Development of attenuated *Vibrio parahaemolyticus* mutant strains as potential live vaccines

Vu Thi Bich Huyen^{a*}, Nguyen Xuan Viet^a, Pham Thi Tam^b, Man Hong Phuoc^c, Huynh Viet Tung^a,
Nguyen Dang Quang^a, Do Thanh Van^d

^aGenetics and Biochemistry, Faculty of Biology, Hanoi National University of Education, 136 Xuan Thuy Street, Cau Giay District, Hanoi City, Vietnam

^bFaculty of Biotechnology, Hanoi Open University, 101 Nguyen Hien Street, Hai Ba Trung District, Hanoi City, Vietnam

^cDepartment of Molecular Microbiology, Institute of Biotechnology, Vietnam Academy of Science and Technology, 18 Hoang Quoc Viet Road, Cau Giay District, Hanoi City, Vietnam

^dCenter for DNA Identification, Institute of Biotechnology, Vietnam Academy of Science and Technology, 18 Hoang Quoc Viet Road, Cau Giay District, Hanoi City, Vietnam

Received 5th September 2019 / Accepted 9th January 2020

Abstract. *Vibrio parahaemolyticus* is a pathogenic Gram-negative bacterium that commonly infects numerous species of marine fishes. In this study, we obtained eight attenuated mutant strains by the selection of rifampicin-resistant *V. parahaemolyticus* from three wild-type strains. Among eight selected strains, four strains (L4650, A650, L4200 and A400) were widely attenuated by virulence analysis. Sequences of two genes, *tllb* and *toxR*, that related to virulence were analyzed. The results showed that seven attenuated strains were different in sequences of the *tllb* gene while there was no difference in sequence of *toxR* gene in comparison with that of wild-type strains. Three attenuated strains (L4650, L4200 and A650) had the nucleotide changes in *tllb* gene that resulted to the changes in the size of deduced amino acid sequences. In addition, *rpoB* gene coding for β -subunit of RNA polymerase that related to Rifampicin resistance was analyzed for all selected strains. Two strains (A650 and L4650) had the nucleotide changes in *rpoB* sequence. To assess the potential use of the L4650 strain as a live attenuated vaccine, the groupers (*Epinephelus coioides*) were intraperitoneally injected with the L4650 strain and later challenged with the virulent wild-type strain at three different doses. Results showed the protection for L4650 strain vaccinated-fish produced relatively earlier than usual at 15 days post-vaccination with relative percent survival (RPS) values from 96.91-100%. At 60 days post-vaccination, the RPS values of vaccinated fish were 96.27-100%. Our results suggest the L4650 strain could become a potential vaccine candidate against *V. parahaemolyticus* for future studies.

Keywords: *Vibrio parahaemolyticus*, *Epinephelus coioides*, attenuated mutant strains, rifampicin, *rpoB*, virulence genes

INTRODUCTION

Vibrio parahaemolyticus is a rod-shaped, curved, Gram-negative bacterium and one of the dangerous pathogens to many marine fish species

such as *Dicentrarchus labrax*, *Amphiprion sebae*, *Epinephelus* spp., *Lates calcarifer*, *Aphanus iberus* (Alaboudi *et al.*, 2016; Alcaide *et al.*, 1999;

*Author for correspondence: Vu Thi Bich Huyen, Department of Genetics and Biochemistry, Faculty of Biology, Hanoi National University of Education, 136 Xuan Thuy Street, Cau Giay District, Hanoi City, Vietnam.
Email – bichhuyenbh88@gmail.com

Khouadja *et al.*, 2013; Marudhupandi *et al.*, 2017; Thuy *et al.*, 2013). The common symptoms of infected fishes are dark skin, pale gills, extensive haemorrhagic septicemia, skin ulcers, fins and tail rot, splenomegaly and enteritis (Austin and Austin, 2007; Khouadja *et al.*, 2013; Paranjpye *et al.*, 2013). The mortality rate of infected fishes could be up to 90%, thus, potentially causing great damages to aquaculture. *V. parahaemolyticus* contains several hemolysin toxins, namely thermolabile hemolysin (TLH), thermostable direct hemolysin (TDH), TDH-related hemolysin (TRH) which are encoded by the *tlh*, *tdh*, and *trh* genes, respectively, in chromosome II (Ahn *et al.*, 2016; Kalburge *et al.*, 2014; Kim *et al.*, 2016; Letchumanan *et al.*, 2016). These hemolysins are considered as important virulence factors of *V. parahaemolyticus*, as they caused a variety of pathogenic consequences, including hemolytic activity, cytotoxicity, cardiotoxicity, and enterotoxicity. Hence, these hemolysins have been widely recognized as potential targets of pathogen detection (Gutierrez *et al.*, 2013; Nelapati and Krishnaiah, 2010; Raghunath, 2014; Yáñez *et al.*, 2015; Zamora-Pantoja *et al.*, 2013). Besides, the *toxR* gene is a conserved regulatory gene of the toxin *tdh* genes in chromosome I and was recognized as a species specific marker for all strains of *V. parahaemolyticus* (Ahn *et al.*, 2016; Nelapati and Krishnaiah, 2010; Zhang, Y. *et al.*, 2017).

Cephalothin, cefuroxime, cefotaxime, ceftazidime, tetracycline, doxycycline, or fluoroquinolone are antibiotics frequently used to treat *Vibrio* diseases in aquaculture (Austin and Austin, 2007). However, the use of antibiotics without careful control might induce the high incidence of antibiotic resistance among bacteria, immune suppression in fishes, and the accumulation of residual antibiotics in fish tissues (Noorlis *et al.*, 2011). Consuming fishes which have accumulated antibiotics negatively affects human health. Therefore, vaccination is an effective solution to control disease and to reduce the use of antibiotics in aquaculture (Somerset *et al.*, 2005).

Bacterial vaccines for fishes include several types such as inactivated vaccines, subunit vaccines, live attenuated vaccines, DNA vaccines, and recombinant vaccines. Previous studies have reported some vaccines against *V. parahaemolyticus*.

Clark *et al.* (2010) created a formalin-inactivated vaccine from *V. harveyi* and *V. parahaemolyticus*. That vaccine was tested on orange-spotted groupers (*E. coioides*) and showed the relative survival percent (RPS) value of 77.6%. The inactivated trivalent vaccine (*V. parahaemolyticus*, *V. alginolyticus* and *Aeromonas hydrophila*) was vaccinated into *Larimichthys crocea*. Transcriptome analysis in spleens at 24 and 72 hours after vaccination showed that vaccine affected the expressions of many genes associated with innate early immune response (Zhang, X. *et al.*, 2017). Inactivated vaccines have a high degree of safety; however, their protection duration for fish is usually not long. For example, the RPS value after 30 days post-vaccination (dpv) of formalin-killed *V. parahaemolyticus* in *E. coioides* was 87.5%, but this value decreased to 41.1% after 60 dpv (Thuy *et al.*, 2013). Outer membrane proteins (OMPs) are antigens of interest for the study of vaccines against *V. parahaemolyticus*. Four genes encoding OMPs (OmpW, OmpV, OmpK, OmpU) of *V. parahaemolyticus* zj2003 were cloned and expressed in *E. coli*. These recombinant OMPs were vaccinated into *L. crocea*. The RPS values in vaccinated-fish were ranging from 80% to 90%, while all control fish died (Mao *et al.*, 2007). Then, four OMPs (LptD, VP0802, VP1243, and VP0966) were demonstrated to make immunogenicity. The VP0802 with highly conserved sequence among major *Vibrio* spp. created the protection to prevent *V. parahaemolyticus* infection with the RPS value of 66.7% (Li *et al.*, 2014). In zebrafish (*Danio rerio*), the vaccination of LamB (an OMP of *V. parahaemolyticus*) resulted in the RPS value of 77.8% (Lun *et al.*, 2014). Similarly, six OMPs (VP2309, VP0887, VPA0548, VP1019, VP1667, and VP2369) were determined as antigens to make immunogenic response against *V. parahaemolyticus* and other *Vibrio* (Peng *et al.*, 2018; Peng *et al.*, 2016). The DNA vaccines were also future potential vaccines for fish. Li *et al.* (2013) created a DNA vaccine named pEGFP-N2-OMP_K (pDNA), which was constructed from the *OmpK* gene of *V. parahaemolyticus* OS4 and pEGFP-N2 vector. The black sea bream (*Acanthopagrus schlegelii* Bleeker) injected with chitosan-pDNA particles had the RPS value of 72.3% after 3 weeks post-vaccination. The result showed that mRNA of the *OmpK* gene existed in

