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Inactivated Whole Vaccine Inhibits Lethal *Vibrio harveyi* Infection in *Oplegnathus punctatus*

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Abstract: Aquaculture plays a key role in food production globally and provides a valuable source of protein and nutrition, addressing a worldwide growing demand. *Oplegnathus punctatus* (spotted knifejaw) is an economically important fish species with a high market value and demand. Previous studies on *O. punctatus* focused mainly on gonadal development, chromosomal microstructure, selective breeding, characterization of immune genes, and viral diseases. There is no published scientific research regarding vibriosis in this fish species. In this study, two potential pathogenic bacteria, *Vibrio harveyi* and *Enterococcus gallinarum*, were isolated from moribund cultured *O. punctatus*. The sequence of the universal 16S rDNA gene was used to identify potential pathogenic bacteria isolated from the moribund *O. punctatus*, and morphological assessments and API20E tests of the bacterial isolates were conducted to verify the identity and biochemical characteristics of the isolates. Injection of *E. gallinarum* did not lead to mortality in *O. punctatus* during the 21 days of observation. In contrast, fish died overnight when challenged with *V. harveyi* at 1.25×10^5 CFU/g body weight, suggesting that the cause of death of the cultured *O. punctatus* was *V. harveyi* infection. Antimicrobial sensitivity analyses revealed that the *V. harveyi* strain NTOU is sensitive to flumequine, doxycycline, oxolinic acid, and amoxycillin. Importantly, we demonstrated for the first time that intraperitoneal administration of an inactivated *V. harveyi* whole-cell vaccine resulted in a high level of protection against *V. harveyi* infection in *O. punctatus*.

Keywords: *Vibrio harveyi*; vaccine; bacteria; *Oplegnathus punctatus*; disease

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1. Introduction

Aquaculture plays a key role in food production globally, and provides a valuable source of protein and nutrition to supply the growing demand worldwide [1]. *Oplegnathus punctatus* (spotted knifejaw) is an economically important fish species with a high market value and demand in Taiwan, Japan, and China [2]. With good flavor and high nutritional value, *O. punctatus* has become an increasingly popular cultural food source in Asian countries. In the wild, *Oplegnathus* spp. are widely distributed in the subtropical and warm temperate waters of the northwestern and central areas of the Pacific Ocean [3]. *O. punctatus* occurs from Hawaii and Guam to the northeastern Pacific, around Korea and Japan, to Vietnam [3].

Artificial breeding technology for *O. punctatus* has been established in Taiwan, and this technological breakthrough has made large-scale farming of *O. punctatus* possible. However, it is important to consider aspects such as high-density intensive farming, poor water quality, overfeeding, and inappropriate farm management, which may result in animal stress, disease outbreaks, and heavy economic losses [4]. While viral infection has been reported in the *O. punctatus* industry [5], bacteria are also causative agents of fish disease. Vibriosis, caused by *Vibrio* species, is a serious problem that leads to substantial economic losses in aquaculture worldwide, and reportedly affects *O. punctatus* culture in China [4].

Antibiotics and chemotherapeutic agents have been used for disease prevention and treatment in aquaculture [6]. However, misuse of antibiotics negatively affects fish and final consumers, and increases the risk of promoting or selecting for the growth of antibiotic-resistant microbes in the environment [6]. Bojarski et al. summarized information from several reports regarding the use of antibiotics in aquaculture, which results in the accumulation of relevant chemicals in the tissues of aquatic animals, effluents, and sediments [7], emphasizing that the issue arose from overuse/misuse of antibiotics during the process of animal production. Moreover, studies have demonstrated that administration of antibiotics could lead to adverse physiological effects in aquatic animals, such as anemic response, hepatotoxic effects, oxidative stress, and death [7].

Vaccination has been used in aquaculture to provide farmed fish with long-term immune responses and protection against pathogenic microbes [8]. Currently, most commercial vaccines that are composed of inactivated pathogens are administered by injection. Prophylactic agents, such as vaccines for the prevention of vibriosis in *O. punctatus*, are still lacking in Taiwan. In this study, we present novel research on the efficacy of administering bacterin to *O. punctatus* to prevent *V. harveyi* infection.

