Comparative Genomics of Different Isolates of Acute Hepatopancreatic Necrosis Disease-causing *Vibrio parahaemolyticus*

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Abstract

Pathogenic *Vibrio parahaemolyticus* is a marine bacterium which is characterized as the main cause of acute hepatopancreatic necrosis disease (AHPND) in aquatic animals including white shrimp. Since it was first reported, this disease has led to a huge reduction of shrimp production worldwide. The primary virulent factor of AHPND-causing *V. parahaemolyticus* (VP_{AHPND}) is identified as binary pirAB toxin genes encoded by a deadly plasmid (pVA1). However, apart from pirAB genes, little is known about genomic factors that contribute to pathogenicity of VP_{AHPND}. In this study, comparative genomic analysis was performed to unravel genomic differences of VP_{AHPND} strains. Six *V. parahaemolyticus* strains were analyzed by whole genome sequencing, and their genome features were compared using several bioinformatics tools. The result showed that all 6 draft genomes exhibited a similar genome size of approximately 5 Mb. Phylogenetic tree based on 16S ribosomal RNA sequence indicated that our studied strains were genetically diverse, but strains in the same group shared highly similar genome patterns. MLST analysis identified 10 sequence types (STs) among VP_{AHPND} strains. All strains contained at least 2 CRISPR elements and phage sequences, meanwhile, 3 out of 6 strains harbored a bacteriocin gene in their genomes. Moreover, *in silico* analysis revealed that all six strains possessed several antimicrobial resistance (AMR) genes, which suggested their antimicrobial resistance characteristics to multiple antibiotics agents. Genomic variation profile of *V. parahaemolyticus* strains was also reported in this study. Functional genes were categorized into bacterial motility and transporter proteins. This finding provided an extended genomic information of AHPND-causing *V. parahaemolyticus*.

Keywords**:** *V. Parahaemolyticus Strains, VP_{AHPND}, Comparative Genomic Analysis, White Shrimp*

1. Introduction

Vibrio parahaemolyticus is a Gram-negative and halophilic bacterium which is commonly found in seawater or estuarine regions. It is known that acute hepatopancreatic necrosis disease (AHPND), a globally infectious disease of marine animals including white shrimp, is mainly caused by some typical strains of *V. parahaemolyticus* (VP_{AHPND}) (Lee et al., 2015). AHPND was first reported in China in 2009 and rapidly spread to other countries such as Mexico, Thailand, Philippines and Vietnam (Kumar et al., 2021; Tran et al., 2013). During 2010 to 2016, AHPND reduced total revenue of shrimp production from China, Southeast Asian countries and Mexico up to US$ 44 billion (Tang & Bondad-Reantaso, 2019). Shrimp infected with AHPND show slough, pale and atrophied hepatopancreas, as well as empty stomach and midgut (Tran et al., 2013).

The primary factor causing AHPND in *V. parahaemolyticus* is identified as pirAB-like toxin genes ($pirAB^{vp}$), encoded by 70 kb-pVA1 plasmid (Dong et al., 2017; Santos et al., 2020; Yu et al., 2020). Praja (2018) stated that $pirAB^{vp}$ genes are only found in AHPND-causing *V. parahaemolyticus* (VP_{AHPND}) and distinguishable from clinical and human-foodborne strains, thus, they have been used to detect AHPND infection using specific primers targeting $pirAB^{vp}$ sequence such as AP4 (Dangtip et al., (2015); Sirikharin et al., 2014;, Sirikharin et al, 2015). However, several studies found that *V. parahaemolyticus* strains containing either full or partial sequence of $pirAB^{vp}$ did not cause AHPND in shrimp (Caro et al., 2020; Phiwsaiya et al., 2017). Hence, it was inferred that other virulence factors, beside $pirAB^{vp}$ toxin, also play roles in virulence of VP_{AHPND}. Secretory systems, biofilm formation, and motility proteins have also been involved in pathogenic processes of *V. parahaemolyticus* (Wang et al., 2022; Wang et al., 2015). Insights into pathogenicity and diversity of microorganisms have been extended thank to whole genome sequencing. Up