fish mid-intestine, liver, kidney, muscle of black sea bream (Li *et al.*, 2013). The DNA vaccine (pEGFP-N1/m-vps) was constructed by inserting the serine protease gene (which hydrolyzed N- α -benzoyl-L-tyrosine p-nitroanilide) into the pEGFP-N1 plasmid and delivered the RPS value of 96.11% in turbot (Liu *et al.*, 2011). The recombinant vaccine and DNA vaccine are potential vaccines for fish. However these vaccines need to be delivered by injection that is not feasible for a large number of under 20 g fishes. Inactivated and live attenuated vaccines have many methods of delivery into the fish body consisting of injection vaccination, immersion vaccination and oral vaccination (Plant and Lapatra, 2011). However, to date, there has been no commercial vaccine against *V. parahaemolyticus*. Especially, the knowledge on the use of live attenuated vaccines against *V. parahaemolyticus* is also very limited.

Random mutations creating virulent bacteria can be caused by using transposon mutations, then resultant bacteria are selected for the loss of virulence (Liu and Bi, 2007). Other random approaches involve the treatment of mutagenesis chemicals such as rifampicin (Rif), novobiocin. Rif is a broad-spectrum, semisynthetic antibiotic that can inhibit bacterial DNA-dependent RNA polymerase (RNAP). Moreover, the bacterial strains being resistance to Rif typically have attenuated virulence (Bhatnagar *et al.*, 1994; Campbell *et al.*, 2001; Hu *et al.*, 2012; LaFrentz *et al.*, 2008). For example, LaFrentz *et al.* (2008) reported the selection of a Rif-resistant and attenuated strain of *Flavobacterium psychrophilum* by repeating passage on media containing increasing Rif concentration. Rainbow trout (*Oncorhynchus mykiss* Walbaum) was injected with an attenuated strain and then produced the antibody to protect the body (LaFrentz *et al.*, 2008). This method has been applied successfully to *Edwardsiella ictaluri*, *Flavobacterium columnare*, *Vibrio harveyi*. Some Rif-resistant strains which are using as live attenuated vaccines for these fish pathogens have been developed and granted licenses to use in aquaculture (Hazen *et al.*, 2009; Hu *et al.*, 2012; Klesius and Shoemaker, 1999; Shoemaker *et al.*, 2007). Bacterial RNAP in complex with transcription inhibitors associated with the antibiotic resistance among bacteria (Murakami, 2015). Rif binds to certain sites of β -subunit,

which is encoded by the *rpoB* gene with 4029 bp and blocks transcription initiation (Campbell *et al.*, 2001). Mutations in the *rpoB* gene were related to Rif resistance of *Mycobacterium tuberculosis*, and the mutation positions would affect the Rif-resistant level (Taniguchi *et al.*, 1996). The Rif-resistant *V. parahaemolyticus* strains in 100 μ g/ml Rif medium had some mutations in the *rpoB* sequence as shown in the previous report (Hazen *et al.*, 2009).

The Rif treatment for virulent strains (wild-type) to select the attenuated strains could develop live attenuated vaccines against fish pathogens was demonstrated. This study aimed to utilize the Rif resistance strategy to *V. parahaemolyticus* with main objectives: (1) generating Rif-resistant strains of *V. parahaemolyticus*, assessing the virulence of Rif-resistant strains by injecting the same challenge dose in groupers (*E. coioides*), then comparing the virulence of Rif-resistant strains and that of the wild-type strains to find attenuated strains, (2) sequencing and aligning the *toxR*, *tliB* sequences of attenuated strains and wild-type strains to determine the mutations in attenuated strains, and (3) assessing the potential of a mutant attenuated strain as a live attenuated vaccine, the groupers were vaccinated with attenuated strain via intraperitoneal injection and challenged with the virulent wild-type strain at three doses.

MATERIALS AND METHODS

Bacterial strains and generation of Rif-resistant strains

Three pathogenic strains of *V. parahaemolyticus*, namely LBT6, A3.3, and B3.13 which were isolated from the diseased groupers in Cat Ba island, Hai Phong city, Vietnam (Huyen *et al.*, 2019) were used as the parental strains (wild-type) to create Rif-resistant strains. These LBT6, A3.3, and B3.13 strains were independently cultured in Brain Heart Infusion Broth (BHI) medium to an OD₆₀₀ of 0.8. Then, 100 μ l of the bacterial suspensions were plated on Thiosulfate Citrate Bile Salts Sucrose (TCBS) agar plates containing 5 μ g/ml Rif (SERVA, Germany), and incubated at 28°C for 2 days. Two of the colonies emerged on the plate of each wild-type strain were randomly selected and separately cultured in BHI medium

containing Rif at 10 µg/ml. Afterward, 100 µl of the bacterial suspension with an OD₆₀₀ of 0.8 were cultured TCBS agar medium containing 10 µg/ml of Rif. The culture process was repeated in BHI and TCBS agar medium containing Rif with increasing concentrations until the final concentration reaching 250 µg/ml. Some colonies growing on the TCBS agar plates containing 250 µg/ml Rif were randomly selected. These were Rif-resistant strains.

The type strain *V. parahaemolyticus* VTCC 12233 purchased from the Institute of Microbiology and Biotechnology, Vietnam National University, Hanoi, Vietnam was included as a control.

Fish

Grouper fingerlings (*E. coioides*) with the mean total length of 60 - 65 mm and the bodyweight of 4.1 ± 0.7 g were purchased from Aquaculture Breeding Center (Cat Hai, Hai Phong, Vietnam) and acclimatized in the laboratory condition for at least two weeks before the experiments. The animals were sustained at 23 - 25°C in seawater and fed commercial dry pellets three times a day.

Virulence assessment

The method of the challenge by subcutaneous injection was used to assess the virulence of bacterial strains (Gao *et al.*, 2014). Groupers were infected via subcutaneous injection with the same dose at 10⁷ CFU/ml and were individually treated with 100 µl/fish of the Rif-resistant strains or 3 wild-type strains (LBT6; A3.3; B3.13). In this trial, groups of 30 groupers were challenged with each strain resuspended in sterile phosphate-buffered saline (PBS; 4.3 mM Na₂HPO₄·7H₂O, 1.4 mM KH₂PO₄, 2.7 mM KCl, 137 mM NaCl, pH 7.2). Besides, the control group (n=30) was injected with 100 µl/fish of sterile PBS. Survival rates of tested fish were recorded daily during 14 days post-infection (dpi) for each strain at the challenge dose tested.

DNA extraction, virulence genes amplification and sequencing

The genomic DNA of three wild-type strains (LBT6, A3.3, B3.13) and some attenuated strains were extracted by using i-genomic™ BYF DNA Extraction Mini Kit (INtRON Inc., Korea). The primers were used to amplify the *toxR*, *tlb*, *tdh*, *trh*

and *rpoB* genes. All additional primers are listed in Table 1. The PCR amplification for each gene was performed in a final volume of 20 µl which contained a DNA template, 2x PCR Master mix Solution (AccuPower®Pfu, Bioneer Inc., Korea), primers, and sterile distilled water. For the PCR, the conditions were as follows: initial denaturation at 94°C for 5 min, 40 cycles of denaturation at 94°C for 50 s, primer hybridization for 30 s and elongation at 72°C for 1.5 min; and finally, 72°C for 5 min.

The *V. parahaemolyticus* VTCC 12233 strain was included as a positive control. Negative control for PCR was a reaction that contained essential components of the amplification reaction except for the template.