2. Materials and Methods

2.1. Fish

Moribund *O. punctatus* (~100 g) were used for isolating pathogens. A total of 90 healthy *O. punctatus* (42.83 ± 7.42 g) obtained from the Aquatic Animal Center (National Taiwan Ocean University, Keelung, Taiwan) were used for susceptibility tests, vaccination, and challenge assays. Animal experiments were performed according to the guidelines of the institutional animal care and use committee of the National Taiwan Ocean University (Approval number: 109045).

The fish were equally divided into three 2 m^3 fiber-reinforced plastic (FRP) tanks supplied with continuous aeration. Commercial feed (Tairoun Products Company, Taipei City, Taiwan) was provided twice a day at 3% of total fish body weight. The water parameters temperature (27 ± 2 °C), dissolved oxygen (5 ± 2 ppm), salinity (33 ± 1‰), pH (7.7 ± 0.2), and NH_3 (<0.5 ppm) were maintained and monitored daily.

2.2. Screening and Isolation of Pathogenic Bacteria

Bacteria were sampled from the trunk kidney, liver, and spleen of diseased fish and cultured onto tryptic soy agar (TSA, BD Difco & BBL, Franklin Lakes, NJ, USA) supplemented with 2% NaCl and thiosulphate-citrate-bile salts-sucrose (TCBS, BD Difco & BBL) agar. Dominant bacteria were isolated and transferred to new TSA agar (+2% NaCl) and preserved at −80 °C in tryptic soy broth (TSB, BD Difco & BBL) supplemented with 20% (*v*/*v*) glycerol.

2.3. Bacteriological Investigation and 16S rDNA Sequencing

Two isolates each were plated on blood agar (TSA *w*/5% Sheep Blood; BD Difco & BBL) or TCBS agar, followed by a 16 h incubation at 28 °C. Morphological characteristics were recorded. Biochemical analyses of the four isolates were conducted using the Analytical Profile Index (API) 20E Test (BIOMÉRIEUX, Marcy-l'Étoile, France).

Pure isolates cultured in TSB were collected and bacterial genomic DNA was extracted using a Presto gDNA Bacteria Advanced Kit (Geneaid, New Taipei City, Taiwan). The 16S rDNA gene was amplified using the primers 16S-F, 5′-AGAGTTTGATCATGGCTCAG-3′, and 16S-R, 5′-GGTTACCTTGTTACGACTT-3′. The polymerase chain reaction (PCR) products were purified and sequenced by Genomicus (New Taipei City, Taiwan).

2.4. Antibacterial Susceptibility Test

An antibacterial susceptibility test was conducted using the disc diffusion test [9]. Nine antibiotics were used (at the indicated doses): oxytetracycline (30 μg); doxycycline

(30 μg); flumequine (30 μg); oxolinic acid (2 μg); amoxicillin (30 μg); ampicillin (10 μg); lincomycin (2 μg); erythromycin (15 μg); and spiramycin (100 μg).

The isolates were washed off the overnight culture on TSA (+2% NaCl) with sterile phosphate-buffered saline (PBS), and the concentration of the bacterial suspension was adjusted to an OD_{600} of 1, which equated to approximately 5×10^7 colony-forming units per milliliter [CFU/mL]). The bacterial suspension (100 μL) was spread on TSA (+2% NaCl) and incubated at 28 °C. The inhibition zones were measured 24 h later.

2.5. Intraperitoneal Injection Challenge Assay

Two challenge trials were performed. To determine which isolates are pathogenic to *O. punctatus*, we first injected fish (42.3 ± 3.2 g, n = 12) intraperitoneally with a bacterial suspension of *Enterococcus gallinarum* at an OD_{600} of 0.5 (1×10^6 CFU/g BW, n = 3), 1 (2×10^6 CFU/g BW, n = 3), or 2 (4×10^6 CFU/g BW, n = 3). A group injected with PBS served as the negative control group. The volume injected was 0.1% of the animal's body weight (BW) (i.e., 0.1 mL was injected into a fish with a BW of 100 g).

