HNUE JOURNAL OF SCIENCE DOI: 10.18173/2354-1059.2020-0039
Natural Sciences 2020, Volume 65, Issue 6, pp. 181-190
This paper is available online at http://stdb.hnue.edu.vn

INACTIVATION OF *Vibrio parahaemolyticus* ISOLATED FROM SEAWATER TO DEVELOP FISH VACCINE

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Abstract. *Vibrio parahaemolyticus* is a gram-negative bacterium which has been widely reported as the cause for hepatic and kidney necrosis in fishes, especially species of high economic value, in many regions across the world. Five pathogenic strains of *V. parahaemolyticus* were isolated from seawater samples collected in Thanh Hoa and Nghe An regions, Vietnam. All those strains were characterized by well-known morphological and biochemical characteristics of *V. parahaemolyticus*. Furthermore, we identified the presence of two common found virulent genes in *V. parahaemolyticus* (i.e. *toxR* và *tlh*) from all isolated strains while the other two genes (i.e. *tdh* and *trh*) were missing. Experimental results indicated LD_{50} values of isolated strains diverged from $10^{5.73}$ to $10^{7.28}$ on tilapia (*Oreochromis niloticus*) and $10^{4.15}$ to $10^{5.15}$ on zebrafish (*Danio rerio*). Then, the strain with the lowest LD_{50} value, named DH64.1, was selected for producing an inactivated vaccine by using formaldehyde. Consequently, the inactivated vaccine was injected on tilapia with survival rate 100% and the lack of any pathogenic symptom. Finally, vaccinated fish that were challenged with DH64.1 strain at three different challenge doses (i.e. 10^6, 10^7, 10^8 CFU/mL). After a 15 days post-vaccination, the relative percentage survival (RPS) of the vaccine was around 88.66-100%.

Keywords: *Oreochromis niloticus*, *Vibrio parahaemolyticus*, inactivated vaccine, LD_{50} value.

1. Introduction

Vibrio parahaemolyticus is a gram-negative bacterium which has been documented as the cause for hepatic and kidney necrosis in 48 fish species in many countries, especially some marine species of high economic value including grouper (*Epionephelus spp*.), sebae clownfish (*Amphiprion sebae*), humphead snapper (*Lutjanus sanguineus*) [1] and can infect for some freshwater fishes including tilapia (*Oreochromis niloticus*) and zebrafish (*Danio rerio*) [2, 3]. In general, infected fishes exhibit symptoms such as the presence of red necrotic lesions in the abdominal muscle and erythema (bloody blotches),

Received May16, 2020. Revised June 20, 2020. Accepted June 27, 2020.
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tissue and appendage necrosis, especially in liver and kidney, with the mortality rate could be up to 90% [4]. *V. parahaemolyticus* genome contains three virulent genes, that cause the lysis of red blood cells to the release of hemoglobin, including thermostable direct hemolysis (*tdh*), thermolabile hemolysin (*tlh*) and thermostable direct hemolysin-related hemolysin (*trh*) [5]. The *toxR* gene is in the *toxRS* operon (Vp - *toxRS*) that regulates the *tdh* gene and several other genes that code for this protein. This gene is a well-known molecular marker for *V. parahaemolyticus*'s identification [6].

To date, using antibiotics are the most common method to prevent and cure *V. parahaemolyticus* infection in fishes. In practice, antibiotics are often mixed with fish foods or immersion bath in order to treat bacterial infections [7]. The excessive usage of antibiotics has resulted in the development of multidrug resistance in many pathogenic bacteria including *Vibrios* [8, 9]. The application of vaccines appears to be an effective, sustainable method in fish farming.