The amplified PCR products of strains were checked on a 1% agarose gel and purified and sequenced at 1st BASE sequencing company, Seri Kembangan, Selangor, Malaysia. The similarity between the sequences of virulence genes and the *rpoB* gene of three wild-type strains and those of other strains in NCBI (National Center for Biotechnology Information) was determined by using the BLAST tool (<https://blast.ncbi.nlm.nih.gov/Blast.cgi>). Then, the sequences of these genes of the attenuated strains were compared with those of three wild-type strains to detect mutant positions by using CLC Genomics Workbench 11.0. The protein sequences were translated from the gene sequences of the *V. parahaemolyticus* strains, then, the protein sequences of the attenuated strains were compared with those of three virulent strains, respectively, by implementing CLC Genomics Workbench ver 11.0.

Determination of LD₅₀ of bacterial strains

Thirty healthy *E. coioides* (4.1 ± 0.7 g) were injected 100 µl/fish of the bacterial suspension via subcutaneous injection with different concentrations (1x10¹ to 1x10⁸ CFU/fish). The control group (n=30) was injected with sterile PBS; 100 µl/fish. To identify mortality rates, the ratio of dead fishes were recorded daily for 14 days after injection. The method of Reed and Muench (1936) was used to determine the LD₅₀ values of the experimental strains (Reed and Muench, 1938).

Vaccination

To assess the potential use of the attenuated strains as vaccine candidates, challenge with the wild-type strain via subcutaneous injection was performed as the previous description (Hu *et al.*, 2012). The most attenuated strain was cultured in BHI medium to an OD₆₀₀ of 0.8, after being washed and suspended in sterile PBS to 10⁵ CFU/ml. Two groups of fish named A and B were divided randomly. All animals of group A were injected at 10⁵ CFU/ml and 100 µl/fish of the most attenuated strain, while all animals of group B (control) were injected sterile PBS with 100 µl/fish. After 15 dpv, a half number of groupers of A and B groups were challenged with a wild-type strain, which was treated by Rif to create the most attenuated strain, at three different doses (5xLD₅₀; 10xLD₅₀ and 100xLD₅₀ CFU/ml; in which LD₅₀ was determined based on the trial of fish infected with the wild-type strain).

Similarly, at 60 dpv, the rest number of fish of groups A and B were challenged with three doses of the wild-type strain. In each trial, there was a mock-infected control group (n=30) that was injected via subcutaneous injection with 100 µl/fish of sterile PBS. Mortality rates of challenged fish were assessed over during periods of 15 days after the challenge. The RPS value (%) of the most attenuated strain was determined as previously described (RPS value=[1– (percentage mortality in immunized fish/percentage mortality in control fish)] × 100 (Amend, 1981).

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 $P < 0.05$.

Table 1. Primers used in this study.

Target gene	Primer	Sequence (5'-3')	T _m (°C)	Expected amplicon (bp)	Reference
<i>toxR</i>	<i>toxR2</i>	ACTCTACCCCCCTAAAAGCA	55.5	1070	This study
	<i>toxR4</i>	CTGCCCCAGTACAACCAACC	58.5		
<i>tllb</i>	<i>tllb1</i>	TGTCGTGGCCATTTTGCTT	55.7	1484	This study
	<i>tllb3</i>	CCGTGATGCCAAAATCAAAA	52.0		
<i>tdlb</i>	<i>tdlbF</i>	GTAAAGGTCTCTGACTTTTGGAC	53.3	269	Bej <i>et al.</i> , 1999
	<i>tdlbR</i>	TGGAATAGAACCITTCATCITCACC	54.5		
<i>trb</i>	<i>trbF</i>	TTGGCITTCGATATTTTCAGTATCT	52.2	500	Bej <i>et al.</i> , 1999
	<i>trbR</i>	TTGGCITTCGATATTTTCAGTATCT	52.8		
<i>rpoB</i>	1110F	GTAGAAATCTACCGCATGATG	50.0	984	Tarr <i>et al.</i> , 2007
	CM32b	CGGAACGGCCTGACGTTGCAT	59.0		Mollet <i>et al.</i> , 1997

RESULTS

Assessment of virulence

From three wild-type strains (LBT6, A3.3; B3.13), we isolated 13 Rif-resistant strains: L1380, L2100, L3600, L3900, L4200, L4650 from LBT6 strain; A400, A500, A650 from A3.3 strain; B5250, B5700, B7050, B7900 from B3.13 strain after selecting on medium containing Rif with increasing concentrations up to 250 µg/ml. The survival rates of fish that were injected with the same dose of three wild-type strains and 13 Rif-resistant strains were determined after 14 dpi.

In the first group, the result of challenge test with the wild-type LBT6 strain and six Rif-

resistant strains (L1380, L2100, L3600, L3900, L4200, L4650) indicated that four strains L3600, L3900, L4200, and L4650 were the attenuated strains (Figure 1). The survival rate of fish challenged with the L4650 strain was the highest (86.67% at 14 dpi) compared with those challenged with other Rif-resistant strains. The survival percentages of groups injected with three strains L4200, L3900, L3600 were significantly different ($p < 0.05$) from that of group injected with the wild-type LBT6 strain (63.33%, 56.67%, and 16.67% respectively vs. 10.0% at 14 dpi). However, there was no significant difference between the survival rates of animals injected with the LBT6 and L1380 and L2100 strains.

In the second group, there were three modest attenuated strains, namely B7050, B7900, and B5700 strains. The survival rates at 14 dpi of fish challenged with these strains were 46.67%, 36.67%, 33.33%, respectively (Figure 2), while survival percentages of fish injected with the wild-type B3.13 and B5250 strains were tiny, of 6.67%.

Similarly, in the third group, the A650 and A400 strains were considered as attenuated strains (Figure 3). The survival rates of fish challenged with these two strains were 81.11% and 63.33% at 14 dpi, respectively. These results were

significantly different from those of fish injected with the wild-type A3.3 and A500 strains (the survival rates of 10.00% at 14 dpi).

In summary, there were 9 Rif-resistant attenuated strains including L3600, L3900, L4200, L4650, B5700, B7050, B7900, A400, A650. The virulence genes and *rpoB* gene in these eight strains (except L3600 strain) and three wild-type strains were detected, sequenced. Then, their sequences were compared to find mutation positions.

Figure 1. The survival rate of fishes injected with the wild-type LBT6 strain and six Rif-resistant strains (L1380, L2100, L3600, L3900, L4200, L4650). Note: Those values with different letters show a significant difference at $p < 0.05$.

Figure 2. The survival rate of fish injected with the wild-type B3.13 strain and four Rif-resistant strains (B7900, B7050, B5700, B5250). Note: Those values with different letters show a significant difference at $p < 0.05$.

Figure 3. The survival rate of fish injected with the wild-type A3.3 strain and Rif-resistant strains (A400, A500, A650). Note: Those values with different letters show a significant difference at $p < 0.05$.

Comparison between the nucleotide sequences of virulence genes and *rpoB* gene in three wild-type strains and those of attenuated strains

PCR results

Virulence genes and *rpoB* gene were amplified using PCR for three wild-type strains and eight attenuated strains (L3900, L4200, L4650, B5700, B7050, B7900, A400, A650). All strains were *tllb*-positive, *toxR*-positive, and *rpoB*-positive. The PCR amplification of these genes obtained 1500 bp, 1000 bp, and 984 bp bands, respectively (Figure 4, Figure 5, Figure 6). Two genes *tdh*, *trh* were not detected in the genome of all bacterial strains.

Figure 4. The agarose gel electrophoresis results of PCR products of *toxR* gene of *V. parahaemolyticus* strains. Lane 1-13: VTCC 12233; negative control; LBT6; L3900; L4200; L4650; B3.13; B5700; B7050; B7900; A3.3; A400, A650; M: 1kb DNA ladder.

Figure 5. The agarose gel electrophoresis results of PCR products of *tllb* gene of *V. parahaemolyticus* strains. Lane 1-13: negative control; VTCC 12233; LBT6; L3900; L4200; L4650; B3.13; B5700; B7050; B7900; A3.3; A400, A650; M: 1kb DNA ladder.

Figure 6. The agarose gel electrophoresis results of PCR products of *rpoB* gene of *V. parahaemolyticus* strains. Lane 1-13: negative control; VTCC 12233; LBT6; L3900; L4200; L4650; B3.13; B5700; B7050; B7900; A3.3; A400, A650; M: 1kb DNA ladder.