In the second trial, 40 healthy fish (weighing 40 ± 10 g) were divided into four groups (n = 10 per group), and three groups were intraperitoneally challenged with *V. harveyi* suspensions at either 6.25×10^3 CFU/g BW, 1.25×10^4 CFU/g BW, or 2.50×10^4 CFU/g BW. The control group was injected with PBS. The volume injected was again 0.1% of BW. Mortality rates were recorded daily for 7 d and the presence of *V. harveyi* was verified by bacterial isolation and PCR analysis of the *toxR* gene using primers V.h_toxR_F, 5′-GAAGCAGCACTCACCGAT-3′, and V.h_toxR_R, 5′-GGTGAAGACTCATCAGCA-3′ [10].

2.6. Vaccine Preparation

V. harveyi was cultured in TSB (+2% NaCl) for 18 h at 28 °C with shaking. The bacterial cells were collected by centrifugation at 5000× g for 10 min. The cells were resuspended in PBS and neutral buffered formalin was added to the bacterial suspension to achieve a final concentration of 1% (v/v) of an ensure bacterial inactivation overnight at 4 °C. Successful inactivation was confirmed by the lack of bacterial growth on TSA (+2% NaCl) after streaking the formalin-treated bacterial suspension on agar. The inactivated bacterial cells were washed twice with PBS, resuspended, and mixed with MONTANIDE ISA763A VG (SEPPIC, Courbevoie, France) at a ratio of 3:7, to reach 2.50×10^7 CFU/mL. The vaccine was administered to the fish via an intraperitoneal injection of bacterin at 1% of BW. The mock vaccination group was injected with a mix of PBS with ISA763A, while the control group was injected with PBS only.

2.7. Efficacy Trial

A total of 112 fish (70 ± 10 g) were equally divided into four groups (28 fish per group) and reared in fresh seawater. Water parameters such as temperature (27 ± 2 °C), pH (7.7 ± 0.2), salinity (33 ± 1‰) and dissolved oxygen (5 ± 1 ppm), were maintained during the experimental period. Additionally, concentrations of ammonia-N and nitrite-N were maintained lower than 0.5 and 0.1 mg/l, respectively. Each group was intraperitoneally injected with the vaccine, adjuvant, or PBS. The fourth group was mock processed without injection. Each group was separately reared in individual 2-t FRP tanks. To test the efficacy of the vaccine, fish were randomly selected from each group on day 14 (9 fish per group), 28 (9 fish per group), and 42 (10 fish per group) and challenged with *V. harveyi* (2.50×10^4 CFU/g BW), as described above. Mortality was recorded for 8 d.

2.8. Statistical Analysis

The survival rates of fish in the challenge tests were calculated using the Kaplan–Meier analysis and the Mantel–Cox test was performed to assess the differences between the groups. p-values < 0.05 were assumed to denote statistical significance. The analyses were conducted using SPSS version 22.0 (IBM, Armonk, NY, USA).

3. Results

3.1. Screening and Isolation of Pathogenic Bacteria

Examination of moribund *O. punctatus* juveniles revealed hemorrhaging in the trunk kidney (Figure 1). Samples from the kidney and spleen were used to isolate pathogenic bacteria by streaking tissues on TSA (+2% NaCl). Two colonies were transferred to new fresh TSA, TBCS, and sheep blood agar, and were characterized using 16S rDNA sequence amplification and sequencing. Isolate numbers 1 (a Gram-negative bacterium) and 2 (a Gram-positive bacterium) were green and yellow on TCBS agar (Figure 2A) and displayed alpha and beta *hemolysis on sheep blood agar,* respectively (Figure 2B).

Figure 1. Representative example of hemorrhaging in the trunk kidney of *Oplegnathus punctatus* cultured in a fiber-reinforced plastic (FRP) tank.