to date, only few research has studied comparative genomics of VP_{AHPND} and several genomic variations were reported. Fu et al. (2017) found that VP_{AHPND} had genome sizes of 5.4-5.9 Mb, which was similar to clinical strain RIMD2210633. Besides, Prithvisagar et al. (2021) discovered that their genomes were genetically diverse due to mutation and recombination events, which provided bacteria adaptation fitness to environmental changes. Moreover, Yu et al. (2020) identified several functional proteins, CRISPR sequences and phages among different *V. parahaemolyticus* strains. We hypothesized that all VP_{AHPND} strains contain variants in their genomes, and these variants could lead to different virulence levels, causing different mortality rates toward shrimp.

In this study, six AHPND-causing *V. parahaemolyticus* strains were sequenced and compared using bioinformatics approaches. Comparative genomic analysis was performed to study genomic differences and phylogenetic relationship of these strains.

2. Objectives

To compare genomes of different AHPND-causing *V. parahaemolyticus* strains.

3. Materials and Methods

3.1 Bacterial strains and culture

Six *V. parahaemolyticus* strains including J36, J39, J41, 7B, SG091, and VPS2 provided by Songkhla Aquatic Animal Health Research and Development Center were streaked on selective Thiosulfate Citrate Bile Salts Sucrose (TCBS) agars and incubated at 37ºC overnight. Single colony of each strain was cultured in Tryptic Soy Broth (TSB) containing 1.5% NaCl at 25ºC, 250 rpm for 16 hrs. Bacterial concentration was determined by bacterial plate count as cfu/ml unit by spreading serially diluted bacterial culture on TSA supplemented with 1.5% NaCl.

3.2 DNA extraction and biomarker validation

Genomic DNA of six bacterial strains was extracted from overnight culture using Vivantis GF-1 Bacterial DNA extraction kit following manufacturer's instruction. The quality of extracted DNA samples was measured with Nanodrop 2000 UV-Vis Spectrophotometer (Thermo Scientific) and confirmed DNA pattern by agarose gel electrophoresis. The DNA samples from those bacterial strains were used to amplify with specific ToxR (Kim et al., 1999) and AP4 primers (Dangtip et al., 2015).

3.3 Whole genome sequencing and genome assembly

The extracted DNA samples of *V. parahaemolyticus* strains were analyzed by Illumina sequencing platform (BGI, China) with paired-end reads of 150 bp. The raw reads were qualified by FastQC and then clean reads were used to generate *de novo* assembled contigs using Spades program. The draft genomes were evaluated by QUAST and BUSCO. Number of CRISPR elements, phages and bacteriocin genes were predicted using CRISPRFinder, PHASTER and BAGEL4, respectively. Besides, sequence type was identified by Multi-Locus Sequence Typing (MLST) website. Phylogenetic tree based on pan-genome analysis was generated using iTOL (https://itol.embl.de/upload.cgi).

3.4 Functional annotation and comparative genomic analysis

All draft genomes were annotated for their functional genes by Rapid Annotations using the Subsystems Technology (RAST) and Prokka in our server. Also, they were predicted against eggnog and KEGG databases. Subsequently, genomic variation of all six genomes were analyzed using Roary scripts.

4. Results and Discussion

4.1 Identification of *V. parahaemolyticus* strains

The PCR amplification with ToxR and pirAB genes using toxR and AP4 primers, respectively confirmed that our strains are *V. parahaemolyticus* that cause AHPND in shrimp, which was shown in specific band of 369 bp for toxR and 230 bp for AP4 (Dangtip et al., 2015; Kim et al., 1999).