The change of virulence gene sequences

The sequences of *toxR* and *tllb* genes from these wild-type strains and eight attenuated strains were also sequenced and compared. For the *toxR* gene, the sequences of eight attenuated strains that were divided by three groups were identical and similar to three wild-type strains *toxR* gene. However, the *tllb* gene had some nucleotide changes in attenuated strains compared to wild-type strains. Mutations were identified by comparing the sequences of the wild-type *tllb* gene and related attenuated strains *tllb* gene using sequence analysis software, CLC Genomics Workbench ver 11.0 (Table 2).

In the first group, there were changes in 21 nucleotides coding for 07 amino acids (codons 6, 21, 144, 283, 286, 296, 305) and 01 stop codon (codon 288) between L4650 and LBT6 strains. Deduced amino acid sequence (DaaSeq) of L4650 strain (287 amino acids) was 130 amino acids shorter than that of the LBT6 strain (417 amino acids). Nucleotide changes at the other positions in the L4650 strain did not result in changes in the DaaSeq. The nucleotide sequence of the *tllb* gene in the L3900 strain was different from the *tllb* gene in wild-type at four positions: nucleotides 34, 40, 234, 1069. Those changes resulted in changes in amino acid 12 (Leu → Phe) and amino acid 14 (Leu → Val). Although the *tllb* gene sequences of the L4200 and LBT6 strains just had differences at nucleotides 1233; and between 1241 and 1242, these led to the replacement of one amino acid at position 411 and the frameshift mutation beginning at position 414 resulting in the absence of stop codon (codon 418). As a consequence, the DaaSeq of L4200 was longer than that of its wild-type strain (Table 2).

In the second group, the *tlb* sequence of B7050 strain differed from that of B3.13 strain at nucleotides 270, 766, 786 and 858. These nucleotide changes resulted in differences of the amino acid at three positions: codons 90; 256 and 262. A unique difference in the B7900 strain at nucleotide 738 resulted in the substitution of amino acid 246, leading to the replacement of phenylalanine with leucine. On the other hand, no change was found in the *tlb* sequence of the B5700 strain (Table 2).

In the third group, the *tlb* sequence of A400 strain had five nucleotides differing from that of A3.3 strain. These changes resulted in four substitutions of amino acids 346, 366, 369 and 399. The comparison of the *tlb* gene sequence between A650 strain and wild-type strain revealed the variations of ten nucleotides. A deletion of nucleotide 1038 and the insertion of one nucleotide between nucleotides 1238 and 1239 resulted in a frameshift mutation from codon 346 to codon 413 where there was the presence of stop codon at position 350. The DaaSeq of A650 strain (349 amino acids) was 68 amino acids shorter than that of the wild-type strain (417 amino acids). These amino acid substitutions at positions 345, 415, 416, 417, 418 were caused by nucleotide changes at positions 1033, 1245, 1247, 1248, 1251, 1252 and 1254 (Table 2).

The change of *rpoB* gene sequence

The sequences of *rpoB* genes from these wild-type strains and eight attenuated strains also were sequenced and compared. The full length of *V. parahaemolyticus* *rpoB* gene is 4029 bp. We used primers 1110F (Tarr *et al.*, 2007) and CM32b (Mollet *et al.*, 1997) to amplify PCR products with bands 984 bp. Then, they were sequenced to obtain nucleotide sequences of the *rpoB* gene region from 1121 position to 2050 position (930 bp).

In the first group (LBT6, L3900, L4200, L4650), nucleotide sequences of the *rpoB* gene region of L3900 and L4200 strains were not different from that of LBT6 strain. The *rpoB* sequence of the L4650 strain had changes in some nucleotide positions compared to the wild-type strain, which resulted in changes of DaaSeq in 28 positions (Table 3). The changed codon 524 was

located in 81 bp rifampicin-resistance determining region (RRDR) (Zaw *et al.*, 2018). No nucleotide change was found in *rpoB* sequences of three attenuated strains (B5700, B7050, B7900) compared with wild-type strain (B3.13) in the second group. In the third group, only A650 strain had a nucleotide change leading to a replacement of amino acid in codon 513 (Gln513→Pro). It was one hotspot of mutation associated with Rif resistance among many bacteria species (Jin and Gross, 1988; Lee *et al.*, 2005; Maughan *et al.*, 2004; Zaw *et al.*, 2018). The A400 strain had no change nucleotide sequence of the *rpoB* gene region compared with the A3.3 strain.

Protection of L4650 strain as an injection vaccine

The protective immune response of *E. coioides* against *V. parahaemolyticus* was formed by intraperitoneal injection with the L4650 attenuated strain, which was created by treatment LBT6 with Rif (Table 2). Virulence analysis in groupers showed that the LD₅₀ value of L4650 (10^{7.2}) was 100-fold higher than that of LBT6. Therefore, the challenge doses with LBT6 would be 7.14 x 10⁵, 1.43 x 10⁶, and 1.43 x 10⁷ CFU/ml.

At 15 dpv, the cumulative mortality of L4650-vaccinated fish that were challenged with LBT6 strain at three different challenge doses was 0.0%, 1.67%, and 3.45%, respectively, corresponding to RPS values of 100%, 98.1%, and 96.91%. The cumulative mortality at dpv after challenging with virulent LBT6 was 0.0%, 1.89% and 3.67% in the L4650-vaccinated fish group at three challenge doses respectively, corresponding to RPS values of 100%, 97.03%, and 96.27%. In contrast, challenging with the wild-type strain maintained the high accumulated mortalities (76.67- 97.33%) in fish injected with PBS as mock immunization during the challenge period. Blue-pigmented bacteria were re-isolated from the dead fishes in the TCBS agar medium with ratios of 98% (96/98) at the 15 dpv and 97% (98/101) and 60 dpv after challenges. We found that they had the phenotypical characteristics of *V. parahaemolyticus*. No mortality of the mock-infected fish groups was also recorded.

Table 2. Comparing the *tlb* sequences between attenuated strains and wild-type strains.

Strain	Mutation			
	Nucleotide position	Amino acid codon	Nucleotide sequence change	Deduced amino acid sequence change
Group I				
L4650	16	6	A → C	Thr → Pro
	61	21	G → A	Glu → Lys
	234	78	C → T	Gly → Gly (silent mutation)
	431	144	A → T	Asn → Ile
	468	156	G → A	Thr → Thr (silent mutation)
	609	203	T → C	Gly → Gly (silent mutation)
	657	219	G → A	Gln → Gln (silent mutation)
	846	282	A → G	Thr → Thr (silent mutation)
	847	283	C → G	Leu → Val
	857	286	C → T	Ala → Val
	862	288	A → T	Lys → Stop codon
	867	289	G → A	Ala → Ala (silent mutation)
	886	296	A → C	Thr → Pro
	914	305	C → A	Thr → Glu
	988	330	T → C	Leu → Leu (silent mutation)
	1006	336	T → C	Leu → Leu (silent mutation)
	1011	337	C → T	Phe → Phe (silent mutation)
	1134	378	G → A	Ala → Ala (silent mutation)
	1140	380	C → T	Ser → Ser (silent mutation)
	1152	384	A → G	Lys → Lys (silent mutation)
	1176	392	C → T	His → His (silent mutation)
	1239	413	C → A	Ala → Ala (silent mutation)
L3900	34	12	C → T	Leu → Phe
	40	14	C → G	Leu → Val
	234	78	C → G	Gly → Gly (silent mutation)
	1069	357	T → C	Leu → Leu (silent mutation)
L4200	1233	411	C → A	Asn → Lys
	1241-1242	414	Insertion 01 nucleotide (C)	Glu → Ala, resulted frameshift mutation, absence of stop codon (codon 418)
Group II				
B5700			None	
B7050	270	90	C → A	Phe → Leu
	766	256	G → T	Val → Phe
	786	262	T → A	Asp → Glu
	858	286	G → A	Ala → Ala (silent mutation)
B7900	738	246	G → T	Leu → Phe
Group III				
A400	1037	346	G → C	Glu → Asp
	1055	352	G → A	Ala → Ala (silent mutation)
	1096	366	G → A	Asp → Asn
	1105	369	T → G	Tyr → Asp
	1195	399	G → T	Arg → Leu
A650	1033	345	G → A	Glu → Lys

A650	1036		G → A	Glu → Asn, resulted frameshift mutation from codon 346 to codon 413, presence of stop codon in codon 350
	1038	346	Deletion 01 nucleotide (A)	
	1238-1239	413	Insertion 01 nucleotide (A)	Ala → His
	1245	415	C → A	Tyr → Stop codon
	1247		G → C	
	1248	416	T → G	Arg → Pro
	1251	417	C → T	Phe → Phe (silent mutation)
	1252		T → A	
	1254	418	A → T	Stop codon → Asn

Table 3. Amino acid change in the region of the *rpoB* gene sequences of attenuated strains compared with those of wild-type strains.

