SUNTEXT REVIEWS

SunText Review of Biotechnology
ISSN: 2766-5097

Open Access
Research Article
Volume 4:1

The *mdr, Toxin* and *Virulence* Genes in Bacteria from Shrimp Fish Aquaculture: New Diagnostic PCR for Vibrio *Parahaemolyticus* Using Chromosomal blaCARB-1, PBP1b and *CatC1 mdr* Genes

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Received date: 28 January 2023; **Accepted date:** 01 February 2023; **Published date:** 05 February 2023

Citation: Chakraborty AK, Halder C, Maity U (2023) The mdr, Toxin and Virulence Genes in Bacteria from Shrimp Fish Aquaculture: New Diagnostic PCR for Vibrio Parahaemolyticus Using Chromosomal blaCARB-1, PBP1b and CatC1 mdr Genes. SunText Rev Biotechnol 4(1): 146.

DOI: https://doi.org/10.51737/2766-5097.2023.046

Abstract

The overuse of antibiotics in aquaculture eventually leads to antimicrobial resistance (AMR) in bacterial strains found in shrimp. About million MDR plasmids were sequenced from diverse bacteria and classified into *bla, aph, aac, aad, tet* and *sul* genes classes. The *Vibrio parahaemolyticus* was the main culprits for scrimp fish contamination and mortality. *The blaPER-1, blaOXA-1, blaNDM-1, dhfr, aacA1, sul1, su12, arr3, aac3'-IId, aac6'-IIa, ANT3'', tetB, qnr1, mphA, catB3 mdr* genes were sequenced from shrimp-derived *V. parahaemolyticus (*pVPS43, pVPH2, pVPS129) *and* V. alginolyticus (pVAS19, pC1394) and surprisingly were 100% homology to *Escherichia, Klebsiella, Acinetobacter, Enterobacter, Shigella species* plasmids suggesting horizontal gene transfer. Plasmid-mediated PirA/B toxin genes used to detect *V. parahaemolyticus* in shrimp and was responsible for acute hepatopancreatic necrosis disease (AHPND). Recently, AHPND-plasmids (pVa, pVA1, pVHvo, pVPE619, pVPGD2014-2 and pVPGX2015-2) were sequenced from *V. parahaemolyticus* as well as *V. owensii* and *V. harveri* but no *mdr* genes was detected.

Similarly, *tdh, trh and tlh* virulence genes also used for diagnosis to cause membrane pore formation and located in both chromosomes and plasmids of Vibrio species. We searched NCBI database of *V. parahaemolyticus* genomic fragments and found very specific chromosomal *mdr* genes like *blaCARB* (carbanicillin specific beta-lactamase), *PBP1B* (Penicillin-binding protein) and *CatC1* (chloramphenicol acetyltransferase) to design PCR primers. Further, few MDR drug efflux genes (macB, MFS, RND, emrD) and rRNA methyl transferases (RlmE, RlmM, RlmN, RsmA) were also detected to cause multi-drug resistance. BLAST search indicated that primers were very specific for *V. parahaemolyticus Ch-1 or Ch-2* and had no similarities to any plasmids. It appeared toxin, virulence and *mdr* genes hardly located in the same *V. parahaemolyticus* plasmid.

Keywords: Vibrio parahaemolyticus; Shrimp aquaculture; mdr genes; blaCARB gene; Penicillin-binding protein; PirAB toxins

Introduction

We are 8000 million people in this Earth and to fed entire population is a hard task as still malnourishment prevails in West Asia, Africa and Latin America [1,2]. The fish food is rich in protein and well tolerated worldwide. Recently, mass aquaculture of Telapia, Carp, Catfish, Trout and Shrimp were taken worldwide [3]. The shrimp was elegantly called fish-chicken and now very much popular in East India due to high demand abroad with good prise [4]. The West Bengal low land area was being hostile for paddy cultivation due to heavy flood in rainy season and such lands were converted into shrimp ponds very quickly. The main problem of shrimp cultivation and export are: (i) healthy and nutritious fish (ii) antibiotic residue like nitrofurans in shrimp (iii) MDR bacterial contamination specifically *Vibrio* species and *Staphylococcus aureus* and (iv) presence of toxin and virulence genes in *Vibrio parahaemolyticus*. *In truth*, farming region, water source, dead fish

removal frequency, antibiotic treatment and virus or bacteria contamination were all found to be significantly associated with shrimp mortality [5,6].