Fish vaccines include inactivated vaccines, recombinant vaccines, DNA vaccines, and live-attenuated vaccines. Nowadays, researches on the development of vaccines for *V. parahaemolyticus* mostly falls into three directions: inactivation, recombination, and live-attenuation. In addition, inactivated vaccines are often made by the use of formaldehyde or other alternatives. An trivalent inactivated vaccine (i.e. *V. alginolyticus*, *V. parahaemolyticus* and *A. hydrophila*) was developed with an RPS (relative percentage survival) tested on yellow croaker (*Pseudosciaena crocea*) was 88.9% [10]. Formaldehyde was used to inactivate *V. harveyi* and *V. parahaemolyticus* resulting in a vaccine with an RPS of 77.6% on orange-spotted grouper (*Epinephelus coioides*) [11]. In Vietnam, a vaccine, called AquaVib, was created using formaldehyde to inactivate three *Vibrio* species (i.e. *V. alginolyticus*, *V. parahaemolyticus* và *V. harveyi*) with an RPS of 70-100% after 7-30 days post-vaccination on cobia (*Rachycentron canadum*) [12]. Similarly, an inactivated vaccine tested on orange-spotted grouper with an RPS value of 87.5% after 30 days post-vaccination [13].

Although the number of studies on the development of *V. parahaemolyticus* vaccines has increased recently, a commercial vaccine is yet to be available. In this study, we (1) isolated *V. parahaemolyticus* from seawater samples collected in some regions in the north of Vietnam, (2) evaluated and selected the virulent strain, and (3) created an inactivated vaccine using a selected strain and finally tested the relative percentage survival (RPS) of the inactivated vaccine on tilapia (*Oreochromis niloticus*). Our study aims to develop an effective and commercially-potential vaccine against *V. parahaemolyticus* on fish farming, especially on fish species of high economic value.

2. Content

2.1. Materials and methods

2.1.1. Materials

Seawater samples were collected from fish farms in the regions of Hai Tien, Da Loc (Thanh Hoa), and Cua Lo (Nghe An). Sampling was performed by a team from the Institute of Biotechnology, Vietnam Academy of Science and Technology.

Especially, a *V. parahaemolyticus* strain named VTCC 12233 was provided by the Institute of Microbiology and Biotechnology, Vietnam National University, Hanoi. Zebrafish with lengths between 3.0 - 3.5 cm and tilapia with lengths between 6.0 - 7.0 cm were provided by the Research Institute for Aquaculture No 1 (Tu Son, Bac Ninh).

2.1.2. Methods

**** Vibrio parahaemolyticus isolation from seawater***

V. parahaemolyticus was isolated on Thiosulfate Citrate Bile Sucrose agar (TCBS) [14]. Methods for evaluating morphological and biochemical characteristics include: fermentation, indole test, catalase test, motility test, and hydrogen sulfide (H_2S) production test [15]. Besides, the strain VTCC 12233 was used as a positive control.

**** Antibiotic susceptibility test***

A stock culture (50 μL, 10^6 CFU/mL) (CFU - Colony-forming unit) of isolated *V. parahaemolyticus* was plated on Brain Heart Infusion (BHI) medium. Then, 5 different types of antibiotic discs (6 mm) including ampicillin 25 µg, gentamycin 30 µg, norfloxacin 10 µg, enrofloxacin 5 µg, and erythromycin 15 µg (Mast Diagnostics, England) were set on the surface of the BHI medium. The disc which did not contain any antibiotics was a negative control. *V. parahaemolyticus* was allowed to grow at 28 ºC for 48 h before the measurements of the diameters of inhibition zones by following Clinical and Laboratory Standards (CLSI, 2006): ≥ 20 mm: Susceptible (S), 15 - 19 mm: Intermediate (I), and < 14 mm: Resistant (R) [16].

**** Genomic DNA isolation***

I-genomic™ BYF DNA Extraction Mini Kit (iNtRON, South Korea) was used to extract genomic DNA following the manufacturer's instructions. Its integrity was checked by running on a 1.5 % agarose gel electrophoresis.

**** PCR and electrophoresis***

A 20 µL-final volume PCR reaction mixture including 10 µL of 2x PCR Master Mix Solution, 1 µL of DNA sample, 2 µL of primers (F/R) (Integrated DNA Technologies, Inc.) and 7 µL of dH_2O was run with the following condition: initial denaturation at 94 ºC for 5 min, 40 cycles of denaturation at 94 ºC or 30 s, primer hybridization for 30 s, elongation at 72 ºC for 30 s and finally, 72 ºC for 10 min. Primers are optimized for primer hybridization temperature when amplifying toxin genes. DNA sample extracted from the VTCC12233 strain was used as a positive control. PCR products were then run on 1.5% agarose gel electrophoresis.