Amino acid change	Nucleotide change	Amino acid change	Nucleotide change
A650			
Gln513 → Pro	A1538 → C		
L4650			
Ser387 → Asn	G1160 → A	Phe614 → Tyr	T1841 → A
Glu393 → Asp	G1179 → C	Ala621 → Thr	G1861 → A, T1863 → C
Thr399 → Ala	A1195 → G, T1197 → G	Lys622 → Asn	G1866 → C
Glu412 → Asp	A1236 → C	Asn624 → Asp	A1870 → G
Gln415 → Glu	C1243 → G	Asp626 → Glu	T1878 → A
Glu422 → Lys	G1264 → A	Glu632 → Asp	G1896 → C
Thr423 → Glu	A1267 → G, C1268 → A	Ile634 → Val	A1900 → G, C1902 → T
Ala434 → Asp	C1301 → A, G1302 → T	Gly643 → Ser	G1927 → A, T1929 → C
Leu492 → Met	C1474 → A	Glu648 → Asp	G1944 → C
Val524 → Ile	G1570 → A, A1572 → C	His649 → Gln	C1947 → G
Leu558 → Val	C1672 → G	Ala650 → Val	C1949 → T
Ala577 → Val	C1730 → T	Ala656 → Ser	G1966 → T, G1968 → C
Phe578 → Tyr	T1733 → A, T1734 → C	Ile663 → Val	A1987 → G
Asp604 → His	G1810 → C, T1812 → C	Ala664 → Gly	C1991 → G

Table 4. Summary of the vaccination results.

Treatment	Challenge dose (CFU fish ⁻¹)	Accumulated mortality (%) after		RPS (%) after	
		15 dpv*	60 dpv*	15 dpv*	60 dpv*
PBS	7.14 x 10 ⁵	76.67 ^a ± 1.01	76.69 ^a ± 1.10		
	1.43 x 10 ⁶	87.67 ^{a,b} ± 2.43	89.67 ^{a,b} ± 2.23		
	1.43 x 10 ⁷	96.67 ^b ± 1.27	97.33 ^b ± 1.29		
L4650	7.14 x 10 ⁵	0.00 ^c ± 0.00	0.00 ^c ± 0.00	100.0 ^d ± 0.00	100.0 ^d ± 0.00
	1.43 x 10 ⁶	1.67 ^c ± 0.02	1.89 ^c ± 0.03	98.10 ^d ± 2.71	97.03 ^d ± 2.32
	1.43 x 10 ⁷	3.45 ^c ± 0.04	3.67 ^c ± 0.03	96.91 ^d ± 2.03	96.27 ^d ± 2.12

The number of fish injected with the mutant attenuated L4650 strain or PBS was determined before each challenge time. Accumulative mortalities (%) for each challenge dose or time were compared, respectively. Those values with different letters show a significant difference at $p < 0.05$. * Days post-vaccination.

DISCUSSION

Rif and novobiocin have been used for the selection and creation of mutant attenuated bacterial strains to develop vaccines for fish (Hu *et al.*, 2012; LaFrentz *et al.*, 2008; Mariam *et al.*, 2004; Pridgeon and Klesius, 2011a, 2011b). In this study, eight attenuated Rif-resistant *V. parahaemolyticus* strains were selected from three wild-type LBT6, B3.13, A3.3 strains. However, the survival percentages of fish challenged with the wild-type strains and attenuated strains were different in three groups. The survival rates of fish challenged with the LBT6, B3.13, A3.3 strains at 14 dpi were tiny of 10.0%, 6.67%, and 10.0% respectively, while those of fish challenged with the attenuated strains were sizable ranging from 56.67% to 86.67% in the first group; and 63.33% and 81.11% in the third group. Nevertheless, the survival rates of fish challenged with the attenuated strains in the second group were lower from 33.33% to 46.67%. These results showed that the impact of Rif to produce attenuated Rif-resistant strains was different. Our results may suggest that L4650, A650, L4200, and A400 strains are potential attenuated strains to be vaccine candidates.

Both *tdh* and *trh* genes were not found in genomes of the wild-type strains (LBT6, B3.13, A3.3) leading to the absence of these genes in eight attenuated strains. Not all strains of *V. parahaemolyticus* carried *tdh* and *trh* genes. In fact, these genes were only observed in 1 - 5% of *V. parahaemolyticus* strains. This result was concordant with the reports of Ahn S. *et al.* (2016), Kim S. *et al.* (2016), and Nguyen *et al.* (2012) (Ahn *et al.*, 2016; Duy and Ly, 2012; Kim *et al.*, 2016). The *tlh* and *toxR* genes were virulence genes that were detected in the genome of virulent wild-type strains. The *tlh* gene having a typical amino acid sequence in the Vibrionaceae was considered as a diagnostic marker for *Vibrio* disease, and the potential factor for fish disease vaccines (Klein *et al.*, 2014; Yáñez *et al.*, 2015).

Rif is a broad-spectrum antibiotic that can hinder DNA-directed RNA polymerase, and thereby, blocking the initiation of transcription and killing bacterium (Campbell *et al.*, 2001). The Rif-resistant bacterial strains were recorded as attenuated strains that might become vaccine

candidates (Bhatnagar *et al.*, 1994). The resistance to Rif was caused by mutations in the *rpoB* gene encoding the β -subunit of the RNA polymerase (Jin and Gross, 1988; Marianelli *et al.*, 2004; Taniguchi *et al.*, 1996). The full-length of the *rpoB* gene was 4029 bp encoding a polypeptide with 1342 amino acids (Campbell *et al.*, 2001). Mutations in the *rpoB* gene were found to be associated with Rif resistance of some bacterial strains such as *Mycobacterium tuberculosis*, *Escherichia coli*, *Acinetobacter baumannii*, *Bacillus subtilis*, *V. parahaemolyticus* (Hazen *et al.*, 2009; Jin and Gross, 1988; Maughan *et al.*, 2004; Perez-Varela *et al.*, 2017; Zaw *et al.*, 2018), and the mutation position would affect the level of the resistance to Rif (Taniguchi *et al.*, 1996). Hazen *et al.* (2009) reported that *V. parahaemolyticus* Rif-resistant strains with concentration at 100 $\mu\text{g/ml}$ had the mutations in the *rpoB* gene (Hazen *et al.*, 2009). The region which contains 81 bp from codon 507 to codon 533 (27 amino acid) is called rifampicin-resistance determining region (RRDR) (Zaw *et al.*, 2018). In RRDR, the most commonly mutated codons are 512, 513, 516, 526, 531. In addition, mutations outside RRDR were also detected in Rif-resistant strains (Zaw *et al.*, 2018). In this study, we found that A650 and L4650 strains had changes in the codon in RRDR (Gln513 $\rightarrow$ Pro, Val524 $\rightarrow$ Ile). There were 27 codons changed in the *rpoB* gene of the L4650 strain. The number of mutations in the *rpoB* gene was higher than that in some previous reports (Hazen *et al.*, 2009; Perez-Varela *et al.*, 2017; Qazi *et al.*, 2014). However, the bacterial strains in those reports were cultured in medium containing Rif with concentrations 40, 50 and 100 $\mu\text{g/ml}$, while L4650 strains were grown over many generations in medium containing Rif with gradually increasing concentrations (from 10 to 250 $\mu\text{g/ml}$). Because of amino acid changes, the structure of the β -subunit of the RNA polymerase could be modified resulting in the affection on the activity of RNA polymerase. Especially, these mutations of different bacterial species can cause global effects on the growth and developmental events (Maughan *et al.*, 2004), reduced virulence (Mariam *et al.*, 2004), and other altered phenotypes (Jin and Gross, 1989). The assessment of characteristics of several Rif-resistant attenuated strains shows that the mutant *Edwardsiella ictaluri*, *Flavobacterium columnare* strains are different from their wild-type strains in terms