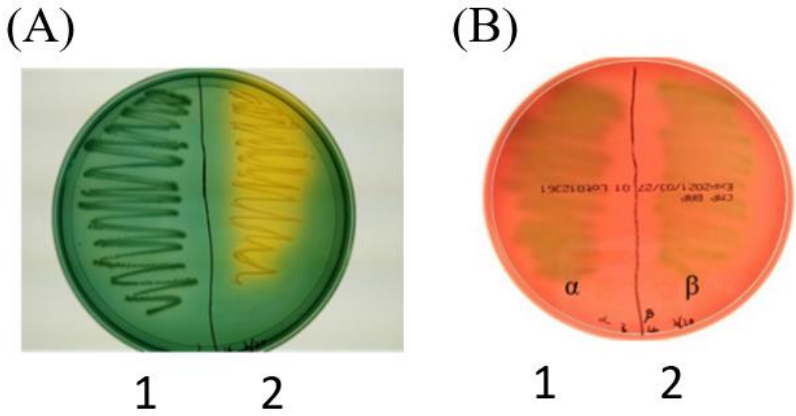


Figure 2. Growth of bacterial isolates on (**A**) TCBS and (**B**) sheep blood agar.

3.2. 16S rDNA Sequencing and Biochemical Characterization

To determine the biochemical characteristics of the Gram-negative isolate, we used API 20E test strips and 16S rDNA sequencing (Table 1). The Gram-negative isolate was positive for lysine decarboxylase, ornithine decarboxylase, urease, indole, citrate, glucose, mannose, sucrose, and amygdaline.

Homology searches of the 16 rDNA sequences amplified from the two isolates were conducted. Isolates 1 and 2 had the highest hit rate and identity with *Vibrio harveyi* (accession no. MT510177_1) and *Enterococcus gallinarum* (accession no. CP046307.1) in the GenBank (Table 2), respectively, confirming the identity of these bacterial isolates. The two isolates were named *Vibrio harveyi* strain NTOU and *Enterococcus gallinarum* strain NTOU, respectively.

Table 1. Biochemical characteristics of isolated *Vibrio harveyi* using the API 20E system.

Biochemical Panel	*Vibrio harveyi*
β-galactosidase (ONPG)	–
Arginine dihydrolase (ADH)	–
Lysine decarboxylase (LDC)	+
Ornithine decarboxylase (ODC)	+
Urease (URE)	+
Tryptophan deaminase (TDA)	–
Gelatinase (GEL)	–
Production of	
H_2S	–
Indole (IND)	+
Acetoin (VP)	–
Fermentation of	
Citrate (CIT)	+
Glucose (GLU)	+
Mannose (MAN)	+
Inositol (INO)	–
Sorbitol (SOR)	–
Rhamnose (RHA)	–
Sucrose (SAC)	+
Melibiose (MEL)	–
Amygdaline (AMY)	+
Arabinose (ARA)	–

Table 2. Homology searches for 16S rRNA sequences of the bacterial isolates in GenBank.

Code	Accession Number	PCR Identification	Identity (%)
1	MT510177_1	*Vibrio harveyi*	99.72
2	CP046307.1	*Enterococcus gallinarum*	99.06

3.3. Susceptibility to Antibiotics

The isolated *E. gallinarum* was resistant to all the antibiotics tested. *V. harveyi* was sensitive to doxycycline, flumequine, oxolinic acid, and amoxycillin, but resistant to oxytetracycline, ampicillin, lincomycin, erythromycin, and spiramycin (Table 3).

Table 3. Antibiotic sensitivity test of the bacterial isolates used in the present study.

Antibiotic / Sample	OTC	DO	UB	OA	AMC	AMP	MY	ERY	SP
Vibrio harveyi—NTOU	R	S	S	S	S	R	R	R	R
Enterococcus gallinarum—NTOU	R	R	R	R	R	R	R	R	R

OTC, oxytetracycline; DO, doxycycline; UB, flumequine; OA, oxolinic acid; AMC, amoxycillin; AMP, ampicillin; MY, lincomycin; ERY, erythromycin; SP, spiramycin; S, susceptible; R, resistant.