Shrimp population and cultures

Commercially, now at least five different types of shrimp fishes were cultivated in Indian ponds. Among them, white leg shrimp (*Litopenaeus vannamei*) and black tiger shrimp (*Penaeus monodon*) mostly cultivated in Asia (Figure 1). The shrimp genomics also has started giving many important transcriptome data [7-9].

Diseases of shrimp aquaculture

Vibrio parahaemolyticus is a marine pathogen and greatly affect shrimp aquaculture. The acute hepatopancreatic necrosis disease (AHPND) is a devastating disease that significantly affects aquaculture production of shrimp fish [10] (Figure 2). Photorhabdus insect-related (Pir) toxin-like genes in plasmid of *V. parahaemolyticus* is the causative agent of AHPND of shrimp. Lee et al elegantly showed that an AHPND-causing strain of *V. parahaemolyticus* contained a 70-kbp plasmid (pVA1; 70kb) with a post-segregational killing system. PirAB toxin was found in plasmid-encoded homologs of the Photorhabdus insect-related (Pir) toxins, PirA and PirB. The toxin is related to Cry toxin and insecticidal Pir homologs were found in *Photorhabdus* and *Xenorhabdus* species chromosomes (FM162591.1, FN667742.1, and FO704550.1), whereas PirABvp is the only toxin to be encoded by a plasmid. Besides *V. owensii*, *V. harveyi* and *V. parahaemolyticus*, similar Tn903-like composite transposons were also detected in plasmid p67vangNB10 (accession no. LK021128) of *V. anguillarum* and also in the whole genomes of various strains of the Harveyi clade (CP006700, CP006701, CP006606 and CP000790) [11]. Recently, *V. owensii* AHPND-plasmid pVH_{vo} was sequenced (accession number: KX268305) and very identical to *V. parahaemolyticus* plasmids. Interestingly, both plasmids had no *mdr* gene. *However, V. harveyi, V. alginolyticus, V. anguillarum, V. splendidus, V. salmonicida, V. vulnificus* and non-AHPND causing *V. parahaemolyticus* that cause vibriosis.

The PirAvp corresponds to domain III of the *Bacillus thuringiensis Cry* toxin and PirBvp corresponds to domains I and II. The *Cry* toxin induces cell death by undergoing a series of processes that include receptor binding, oligomerization, and pore forming [12]. The *B. thuringiensis* Cry1A toxin domain III first interacted with the GalNAc sugar on the aminopeptidase N (APN) receptor facilitating further binding of domain II to another region of the same receptor. The APN-bound *Cry* toxin subsequently binds to another receptor, cadherin, which facilitates the proteolytic cleavage of its domain Iα1 helix. This cleavage induces the formation of Cry oligomer, which has pore-forming activity [13]. Interestingly, such interactions trigger an alternative signal transduction pathway activating protein kinase G and adenylyl cyclase to increase cellular cAMP concentration destabilizing the cytoskeleton and ion channels on the membrane to cause cell death [14]. It was postulated that PirAvp/PirBvp system used a similar strategy to kill host cells [15]. *V. cholerae* neuraminidase (EC 3.2.1.18) releases sialic acid from higher gangliosides present on eukaryotic cells surface, exposing ganglioside GM1, which is the cholera toxin receptor and thus activates cholera toxin function [16].

Contaminated sea food human diseases

Human seafood-associated bacterial gastroenteritis is caused by *Vibrio parahaemolyticus* in many countries including United States and India [17]. The diseases produced by *Vibrio* bacteria is known as vibriosis and the symptoms include watery diarrhea, vomiting, abdominal cramping, nausea, fever, and chills [18]. The *Vibrio* species are halophilic bacteria that are ubiquitous in sea, coastal areas and fish ponds. Many are pathogenic to human and marine animals, and three species, *Vibrio parahaemolyticus, Vibrio vulnificus and Vibrio cholerae* are responsible for seafood-related human illness [19]. The *V. vulnificus* and *V. parahaemolyticus* are naturally occurring estuarine bacteria, that causes seafood-borne mortality in USA. It was reported by the United States Centre for Disease Control and Prevention (CDC) that the incidence of *Vibrio* infections increased dramatically since 2001 [20]. In August 2012, a *V. parahaemolyticus* outbreak involving 6 persons occurred in Maryland and the outbreak isolates were linked to the O3:K6 pandemic clone of *V. parahaemolyticus* that had been observed throughout the world [21]. In July 30, 2014, ABC News reported several cases of *V. vulnificus* occurrence in Florida, where 32 people had contacted the bacteria and 10 had died according to the Florida Department of Health.