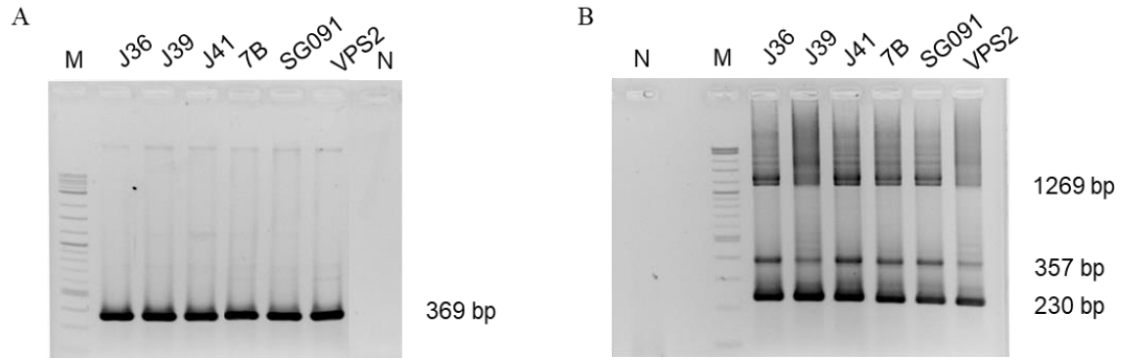

Figure 1 PCR detection of toxR and pirAB genes of *V. parahaemolyticus* strains. PCR amplicon(s) of toxR-369 bp (A), and pirAB-1269, 357, 230 bp (B) genes. M: 1 kb Plus DNA Ladder (NEB)

4.2 Comparative genomic analysis

As expected, our *V. parahaemolyticus* strains exhibited a genome size of 5.3-5.7 Mb, which was similar to clinical strain RIMD2210633 and other *V. parahaemolyticus* genomes in NCBI database. Number of rRNA, tRNA, CRISPR and phage sequences were not different between six genomes (Table 1). 7B, SG091, and VPS2 were found contain a bacteriocin gene in their genomes which belonged to sactipeptides class.

Table 1 General features of *V. parahaemolyticus* strains

Genomic feature	J36	J39	J41	7B	SG091	VPS2
Size	5404457	5386569	5538812	5530246	5518232	5737862
rRNA	6	7	6	5	5	5
tRNA	114	117	124	126	116	117
CRISPR	3	2	2	2	2	2
Phage	2	2	2	2	2	2
Bacteriocin	0	0	0	1	1	1

All 6 draft genomes, together with reference sequences from NCBI, were analyzed, then phylogenetic tree was constructed. Phylogenetic tree and multiple genome alignment showed that J36, J39, and J41, as well as 7B and SG091 were closely related, as they shared highly similar genomic components (Figure 2). MLST analysis identified 3 sequence types (STs) including ST 247 (found in J36, J39, and J41), ST 970 (in 7B and SG091), and ST 1166 (VPS2). ST 247 was found in a Thai VP_{APHND} strain PSU5579, whereas ST 1166 was identified in a Vietnam strain, and ST 970 was the most common ST across multiple sources of VP_{AHPND} (Yu et al., 2020). This broad sequence typing agrees with previous studies on high diversity of *V. parahaemolyticus* genomes.

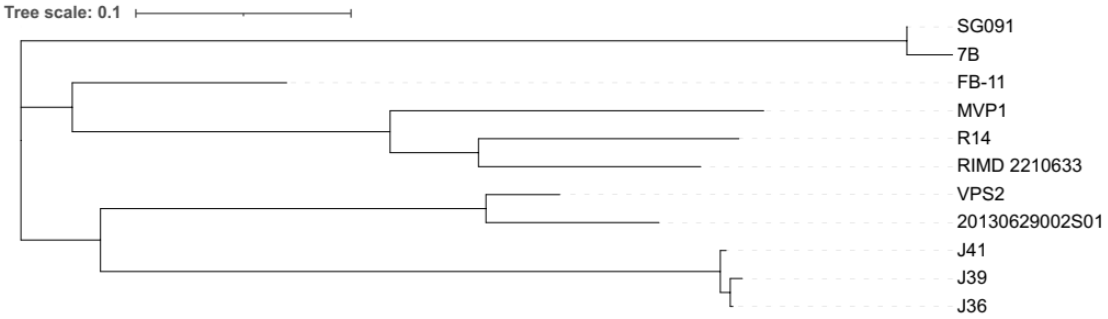

Figure 2 Phylogenetic relationship between *V. parahaemolyticus* strains and RIMD 2210633 reference.