3.4. Lethality Tests

To determine whether *E. gallinarum* is lethal to *O. punctatus*, juvenile *O. punctatus* were intraperitoneally injected with various doses of *E. gallinarum* and the mortality rate was recorded. Notably, no mortality was observed in these fish, regardless of the dose used, but an enlarged spleen was common (Figure 3). These results indicated that the etiological agent of death in *O. punctatus* may not be an *E. gallinarum* infection.

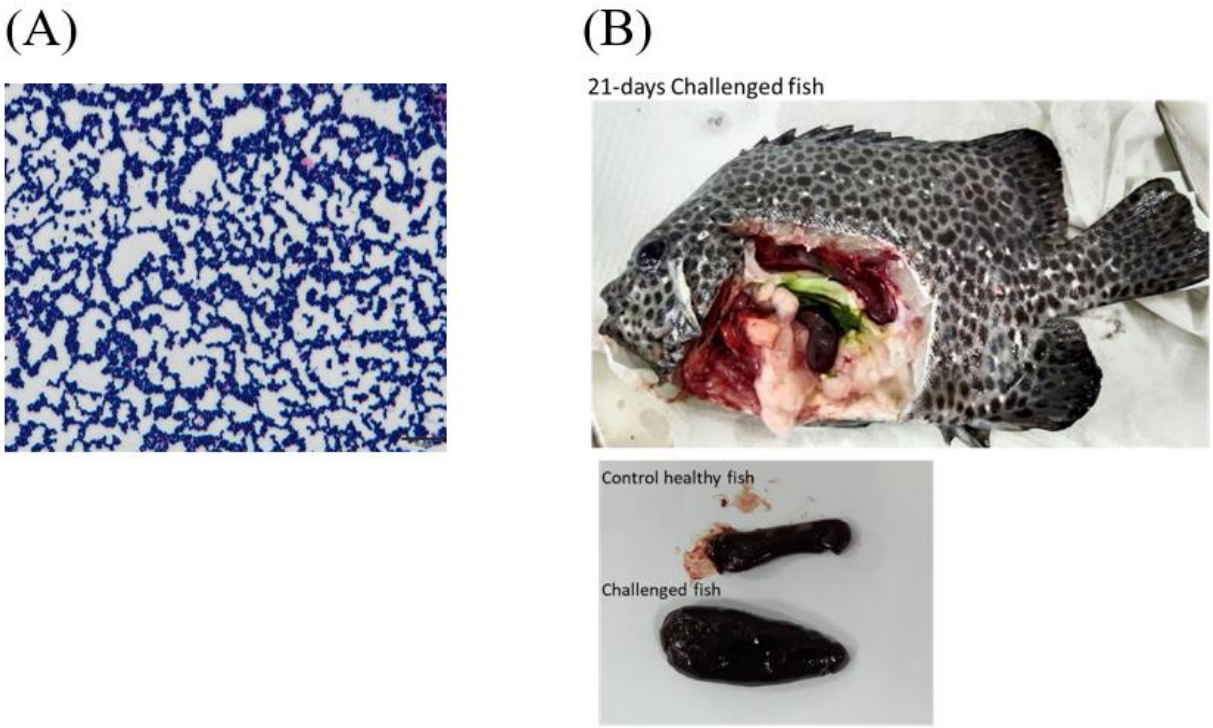


Figure 3. (**A**) Results of Gram staining for *Enterococcus gallinarum* isolated from moribund *Oplegnathus punctatus*. (**B**) *O. punctatus* intraperitoneally injected with *E. gallinarum* showed spleen enlargement 21 days post injection (dpi).

We then challenged fish with *V. harveyi* at doses of 6.25×10^4 CFU/g BW, 1.25×10^5 CFU/g BW, and 2.50×10^5 CFU/g BW. No mortality was recorded in the group challenged with the lowest dose of bacteria, but was observed 18–24 h post bacterial injection in the groups injected with higher doses (Table 4). The cumulative mortality rates were 80% and 50% for the fish challenged with 2.50×10^5 CFU/g BW and 1.25×10^5 CFU/g BW, respectively. *V. harveyi* was successfully re-isolated from the challenged fish, and the *toxR* was detected in the original isolate and in the bacteria retrieved from the dead fish (Figure 4A). *O. punctatus* exposed to *V. harveyi* displayed internal hemorrhaging in the trunk kidney and ascites in the abdomen (Figure 4B).