of the structure of lipopolysaccharide (LPS) and compositions of outer membrane (Arias *et al.*, 2003; Shoemaker *et al.*, 2007; Zhang *et al.*, 2006). On the other hand, the mutant strains of *Flavobacterium psychrophilum* and *Vibrio harveyi* have the LPS profile similar to their wild-type strains and differ from the wild-type strain in terms of growth profile and whole-cell lysate (Hu *et al.*, 2012; LaFrentz *et al.*, 2008). The spontaneous resistance to Rif is often accompanied by the variations that change the fundamental structures of some proteins, however, the exact mechanism of attenuation of virulence in bacteria has not been clarified (Hu *et al.*, 2012). Perez-Varela M. *et al.* (2017) suggested that mutations in the β -subunit of RNA polymerase impaired the surface-associated motility and virulence of *Acinetobacter baumannii* (Perez-Varela *et al.*, 2017).

In the present study, we studied the difference in the virulence gene sequences (*toxR* and *tllb*) of attenuated Rif-resistant *V. parahaemolyticus* strains and wild-type strains. The results of this study showed that the *toxR* sequences of attenuated strains were not different from the sequences of the wild-type strains, whereas the *tllb* gene saw some nucleotide changes in attenuated strains compared to their wild-type strains. Those results suggested that the *toxR* gene is more conserved than the *tllb* gene. The *tllb* gene sequence was typical in the Vibrionaceae, therefore, it was considered as a diagnostic marker for *Vibrio* disease and the potential factor for fish disease vaccines (Klein *et al.*, 2014; Yáñez *et al.*, 2015). There are similarities in the result of the assessment of virulence and the result of the comparison of the nucleotide sequences of *tllb* genes in three wild-type strains and the attenuated strains. The attenuated strains (L4650, A650, and L4200) which showed higher survival rates of challenged fish (86.67%; 81.11% and 63.33%, respectively) had nucleotide changes leading to the changes in the size of DaaSeq. The attenuated B5700 strain which had no change in nucleotides of *tllb* gene also had lower survival rates of challenged fish. The attenuated L4650 strain with mutations in *tllb* and *rpoB* genes was a potential. However, the mechanism causing mutation in the *tllb* gene has not been clear. This result may suggest that virulence attenuation of the Rif-resistant strains may be caused by the inhibition of RNA

polymerase and the mutations in *tllb*, *rpoB* gene sequences of those strains.

The Rif-resistant strains which had attenuated virulence were used successfully as potential live attenuated vaccines. (Hu *et al.*, 2012; Klesius and Shoemaker, 1999; LaFrentz *et al.*, 2008; Shoemaker *et al.*, 2007). The injection is an effective method that could be used in the delivery of vaccines for fish because the intraperitoneal injection approach usually induces systematized immune responses that strongly protect the body (Plant and Lapatra, 2011). Similarly, groupers intraperitoneally injected with the Rif-resistant attenuated L4650 strain were conferred a protective immune response against the wild-type strains. Virulence assessment indicated that the LD₅₀ value of L4650 was 100-fold smaller than that of the LBT6 strain. The cumulative mortality rates of control fish injected with PBS at three different doses (5x LD₅₀, 10x LD₅₀ and 100x LD₅₀) were high (from 66.67% to 97.33%) and shared the similar results at 15 dpv and 60 dpv. In contrast, L4650-vaccinated fish had an immune response against wild-type *V. parahaemolyticus* LBT6. In the high challenge dose, the RPS values of vaccinated-fish were 96.91% (at 15 dpv) and 96.27% (at 60 dpv), while those values of fish challenged with low dose were 100% (at 15 dpv and 60 dpv). However, the results showed there was no significant difference ($p>0.05$) between the RPS values at different doses and different challenge time. Importantly, at 15 dpv, fish vaccinated with L4650 strain exhibited the high RPS values (from 96.91% to 100%), indicating that the antibody was produced quite early. A similar study that has been carried on *E. coioides* in Thailand showed that intraperitoneal injection of vaccine using formalin-inactivated *V. parahaemolyticus* provided the highest protection against bacterial infection with RPS value up to 77.6% (Clark *et al.*, 2010). In other study, vaccination using formalin-killed bacteria *V. parahaemolyticus* mixed with adjuvant FIA provided good protection in grouper *E. coioides*. At 30 dpv, the vaccinated groups showed clear protection against *V. parahaemolyticus* with a cumulative mortality of 10%, whereas high mortality was observed in control groups (70-90%) which corresponded to RPS value of 87.5%. However, the RPS value was reduced to 41.1% after 60 dpv (Thuy *et al.*, 2013). Although

inactivated bacterial strains were often used to produce commercial fish vaccines because of their safety, the immune-mediated protective responses resulted from inactivated strains were not optimal, especially for the protection in long terms (Sommerset *et al.*, 2005). In contrast, the live attenuated vaccines appeared to be more effective in creating protective immune responses than killed bacterial strains, especially the vaccines for the intracellular pathogens (Titball, 2008). Furthermore, the *V. parahaemolyticus* L4650 strain was evaluated for the stability of virulence attenuation in groupers (*E. coioides*). The L4650 strain was cultured in TCBS in long terms and injected into grouper at five different times. Injection times spaced 2 months apart in which the survival rates of injected fish were not significantly different among each time. Thus, the virulence attenuation of the *V. parahaemolyticus* L4650 strain might be stable (Huyen *et al.*, 2018). Those results suggest that attenuated Rif-resistant strains of *V. parahaemolyticus* are effective approaches for development for fish vaccines.

In summary, the results of the present study show that some attenuated strains of *V. parahaemolyticus* were created by Rif treatment for wild-type strains in media containing increasing Rif concentration. There were some nucleotide changes in the sequences of *tlh*, *rpoB* genes of attenuated strains compared to those of their wild-type strains. *E. coioides* vaccinated with the attenuated L4650 strain conferred a highly protective immune response against *V. parahaemolyticus*, which suggested that the L4650 strain was potential strain which could be used as a live attenuated vaccine candidate in aquaculture.

ACKNOWLEDGEMENTS

This study was funded from Hanoi Open University (No. V2018.01-01) and Hanoi National University of Education (No. SPHN-18-03), Hanoi, Vietnam.

REFERENCES

Ahn, S., Chung, H. Y., Lim, S., Kim, K., Kim, S., Na, E. J., Caetano-Anolles, K., Lee, J. H., Ryu, S., Choi, S. H., & Kim,