Table 1. List of PCR primers used in this study

Gene	Primer	Primer sequence (5' – 3')	Tm (ºC)	Product size (bp)	References
toxR	*toxR*2	ACTCTACCCCCCTAAAAGCA	55.5	1070	[17]
	*toxR*4	CTGCCCCAGTACAACCAACC	58.5		
tlh	*tlh*1	TGTCGTGGCCATTTTGCTT	55.7	1484	[17]
	*tlh*3	CCGTGATGCCAAAATCAAAA	52.0		
tdh	*tdh*F	GTAAAGGTCTCTGACTTTTGGAC	53.3	269	[18]
	*tdh*R	TGGAATAGAACCTTCATCTTCACC	54.5		
trh	*trh*F	TTGGCTTCGATATTTTCAGTATCT	52.2	500	[18]
	*trh*R	TTGGCTTCGATATTTTCAGTATCT	52.8		

**** LD50 determination method***

LD_{50} values were determined by following a method of Reed and Muench (1938). Treated tilapia was injected via subcutaneous injection of 0.2 mL/fish of a bacterial stock (10^1 - 10^9 CFU/mL) while control fish was injected with 0.2 mL/fish of PBS. Treated and control zebrafish were infected by using a bath immersion method with the same bacterial stock (10^1 - 10^9 CFU/mL) or PBS, respectively, in 24 h. Survival rates of experimental fishes were measured in day 7 (with zebrafish) and day 14 (with tilapia) after infection.

LD_{50} values were calculated by the following equation [19]: $LD_{50} = 10^{(a + x)}$

10^a is the concentration of injected bacteria with 50% of survival rate after experimental time.

$x = (P_a – 50)/(P_a – P_u)$ with Pa and Pu is the mortality at dilution next above 50% and mortality next below 50%.

**** Inactivation method using formaldehyde***

Isolated bacteria were inactivated by using formaldehyde as described previous studies [13]. Then, inactivated bacteria with a concentration of 10^8 CFU/mL were used to validate the inactivation process by being grown on TCBS agar and BHI agar for 24 h.

**** Relative percent survival (RPS) assessment***

To assess the effect of inactivated bacteria as vaccine candidates, challenge with the wild-type strain via subcutaneous injection was performed as the previous description of Hu *et al*. (2012) [20]. Treated tilapia fish was injected with 0.2 mL of the inactivated vaccine while control fish was injected by 0.2 mL of phosphate-buffered saline (PBS). After 15 days post-vaccination, vaccinated fishes and control fishes were injected with 0.2 mL of virulent *V. parahaemolyticus* at different concentrations of 10^6, 10^7, and 10^8 CFU/mL. RPS (Relative Percentage Survival) was then calculated with the following equation [21].

$$RPS\ (\%) = \frac{1 - \%vaccinated\ dead\ fish}{\%\ unvaccinated\ dead\ fish} \times 100$$

**** Statistical analyses***

All statistical analyses were determined with analysis of variance (ANOVA) of the SPSS software (Version 22.0, SPSS Inc.), significance was determined as $P < 0.05$.

2.2. Results

2.2.1. Isolation of *Vibrio parahaemolyticus*

**** Colony morphologies, biochemical characteristics and antibiotic resistance of isolated strains***

Five bacterial strains were isolated on TCBS medium including DH6.1, DH10.1 (from Hai Tien, Thanh Hoa), DH48.3 (from Da Loc, Thanh Hoa), and DH64.1, DH64.2 (from Cua Lo, Nghe An). All those five strains exhibited well-known characteristics of

V. parahaemolyticus such as dark-green, a circular colony with a diameter of 2.0 - 3.0 mm, gram-negative and curved shape.