Table 4. Mortality of juvenile *Oplegnathus punctatus* challenged with the *Vibrio harveyi* isolate via intraperitoneal injection.

Bacterial Dose (CFU)	No. of Fish Dead at:			Cumulative Mortality (%)
	1 Day	2 Days	3–7 Days	
2.50×10^5 CFU/g BW	8	0	0	80%
1.25×10^5 CFU/g BW	4	1	0	50%
6.25×10^4 CFU/g BW	0	0	0	0%
Control (PBS)	0	0	0	0%

CFU, colony forming unit; BW, body weight.

3.5. Protection against V. harveyi in O. punctatus Induced by Inactivated Vaccine

As shown in Figure 5, inactivated bacterin exhibited immunoprotection at 14, 28, and 42 d post immunization (dpi). A significantly higher survival rate was noticed in the "vaccine" group (33.3%) than in the control group (survival rate 0%) at 14 dpi. Similarly, the survival rate after the challenge with *V. harveyi* was 11.1% for the PBS-injected control group, but 55.6% for the vaccine group at 28 dpi. By 42 dpi, only 40% of fish had died in the

vaccine group after *V. harveyi* challenge, whereas the survival rate was low in the control group (10%).

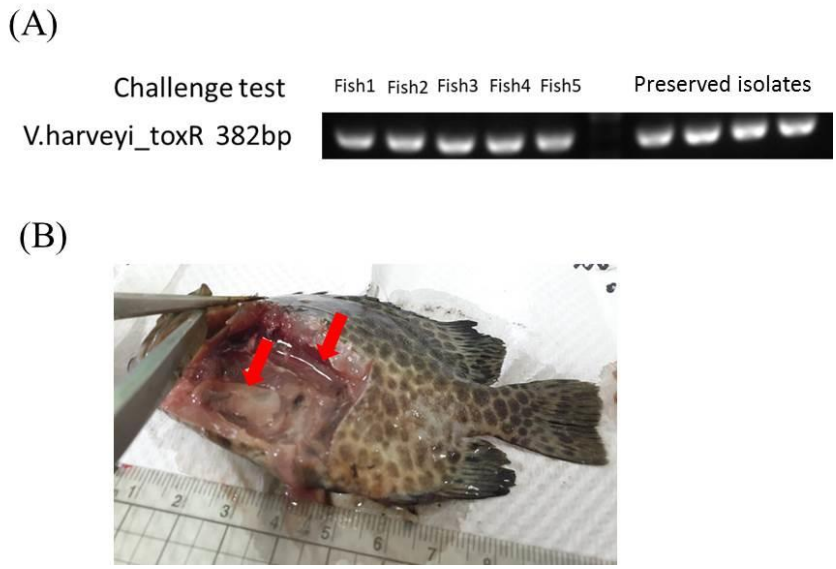
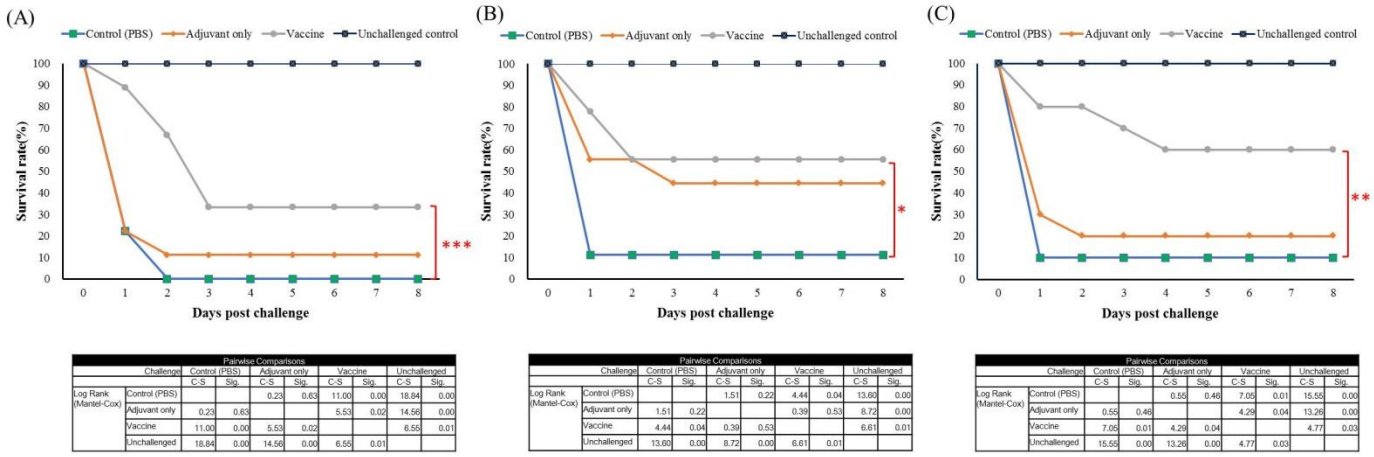