- H. 2016. Complete genome of *Vibrio parahaemolyticus* FORC014 isolated from the toothfish. *Gut Pathogenesis* 8: 59-64.
- Alaboudi, A. R., Ababneh, M., Osaili, T. M., & Al Shoul, K. 2016. Detection, identification, and prevalence of pathogenic *Vibrio parahaemolyticus* in fish and coastal environment in Jordan. *Journal of Food Science* 81: 130-134.
- Alcaide, E., Amaro, C., Todoli, R., & Oltra, R. 1999. Isolation and characterization of *Vibrio parahaemolyticus* causing infection in Iberian toothcarp *Aphaniusiberus*. *Diseases of Aquatic Organisms* 35: 77-80.
- Amend, D. F. 1981. Potency testing of fish vaccines. International symposium on fish biologics: serodiagnostics and vaccines. *Developments in Biological Standardization* 49: 447-454.
- Arias, C. R., Shoemaker, C. A., Evans, J. J., & Klesius, P. H. 2003. A comparative study of *Edwardsiella ictaluri* parent (EILO) and *E. ictaluri* rifampicin-mutant (RE-33) isolates using lipopolysaccharides, outer membrane proteins, fatty acids, Biology, API 20E and genomic analyses. *Journal of Fish Diseases* 26: 415-421.
- Austin, B. & Austin, D. A. 2007. Bacterial fish pathogens. Disease of farmed and wild fish. Chichester, UK: Springer/Praxis Publishing.
- Bej, A. K., Patterson, D. P., Brasher, C. W., Vickery, M. C., Jones, D. D., & Kaysner, C. A. 1999. Detection of total and hemolysin-producing *Vibrio parahaemolyticus* in shellfish using multiplex PCR amplification of *tlh*, *tdh* and *trh*. *Journal of Microbiological Methods* 36(3): 215-225.
- Bhatnagar, N., Getachew, E., Straley, S., Williams, J., Meltzer, M. & Fortier, A. 1994. Reduced virulence of rifampicin-resistant mutants of *Francisella tularensis*. *The Journal of Infectious Diseases* 170: 841-847.
- Campbell, E. A., Korzhcheva, N., Mustaev, A., Murakami, K., Nair, S., & Goldfarb, A. 2001. Structural mechanism for rifampicin inhibition of bacterial RNA polymerase. *Cell* 104: 901-912.
- Clark, J. S., Tangtrongpiros, J., & Chansue, N. 2010. Establishing safety and efficacy of an injectable form of a *Vibrio* vaccine for the orange-spotted grouper. *Aquaculture Asia Pacific* 6: 30-33.
- Duy, N. V. & Ly, N. T. C. 2012. Isolation and detection of toxin genes of *Vibrio parahaemolyticus* in fresh seafood in Nha Trang. *Journal of Seafood Science and Technology (in Vietnamese)* 2/2012: 42-47.
- Gao, Y., Wu, H., Wang, Q., Qu, J., Liu, Q., Xiao, J., & Zhang, Y. 2014. A live attenuated combination vaccine evokes effective immune-mediated protection against *Edwardsiella tarda* and *Vibrio anguillarum*. *Vaccine* 32: 5937-5944.
- Gutierrez W. C. K., Klein S. L., & Lovell, C. R. 2013. High frequency of virulence factor genes *tdh*, *trh*, and *tlh* in *Vibrio parahaemolyticus* strains isolated from a pristine estuary. *Applied and Environmental Microbiology* 79: 2247-2252.
- Hazen, T. H., Kennedy, K. D., Chen, S., Yi, S. V., & Sobecky, P. A. 2009. Inactivation of mismatch repair increases the diversity of *Vibrio parahaemolyticus*. *Environmental Microbiology* 11: 1254-1266.
- Hu, Y. H., Deng, T., Sun, B. G., & Sun, L. 2012. Development and efficacy of an attenuated *Vibrio harveyi* vaccine candidate with cross protectivity against *Vibrio alginolyticus*. *Fish and Shellfish Immunology* 32: 1155-1161.
- Huyen, V. T. B., Viet, N. X., Tham, P. T., Tung, H. V., & Phuoc, M. H. 2019. Biochemical characteristics and genetics of *Vibrio parahaemolyticus* strains caused hepatic necrosis disease for groupers in Cat Ba, Hai Phong. *Veterinary Science and Techniques Journal (in Vietnamese)* 26 (7/2019): 62-73.
- Huyen, V. T. B., Viet, N. X., Tham, D. T. H., Phuoc, M. H., & Tam, P. T. 2018. Assessment of stability and immunization response of *Vibrio parahaemolyticus* L4650 attenuated strain

- to support vaccine production against hepatic and kidney necrosis disease for marine fish. *Science and Technology Journal of Agriculture and Rural Development (in Vietnamese)* 21/2018: 79-85.
- Jin, D. J. & Gross, C. A. 1989. Characterization of the pleiotropic phenotypes of rifampin-resistant *rpoB* mutants of *Escherichia coli*. *Journal of Bacteriology* 171: 5229-5231.
- Jin, D. J. & Gross, C. A. 1988. Mapping and sequencing of mutations in the *Escherichia coli rpoB* gene that lead to rifampicin resistance. *Journal of Molecular Biology* 202: 45-58.
- Kalburge, S. S., Polson, S. W., Boyd Crotty, K., Katz, L., Turnsek, M., Tarr, C. L., Martinez-Urtaza, J., & Boyd, E. F. 2014. Complete genome sequence of *Vibrio parahaemolyticus* environmental strain UCM-V493. *Genome Announcements* 2: 1-2.
- Khouadja, S., Lamari, F., & Bakhrouf, A. 2013. Characterization of *Vibrio parahaemolyticus* isolated from farmed sea bass (*Dicentrarchus labrax*) during disease outbreaks. *International Aquatic Research* 5: 1-11.
- Kim, S., Chung, H. Y., Lee, D. H., Lim, J. G., Kim, S. K., Ku, H. J., Kim, Y. T., Kim, H., Ryu, S., Lee, J. H., & Choi, S. H. 2016. Complete genome sequence of *Vibrio parahaemolyticus* strain FORC_008, a foodborne pathogen from a flounder fish in South Korea. *Pathogen and Disease* 74(5): ftw044.
- Klein, S. L., Gutierrez West, C. K., Mejia, D. M., & Lovell, C. R. 2014. Genes similar to the *Vibrio parahaemolyticus* virulence-related genes *tdh*, *tlh*, and *vsc2* occur in other *Vibrionaceae* species isolated from a pristine estuary. *Applied and Environmental Microbiology* 80: 595-602.
- Klesius, P. H. & Shoemaker, C. A. 1999. Development and use of modified live *Edwardsiella ictaluri* vaccine against enteric septicemia of catfish. *Advances in Veterinary Medicine* 41: 523-537.
- LaFrentz, B. R., LaPatra, S. E., Call, D. R., & Cain, K. D. 2008. Isolation of rifampicin resistant *Flavobacterium psychrophilum* strains and their potential as live attenuated vaccine candidates. *Vaccine* 26: 5582-5589.
- Lee, A. S., Lim, I. H., Tang, L. L., & Wong, S. Y. 2005. High frequency of mutations in the *rpoB* gene in rifampin-resistant clinical isolates of *Mycobacterium tuberculosis* from Singapore. *Journal of Clinical Microbiology* 43: 2026-2027.
- Letchumanan, V., Ser, H. L., Chan, K. G., Goh, B. H., & Lee, L. H. 2016. Genome sequence of *Vibrio parahaemolyticus* VP103 strain isolated from shrimp in Malaysia. *Frontiers in Microbiology* 7: 1496.
- Li, C., Ye, Z., Wen, L., Chen, R., Tian, L., Zhao, F., & Jianyi, P. 2014. Identification of a novel vaccine candidate by immunogenic screening of *Vibrio parahaemolyticus* outer membrane proteins. *Vaccine* 32: 6115-6121.
- Li, L., Lin, S. L., Deng, L., & Liu, Z. G. 2013. Potential use of chitosan nanoparticles for oral delivery of DNA vaccine in black seabream *Acanthopagrus schlegelii* Bleeker to protect from *Vibrio parahaemolyticus*. *Journal of Fish Diseases* 36: 987-995.
- Liu, R., Chen, J., Li, K., & Zhang, X. 2011. Identification and evaluation as a DNA vaccine candidate of a virulence-associated serine protease from a pathogenic *Vibrio parahaemolyticus* isolate. *Fish and Shellfish Immunology* 30: 1241-1248.
- Liu, Y. & Bi, Z. 2007. Potential use of a transposon Tn916-generated mutant of *Aeromonas hydrophila* J-1 defective in some exoproducts as a live attenuated vaccine. *Preventive Veterinary Medicine* 78: 79-84.
- Lun, J., Xia, C., Yuan, C., Zhang, Y., Zhong, M., Huang, T., & Hu, Z. 2014. The outer membrane protein, LamB (maltoporin), is a versatile vaccine candidate among the *Vibrio* species. *Vaccine* 32: 809-815.
- Mao, Z., Yu, L., You, Z., Wei, Y., & Liu, Y. 2007. Cloning, expression and immunogenicity analysis of five outer membrane proteins of *Vibrio parahaemolyticus* zj2003. *Fish and Shellfish Immunology* 23: 567-575.
- Mariam, D. H., Mengistu, Y., Hoffer, S. E., & Andersson, D. I. 2004. Effect of *rpoB* mutations conferring rifampin resistance on fitness of *Mycobacterium tuberculosis*. *Antimicrobial Agents and Chemotherapy* 48: 1289-1294.
- Marianelli, C., Ciuchini, F., Tarantino, M., Pasquali, P., & Adone, R. 2004. Genetic bases of the rifampin resistance phenotype in *Brucella* spp. *Journal of Clinical Microbiology* 42: 5439-5443.
- Marudhupandi, T., Ajith Kumar, T. T., Prakash, S., Balamurugan, J., & Dhayanithi, N. B. 2017. *Vibrio parahaemolyticus* a causative bacterium for tail rot disease in ornamental fish, *Amphiprion sebae*. *Aquaculture Reports* 8: 39-44.
- Maughan, H., Galeano, B., & Nicholson, W. L. 2004. Novel *rpoB* mutations conferring rifampin resistance on *Bacillus subtilis*: Global effects on growth, competence, sporulation, and germination. *Journal of Bacteriology* 186: 2481-2486.
- Mollet, C., Drancourt, M., & Raoult, D. 1997. *rpoB* sequence analysis as a novel basis for bacterial identification. *Molecular Microbiology* 26: 1005-1011.
- Murakami, K. S. 2015. Structural biology of bacterial RNA polymerase. *Biomolecules* 5: 848-864.
- Nelapati, S. & Krishnaiah, N. 2010. Detection of total and pathogenic *Vibrio parahaemolyticus* by polymerase chain reaction using *toxR*, *tdh* and *trh* genes. *Veterinary World* 3(6): 268-271.
- Noorlis, A. T. C., Ghazali, F. M., Wong, W. C., Tunung, R., Nakaguchi, Y., Cheah, Y. K., Pui, C. F., Son, R., Tuan Zainazor, T. C., & Nishibuchi, M. 2011. Antibiotic resistance and biosafety of *Vibrio cholerae* and *Vibrio parahaemolyticus* from freshwater fish at retail level. *International Food Research Journal* 18: 1523-1530.
- Paranjpye, R. N., Myers, M. S., Yount, E. C., & Thompson, J. L. 2013. Zebrafish as a model for *Vibrio parahaemolyticus* virulence. *Microbiology* 159: 2605-2615.
- Peng, B., Lin, X. P., Wang, S. N., Yang, M. J., Peng, X. X., & Li, H. 2018. Polyvalent protective immunogens identified from outer membrane proteins of *Vibrio parahaemolyticus* and their induced innate immune response. *Fish and Shellfish Immunology* 72: 104-110.
- Peng, B., Ye, J. Z., Han, Y., Zeng, L., Zhang, J. Y., & Li, H. 2016. Identification of polyvalent protective immunogens from outer membrane proteins in *Vibrio parahaemolyticus* to protect fish against bacterial infection. *Fish and Shellfish Immunology* 54: 204-210.
- Perez-Varela, M., Corral, J., Vallejo, J. A., Rumbo-Feal, S., Bou, G., Aranda, J., & Barbe, J. 2017. Mutations in the beta-subunit of the RNA polymerase impair the surface-associated motility and virulence of *Acinetobacter baumannii*. *Infection and Immunity* 85: e00327-00317.
- Plant, K. P. & LaPatra, S. E. 2011. Advances in fish vaccine delivery. *Developmental and Comparative Immunology* 35: 1256-1262.
- Pridgeon, J. W. & Klesius, P. H. 2011a. Development and efficacy of novobiocin and rifampicin-resistant *Aeromonas hydrophila* as novel vaccines in channel catfish and Nile tilapia. *Vaccine* 29: 7896-7904.
- Pridgeon, J. W. & Klesius, P. H. 2011b. Development of a novobiocin-resistant *Edwardsiella ictaluri* as a novel vaccine in channel catfish (*Ictalurus punctatus*). *Vaccine* 29: 5631-5637.
- Qazi, O., Rahman, H., Tahir, Z., Qasim, M., Khan, S., Ahmad Anjum, A., Yaqub, T., Tayyab, M., Ali, N., & Firyal, S. 2014. Mutation pattern in rifampicin resistance determining region of *rpoB* gene in multidrug-resistant *Mycobacterium*

