different sample types (animal, water, and sediment), suggesting that sample type influences isolation efficiency. The formation of green *Vibrio* colonies (likely representing *V. vulnificus* and *V. parahaemolyticus*) varied

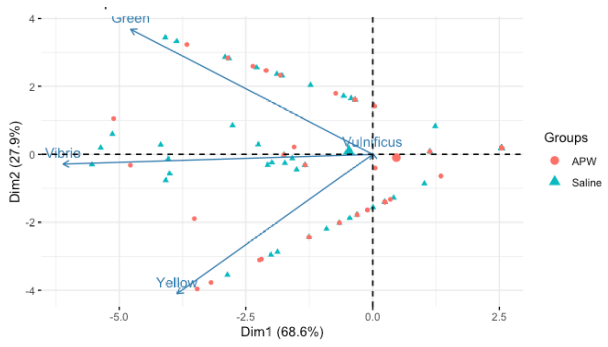

Fig. 4. The PCA biplot

significantly across the method used, while the formation of yellow colonies did not. This indicates that the green colonies vary depending on whether direct plating or enrichment is used. Principal Component Analysis (PCA) revealed that the variables "Green" (green colonies) and "Yellow" (yellow colonies) contributed significantly to the variation in the data, highlighting greater diversity in the direct plating.

In the aquaculture samples analyzed, the sediment samples generally showed higher *Vibrio* counts compared to water samples from the same location. The highest sediment count was observed in sample sediment 190,000 CFU per mL. In contrast, the water samples exhibited relatively lower counts of vibrios, with the highest water count observed at 3,400 CFU per mL in the aquaculture pond water. The findings suggest that sediment serves as a more favorable environment for the survival and growth of vibrios, such as *V. vulnificus,* compared to water.

The time required for enrichment can be significantly reduced with direct plating. The typical 18-24 h enrichment period can be eliminated by using direct plating. Another advantage is the cost: the saline producing cost is 100 times lower than that of the specific broth used for enrichment. In aquaculture settings, where the routine monitoring of vibrios is essential, direct plating offers a diverse range of vibrios at minimal expenses.

Aquaculture is a rapidly growing food production sector, but the emerging pathogens like *V. vulnificus* pose a significant threat to those involved in the industry. The conventional isolation procedure for *V. vulnificus* from aquaculture systems requires modification due to the dynamic nature of this pathogen. This study suggests that using physiological saline instead of APW enrichment can enhance the isolation of *V. vulnificus* by reducing competition from other *Vibrio* species, particularly *V. parahaemolyticus*.

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