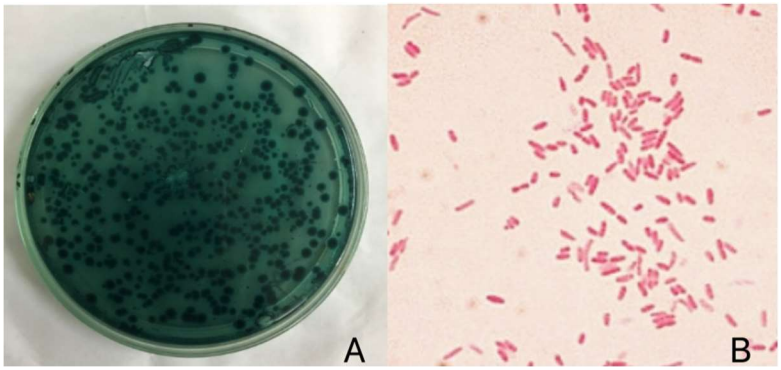

Figure 1. DH6.1 strain. (A) DH6.1 on TCBS medium and (B) Gram stain result

Biochemical results indicated all isolated bacteria could survive high salinity conditions (i.e. 3 - 6%), bacterial motility, glucose fermentation and lack of H_2S production and lactose fermentation. All these results concur with characteristics of the positive control strain *V. parahaemolyticus* VTCC 12233 as described by Thuoc (2009) [15].

Table 2. Antibiotic resistance abilities of some isolated V. parahaemolyticus strains

Strain	ENR	NOR	ERY	AMP	GEN	Control
VTCC 12233	S	S	R	S	S	R
DH6.1	S	R	I	R	I	R
DH10.1	S	S	R	R	I	R
DH48.3	S	S	I	R	I	R
DH64.1	I	S	R	R	R	R
DH64.2	S	S	R	R	I	R

ENR-Enrofloxacin (5 µg); NOR- Norfloxacin (10 µg); ERY- Erythromycin (15 µg); AMP- Ampicilin (25 µg); GEN- Gentamicin (30 µg); S: Susceptible; I: Intermediate, R: Resistant.

Isolated strains were tested for their antibiotic resistance abilities with 5 common antibiotics (results are shown in Table 2). First, all isolated strains could survive with ampicillin. In particular, the strain DH64.1 resisted 3/5 tested antibiotics while three other strains (i.e. DH6.1, DH10.1, and DH64.2) could withstand 2/5 tested antibiotics. The antibiotic resistance ability of *V. parahaemolyticus* has been documented in many previous studies. For instance, when studying the antibiotic resistance abilities of 31 bacterial strains on 16 different antibiotics has been reported a multidrug resistance phenomenon with a multiple antibiotic es of with 12 antibiotics and result in these bacteria were highly resistant to many tested antibiotics including streptomycin (86.2%), ampicillin (49.6%), cefazolin (43.5%), cephalothin (35.9%) as well as kanamycin (22,1%) [23].