Figure 4. (**A**) Polymerase chain reaction (PCR) detection of the virulence gene *toxR* from *Vibrio harveyi* isolated from artificially infected fish (fish 1–5) and preserved isolates. (**B**) *Oplegnathus punctatus* exposed to *V. harveyi* displaying hemorrhaging in the trunk kidney and ascites in the abdomen.



Pairwise Comparisons

	Challenge	Control (PBS)		Adjuvant only		Vaccine		Unchallenged	
		C-S	Sig.	C-S	Sig.	C-S	Sig.	C-S	Sig.
Log Rank (Mantel-Cox)	Control (PBS)			0.23	0.63	11.00	0.00	18.84	0.00
	Adjuvant only	0.23	0.63			5.53	0.02	14.56	0.00
	Vaccine	11.00	0.00	5.53	0.02			6.55	0.01
	Unchallenged	18.84	0.00	14.56	0.00	6.55	0.01		

Pairwise Comparisons

	Challenge	Control (PBS)		Adjuvant only		Vaccine		Unchallenged	
		C-S	Sig.	C-S	Sig.	C-S	Sig.	C-S	Sig.
Log Rank (Mantel-Cox)	Control (PBS)			1.51	0.22	4.44	0.04	13.60	0.00
	Adjuvant only	1.51	0.22			0.39	0.53	8.72	0.00
	Vaccine	4.44	0.04	0.39	0.53			6.61	0.01
	Unchallenged	13.60	0.00	8.72	0.00	6.61	0.01		

Pairwise Comparisons

	Challenge	Control (PBS)		Adjuvant only		Vaccine		Unchallenged	
		C-S	Sig.	C-S	Sig.	C-S	Sig.	C-S	Sig.
Log Rank (Mantel-Cox)	Control (PBS)			0.55	0.46	7.05	0.01	15.55	0.00
	Adjuvant only	0.55	0.46			4.29	0.04	13.26	0.00
	Vaccine	7.05	0.01	4.29	0.04			4.77	0.03
	Unchallenged	15.55	0.00	13.26	0.00	4.77	0.03		

Figure 5. Kaplan–Meier survival curves for *Oplegnathus punctatus* vaccinated with phosphate-buffered saline (PBS), adjuvant ISA763A, or bacterin and then challenged with *Vibrio harveyi* on day (**A**) 14 (n = 9), (**B**) 28 (n = 9), or (**C**) 42 (n = 10) post immunization. Differences between the groups were assessed using the Mantel–Cox test for pairwise comparisons. * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$.

4. Discussion

Since technical difficulties in the breeding of larvae have recently been resolved, *O. punctatus* is a newly emerging aquaculture species. Previous studies on *O. punctatus* focused mainly on sex chromosome evolution [11], gonadal development [12], chromosomal microstructure [13], selective breeding [14], characterization of immune genes [4,15], and viral diseases [16,17]. Although pathogens such as *Vibrio anguillarum* were thought to cause disease in *O. punctatus*, there was no published scientific research regarding vibriosis in this fish species.