Drug resistance in Vibrios and MDR plasmids

In the past, most *Vibrio* species were susceptible to common antibiotics of veterinary and human significance [22,23]. Nevertheless, several investigations reported that both *V. parahaemolyticus* and *V. vulnificus* were resistant to ampicillin [24,25]. But excessive use of oral antibiotics in human as well as in agriculture, and aquaculture systems, antibiotic resistance was emerged permanent and evolved in many bacterial genera (*Klebsiella, Salmonella, Escherichia, Staphylococcus, Acinetobacter, Pseudomonas*) including *Vibrio* during the past few decades [26,27]. Bacterial resistance to common antibiotics has reached frightening levels in many countries which can lead to failure of the available treatment options for common infections [28]. Thus, the development of alternative biocontrol agents is urgently needed.

Large plasmids (>95kb) were also detected in many antibiotic-resistant *Salmonella* isolates and *E. coli* isolates derived from fishes. Conjugation experiments showed the successful transfer of all or part of the antibiotic resistance phenotypes among the *Salmonella* species, *Vibrio* species and *E. coli* food isolates [29,30]. Sequencing results from plasmids of *Vibrio* species isolated from shrimp revealed that the integrons harboured various gene cassettes, including aadA1, aadA2, and *ANT* (resistance to streptomycin), *aac*3"/6" (resistance to aminoglycosides), the dihydrofolate reductase gene cassette dhfrA17 (trimethoprim resistance), the beta-lactamase gene *bla*PER-1 (ampicillin resistance), and *cat*B3 (chloramphenicol resistance) [31-33]. The β-lactamases cleave penicillin whereas catB3 or aacA1 acetylate antibiotics like aminoglycosides and chloramphenicol. The *Vibrio parahaemolyticus* plasmid-bearing *blaPER-1* has no similarity to *blaCARB-1* gene that located chromosomally but has very similarity to *blaPSE* gene with extended 27 amino acids at the *N-terminus (see, plasmids pVPH1, pVAS19, pVPS43 and pVPS129)*. The *dhfr* gene was responsible for trimethoprim resistance and sul1/2/3 gave resistance to methotrexate. Thus, old antibiotics like ampicillin, oxacillin, streptomycin and tetracycline will not cure bacterial diseases in aquaculture.

In Thailand, oxytetracycline resistant *Aeromonas* species (4-128µg/ml) and *Lactococcus* species (~120µg/ml) were isolated from white leg shrimp (>25% samples) as well as black tiger shrimp (>10% samples). The TET resistance was found to be conferred by the genes tet(A), tet(C), tet(D), tet(E), tet(M) and tet(S) [34]. Shrimp aquaculture *V. parahaemolyticus* isolates from Southern province of India revealed seven plasmids of 0.75, 1.2, 6, and 8 kb sizes and 3 plasmids greater than 10 kb. The bacteria were resistant to ampicillin (100), polymyxin (100), oxytetracycline (30), streptomycin (30), chloramphenicol (20-60), trimethoprim (10-60), nalidixic acid (100) but during October-January post monsoon season such resistance pattern showed inconsistent [35]. However, both *mdr* genes and *toxin* (PirAB) genes were rarely located in same plasmids of *Vibrio parahaemolyticus* and such data was limited in the database. The Mexico AHPND-causing *V. parahaemolyticus* strain (13-306D/4 and 13-511/A1) were reported to carry tetB gene coding for tetracycline resistance gene, and *V. campbellii* from China was found to carry multiple antibiotic resistance genes [36].