**** Identification of virulent genes by PCR***

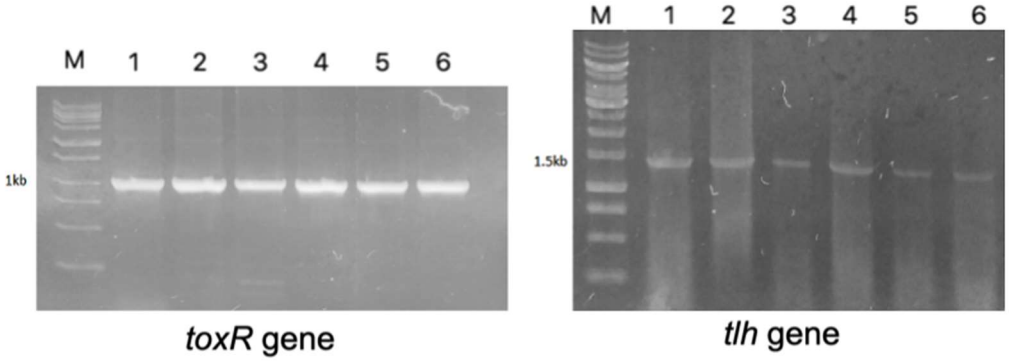

Figure 2. PCR products of toxR and tlh gene on 1.5% agarose

Line 1-6: DH6.1, DH10.1, DH48.3, DH64.1, DH64.2, VTCC 12233; M: 1 kb DNA ladder

Genomic DNA samples from isolated *V. parahaemolyticus* were used to identify the presence of virulent genes (e.g. *toxR*, *tlh*, *tdh,* and *trh*) by the mean of PCR method. Results indicated the presence of *toxR* and *tlh* in samples' genome (bands with related sizes to *toxR* and *tlh*, 1000 bp and 1500 bp, respectively, Fig. 2) while no band was obtained corresponding to *tdh* and *trh* gene. An earlier study by Iida et al. (1997) suggested that not all strains of *V. parahaemolyticus* carry the gene *tdh*, *trh* [24]. Indeed, it has been suggested that only 1 - 5% of *V. parahaemolyticus* actually contain *tdh* and *trh* gene in their genome [6]. In contrast, *toxR* gene is a well-known molecular marker for *V. parahaemolyticus*'s identification [6]. Together, morphological, biochemical, and molecular results strongly proved all five isolated bacterial strains were *V. parahaemolyticus*.

2.2.2. Analysis of virulence characteristics of isolated bacteria

Virulence characteristics of isolated bacteria were defined through their LD50 values on tilapia and zebrafish. The results are shown in Figure 3 and Figure 4.

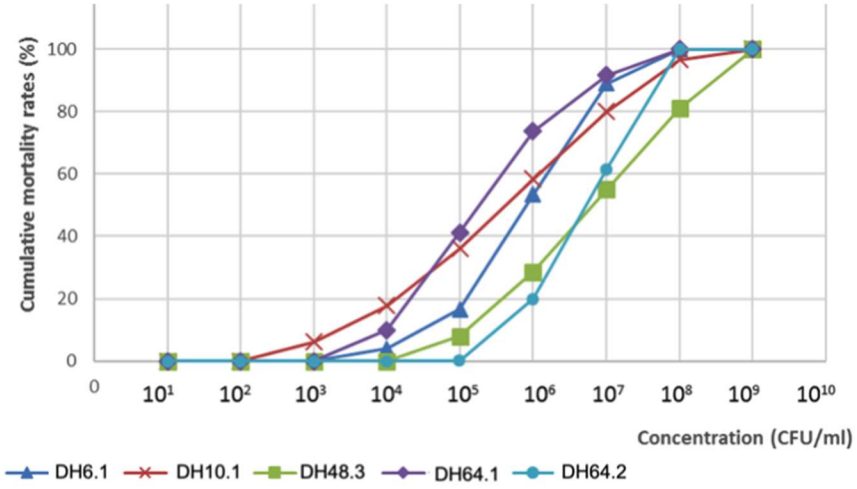

Figure 3. Cumulative mortality rates of tilapia after infections

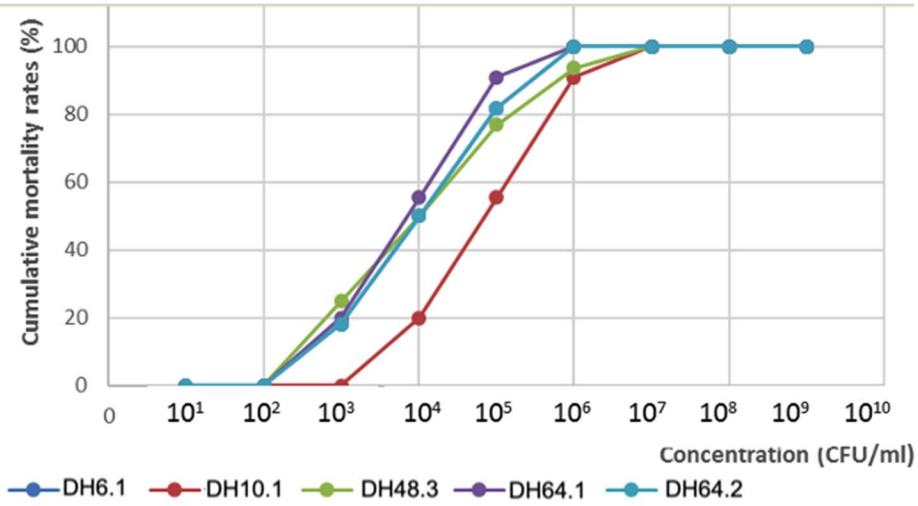

Figure 4. Cumulative mortality rates of zebrafish after infections

Consequently, LD_{50} values were calculated upon these cumulative mortality rates. The results of LD_{50} calculations are presented in Table 3.