In this study, the sequence of the universal 16S rDNA gene was used to identify potential pathogenic bacteria that had been isolated from moribund *O. punctatus*. Two bacterial

species, *V. harveyi* and *E. gallinarum*, were identified and their morphology, biochemical identification were examined to verify the identity of the two isolates. API20E characteristics were additionally analyzed for the pathogenic *V. harveyi* lethal to *O. punctatus*.

During the first challenge, we found that injecting *O. punctatus* with *E. gallinarum* did not lead to mortality during the 21 days of observation. *E. gallinarum* is responsible for autoimmunity [18] and spontaneous bacterial peritonitis [19] in humans. Our results suggest that this bacterial species is not the cause of death in *O. punctatus*, although it does cause splenomegaly.

We then tested whether *V. harveyi* was the causative agent for the death of the cultured *O. punctatus*. Indeed, fish injected with a bacterial suspension of *V. harveyi* died overnight post challenge. *V. harveyi* is one of the *Vibrio* species most frequently isolated [20] as infecting aquacultured animals, including shrimp and marine fish [21,22]. However, this disease has not been previously reported in *O. punctatus*. To the best of our knowledge, ours is the first report of *V. harveyi* infecting juvenile *O. punctatus* and inducing mortality with hemorrhaging in the kidney.

The virulence gene *toxR* was first discovered in *V. cholerae* as a transcriptional regulator of the cholera toxin (encoded by the *ctx* gene) of *V. cholerae* [23,24]. *ToxR* was found to encode a transmembrane protein for regulating *ctx* [25] and other genes such as *tcp*, which encodes toxin co-regulated pilus, and *ompU* and *ompT*, which encode major outer membrane proteins in *V. harveyi* [26]. Detecting the presence of *toxR* was demonstrated to be an effective method for identifying the *V. harveyi* isolate [10]. A PCR analysis in the present study revealed that *toxR* was present in the isolated *V. harveyi*. Together with the 16S rDNA sequence, this verified that the pathogenic bacterium was *V. harveyi*.

Antimicrobial sensitivity analysis revealed that the *V. harveyi* strain NTOU was sensitive to flumequine, doxycycline, oxolinic acid, and amoxycillin, and these antibiotics could be used as emergency treatments for the disease. However, prophylactic agents, such as an efficient vaccine for the prevention of *V. harveyi* infection in *O. punctatus*, represent the best strategy for controlling this microbe in the species. Inactivated vaccines are generally administered via injection to attain a high efficacy [27]. It was demonstrated that a formalin-inactivated vaccine could provide good protection against *V. harveyi* infection in *Epinephelus coioides* (orange-spotted grouper) [27]. The oil adjuvant ISA763A VG, with outer membrane porin F (OmpF) from *Yersinia ruckeri*, was also used in the subunit vaccine and was shown to provide good protection for channel catfish [28]. This study demonstrated that intraperitoneal administration of an inactivated *V. harveyi* whole-cell vaccine resulted in a high level of protection against *V. harveyi* infection in *O. punctatus*.

5. Conclusions

In summary, we identified *Vibrio harveyi*, but not *Enterococcus gallinarum*, as the causative agent of the death of cultured *Oplegnathus punctatus*. The *V. harveyi* strain NTOU was only sensitive to flumequine, doxycycline, oxolinic acid, and amoxycillin. An inactivated *V. harveyi* vaccine provided good protection against *V. harveyi* These results provide a foundation for the development of effective prevention measures against this disease.

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Institutional Review Board Statement: Animal experiments were performed according to the guidelines of the institutional animal care and use committee of the National Taiwan Ocean University (Approval number: 109045).

Informed Consent Statement: Not applicable.

Data Availability Statement: The authors confirm that the data supporting the findings of this study are available within the article.

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Conflicts of Interest: The authors declare no conflict of interest.

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