Table 2 Genomic variation profile of *V. parahaemolyticus* genomes. Blue: present, yellow: absent

System	Gene	7B	J36	J39	J41	SG091	VPS2
Bacterial toxins	putative hemolysin						
	LDH						
	collagenase						
Antimicrobial resistance genes	beta-lactamase						
	chloramphenicol acetyltransferase						
	antibiotic acetyltransferase						
	putative multidrug efflux membrane fusion protein						
Motility	pilMNOP						
	flgABCDEFGHIJKLMN						
	motAB						
	cheARVWY						
	flaD						
Transporters	ompK precursor						
	ompA						
	ompU						
Enzymes	putative peptide methionine sulfoxide reductase						
Secretory systems	secA						
	secB						
	secD						
	secF						

Comparative genomic analysis revealed 4,119 absent/present genes in *V. parahaemolyticus* draft genomes. It was shown that all studied strains carried multiple antimicrobial resistance (AMR) genes (Table 2). This suggested multidrug-resistance characteristics of our *V. parahaemolyticus* strains. In consistence, it was well-documented that *V. parahaemolyticus* is multidrug-resistant bacterium, either pathogenic or non-pathogenic strains (Jeamsripong et al., 2020; Jingjit et al., 2021; Prithvisagar et al., 2021; Wang et al., 2022). Particularly, most strains from any sources were resistant to beta-lactam antibiotics (Ha et al., 2023; Li et al.,

2016; Silvester et al., 2015; Venggadasamy et al., 2021). Furthermore, we found a polar flagellin gene (flaD) was absent in the genomes of J36, J39 and J41. FlaD is a subunit protein involved in formation of polar flagella structure that is responsible for swimming motility of bacteria. Although there has been no study on role of flaD in *V. parahaemolyticus*, McGee et al. (1996) showed that flaD mutants (5'-end deletion or full-gene deletion of flaD) did not significantly affect virulence and motility of *V. anguillarum*, implying that it might not be a major factor that contribute to virulence.

The absence of outer membrane ompU was also found in J36, J39 and J41 genomes, meanwhile, the lack of ompK precursor was detected in SG091 and VPS2. Previous studies have demonstrated potential of ompK and ompU as effective vaccine candidates (Inoue et al., 1995; W. Wang et al., 2021) as ompK is receptor of broad-host-range vibriophage and ompU plays important role in host response and pathogenesis of *Vibrio* spp. In *V. parahaemolyticus*, Gulati et al. (2019) demonstrated that ompU modulated innate immunity via activation of macrophages and monocytes. Moreover, ompU was responsible for bacterial survival under stress conditions and important for *in vivo* colonization (Whitaker et al., 2012). Therefore, ompK and ompU might be crucial virulence-associated genes and lack of these genes may affect virulence of our *V. parahaemolyticus* strains.

5. Conclusion

In this study, six AHPND-causing *V. parahaemolyticus* strains were sequenced and analyzed. Pan-genomic analysis showed high diversity between our VP_{AHPND} strains, but strains in the same group exhibited great similarities in genome components. We found that all six strains carried several AMR genes, confirming broad resistance of this bacterium to multi-antimicrobial agents. *In silico* analysis identified virulence genes including outer membrane proteins ompK and ompU existing in our VP_{AHPND} genomes. This indicated roles of these genes in virulence of *V. parahaemolyticus* strains. In conclusion, our findings provided more genomic information about VP_{AHPND}, and it might be helpful for further research on pathogenicity of VP_{AHPND}, which could be applicable for diagnostic test kit.

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