- tuberculosis* isolates from Pakistan. *International Journal of Mycobacteriology* 3: 173-177.
- Raghunath, P. 2014. Roles of thermostable direct hemolysin (TDH) and TDH-related hemolysin (TRH) in *Vibrio parahaemolyticus*. *Frontiers in Microbiology* 5: 805-809.
- Reed, L. J. & Muench, H. 1938. A simple method for estimating fifty percent endpoints. *American Journal of Hygiene* 27: 493-497.
- Shoemaker, C. A., Klesius, P. H., & Evans, J. J. 2007. Immunization of eyed channel catfish, *Ictalurus punctatus*, eggs with monovalent *Flavobacterium columnare* vaccine and bivalent *F. columnare* and *Edwardsiella ictaluri* vaccine. *Vaccine* 25: 1126-1131.
- Sommerset, I., Krossoy, B., Biering, E., & Frost, P. 2005. Vaccines for fish in aquaculture. *Expert Review of Vaccines* 4: 89-101.
- Taniguchi, H., Aramaki, H., Nikaido, Y., Mizuguchi, Y., Nakamura, M., Koga, T., & Yoshida, S. 1996. Rifampicin resistance and mutation of the *rpoB* gene in *Mycobacterium tuberculosis*. *FEMS Microbiology Letters* 144: 103-108.
- Tarr, C. L., Patel, J. S., Puhr, N. D., Sowers, E. G., Bopp, C. A., & Strockbine, N. A. 2007. Identification of *Vibrio* isolates by a multiplex PCR assay and *rpoB* sequence determination. *Journal of Clinical Microbiology* 45: 134-140.
- Thuy, N. T. T., Dung, N. H., & Wergeland, H. I. 2013. Specific humoral immune response and protection against *Vibrio parahaemolyticus* in orange-spotted grouper *Epinephelus coioides*. *International Journal of Marine Science* 4(1): 24-35.
- Titball, R. W. 2008. Vaccines against intracellular bacterial pathogens. *Drug Discovery Today* 13: 596-600.
- Yáñez, R., Bastías, R., Higuera, G., Salgado, O., Katharios, P., Romero, J., Espejo, R., & García, K. 2015. Amplification of *tlh* gene in other *Vibrionaceae* specie by specie-specific multiplex PCR of *Vibrio parahaemolyticus*. *Electronic Journal of Biotechnology* 18: 459-463.
- Zamora-Pantoja, D. R., Quñones-Ramírez, E. I., Fernández, F. J., & Vázquez-Salinas, C. 2013. Virulence factors involved in the pathogenesis of *Vibrio parahaemolyticus*. *Reviews in Medical Microbiology* 24: 41-47.
- Zaw, M. T., Emran, N. A., & Lin, Z. 2018. Mutations inside rifampicin-resistance determining region of *rpoB* gene associated with rifampicin-resistance in *Mycobacterium tuberculosis*. *Journal of Infection and Public Health* 11: 605-610.
- Zhang, X., Mu, Y., Mu, P., Ao, J., & Chen, X. 2017. Transcriptome analysis reveals comprehensive insights into the early immune response of large yellow croaker (*Larimichthys crocea*) induced by trivalent bacterial vaccine. *PLoS One* 12: e0170958.
- Zhang, Y., Arias, C., Shoemaker, C., & Klesius, P. 2006. Comparison of lipopolysaccharide and protein profiles between *Flavobacterium columnare* strains from different genomovars. *Journal of Fish Diseases* 29: 657-663.
- Zhang, Y., Gao, H., Osei-Adjei, G., Zhang, Y., Yang, W., Yang, H., Yin, Z., Huang, X., & Zhou, D. 2017. Transcriptional regulation of the type VI secretion system 1 genes by quorum sensing and *toxR* in *Vibrio parahaemolyticus*. *Frontiers in Microbiology* 8: 2005-2017.