Table 3. LD_{50} values of isolated strains on tilapia and zebrafish

Strain		DH6.1	DH10.1	DH48.3	DH64.1	DH64.2
LD_{50}	Tilapia	$10^{6.09}$	$10^{6.37}$	$10^{7.19}$	$10^{5.72}$	$10^{7.27}$
	Zebrafish	$10^{4.5}$	$10^{5.15}$	$10^{5.0}$	$10^{4.15}$	$10^{5.0}$

Figure 5. Pictures of dead tilapia (left) and zebrafish (right) after infection

The virulence assessment of bacterial strains was performed on both zebrafish and tilapia. Zebrafish are small in size, so the infection with bacteria was used bath immersion method. So, LD_{50} values regarding zebrafish were relatively smaller than those of tilapia. This observation could due to the differences in sizes and infection methods between the two fish species. Importantly, the DH64.1 strain appeared to have the lowest LD_{50} value on both tilapia and zebrafish. Thus, this strain was selected to develop an inactivated vaccine.

2.2.3. Relative percent survival (RPS) assessment

Treated fish was vaccinated with 0.2 mL of inactivated vaccine (DH64.1 strain, 10^8 CFU/mL). After two weeks of vaccination, 100% of vaccinated fishes survived

without any pathogenic symptom. This result indicated that the inactivated DH64.1 vaccine was safe for the tested fishes. Then, vaccinated fishes were injected with virulent DH64.1 bacterium with the challenge dose 10^6, 10^7, 10^8 CFU/mL. The relative percent survival (RPS) results of experimental fishes after 14 days of challenge are shown in Table 4.

Table 4. Relative percent survival rate in tilapia

Treatment		Challenge dose (CFU/mL)	Accumulated mortality (%)	RPS (%)
Tilapia	PBS	10^6	$41.11^a \pm 3.14$	
		10^7	$78.89^b \pm 4.16$	
		10^8	$97.78^b \pm 1.57$	
	Vaccine DH64.1	10^6	$0.00^c \pm 0.00$	$100.0^e \pm 0.00$
		10^7	$6.67^{c,d} \pm 2.72$	$91.71^{e,f} \pm 3.04$
		10^8	$11.11^d \pm 1.57$	$88.66^f \pm 1.41$

Accumulative mortalities (%) for each challenge dose were compared, respectively. Those values with different letters show a significant difference at $p < 0.05$

The RPS of inactivated DH64.1 strain for tilapia were 88.66 - 100%. The accumulated mortality of vaccinated fishes were 0.0 - 11.11% while these of unvaccinated fishes were 41.11 - 97.78% In this study, the RPS of vaccine is high and similar to that of some published studies. In 2004, a research team developed an inactivated-trivalent vaccine (i.e. *V. alginolyticus*, *V. parahaemolyticus* and *A. hydrophila*) with an RPS of 88.9% [10]. Similarly, Clark et al. (2010) developed an inactivated vaccine with *V. harveyi* and *V. parahaemolyticus*, and obtained an RPS of 77.6% on orange-spotted grouper [11]. Recently, Thuy et al. (2013) developed the inactivated vaccine *V. parahaemolyticus* V3 with an RPS value of 87.5% after 30 days post-vaccination [13].

3. Conclusions

From seawater samples collected from the regions of Thanh Hoa and Nghe An, we successfully isolated 5 strains of *V. parahaemolyticus* with well-known morphological, biochemical, and molecular characteristics. LD_{50} values of isolated bacteria varied from $10^{5.73} – 10^{7.28}$ and $10^{4.15} – 10^{5.15}$ on tilapia and zebrafish, respectively. The DH64.1 strain was experimentally tested for its ability to activate an immune response in tilapia as an inactivated vaccine candidate with RPS values of 88.66-100%. This strain could be used as a potential material for future vaccine production to prevent *V. parahaemolyticus* on fishes.

Acknowledgment. This study was funded from Hanoi National University of Education (No. SPHN-18-03), Hanoi, Vietnam.

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