Control of bacteria in shrimp ponds

In aquaculture, several strategies have already been applied to control *Vibrio* strains, including chemicals, probiotics, antibiotics, natural products from plants, including plant oils. The FDA approved oxacillin, florfenicol, erythromycin, oxytetracycline, sulfamerazine, and combination drugs, sulfadimethoxine and ormetoprim in fish aquaculture [37]. The malachite green and chloramphenicol uses have reduced due to toxicities and antibiotic residue in shrimp. Similarly, floroquinolones were important drugs for human use and its use was contradicted in aquaculture due to development of drug resistance bacteria. The FDA also controls the use of nitrofurazone which may induce tumour in mammary glands [38,39]. Quiroz-Guzmán et al showed that after 120 hrs post infection shrimp fed with a diet containing 2% of a mix with *Curcuma longa* and *Lepidium meyenii* (TuMa) and a diet containing 0.2% of vitamin C (Vit-C) showed a significantly higher survival (85%) of shrimp fishes as compared to the other treatments [40]. The *Gracilaria* spp. (Gracilariaceae family) and *Sargassum* spp. (family Sargassaceae) have been used most for in vitro and in vivo experiments to control *Vibrio* species in shrimp ponds. Among the terrestrial plants, *Eucalyptus camaldulensis*, *Psidium guajava*, *Rhodomyrtus tomentosa*, and *Syzygium cumini* (Myrtaceae family) had significant activity against Vibrios [41,42]. Hannan et al. from Bangladesh screened twenty-one ethyl acetate plants extracts of which *Emblica officinalis* and *Allium sativum* were found strong inhibitory to *Vibrio alginolyticus in vivo* shrimp culture at 10mg/g feed [43]. The antimicrobial peptide, vibriocin (18 KDa of molecular mass) was very effective controlling pathogenic *Vibrio harveyi* [44]. The peptide acted stable in a wide range of pH, temperature, UV radiation, solvents and chemicals utilized [45]. Chakraborty et al discovered a CU1 phyto-antibiotic that killed Mdr bacteria targeting RNA polymerase enzyme [46,47].

Other fish aquaculture

Nile tilapia (*Oreochromis niloticus*) cultivated in major aquaculture worldwide because the fish was easy to cultivate, adapts to a wide range of environmental conditions, grows fast with tolerant to stress and diseases [48]. The *Oreochromis* species annual production reached >50MT world-wide. The reports suggested such fish was highly contaminated with MDR *Aeromonas veronii* [49,50]. Examples of emerging viruses in aquaculture include rhabdoviruses, orthomyxoviruses, reoviruses, iridoviruses, nodavirus and herpesvirus. The TiLV (OM1 and OM2) virus isolated from tilapia fishes shared 94.30% and 95.52% nucleotide identity with the TiLV isolated from West Bengal, India (MF502419.1) and Israel (KJ605629.1). However, use of MYXV (Myxoma virus) and RHDV (rabbit haemorrhagic disease virus) was proposed as a potential BCA (Biological Control Agents) for common carp (*Cyprinus species)* which are regarded as the most devastating invasive fish in Australia [51]. *Vibrio cholerae*, *V. parahaemolyticus*, and *V. vulnificus* were identified in blue crab aquaculture [52]. The acute hepatopancreatic necrosis disease (AHPND), also known as early mortality syndrome (EMS) was causing significant losses in shrimp production in the Southeast Asian countries due to PirAB toxins. This disease is caused by *V. parahaemolyticus* and affects the hepato-pancreas of infected

shrimp with mortality up to 100%, in *Litopenaeus vannamei* and *Penaus monodon [53]*.

Materials & Methods

Growth of Vibrio species

The new chromogenic TCBS medium consists of 10 g of peptone, 10 g of sea salts mixture, 10 g of ox bile, 10 g of sodium thiosulfate, 5 g of yeast extract, 5 g of sodium citrate, 2.2 g of sodium carbonate, 2 g of lactose, 0.5 g of sodium pyruvate and 1000ml with water and P^H adjusted to 8.6 and autoclaved at 15psi/15min [54].

Isolation of MDR bacteria and fish aquaculture

The Vibrio strains metabolize sucrose efficiently. The *V. cholerae* forms yellow colonies on TCBS agar, whereas other pathogenic species like *V. parahaemolyticus and V. vulnificus* produce green colonies in TSB agar plate [55]. The pictures of colonies were taken in chromogenic agar plate and then confirmed by 16S rRNA sequencing.

For experiment, each group of fish (n = 6/tank) were acclimatized in aquaria (120 × 30 × 45 cm) supplied with 120L freshwater and maintained at 35°C-37^{0}C with aeration for about 2 weeks. The fish were fed with a commercial diet (PT Central Protein, Prima) twice daily at a rate of 2% body weight. Water was 50% replaced and uneaten feed was siphoned daily. The bacteria were grown in Trypticase Soy Broth (TSB) overnight and the density of the bacterial suspension was enumerated using spread plate method on TSA. Each fish from the four groups was intraperitoneally injected with 0.1 ml bacterial suspension with a mean density of 1.0 × 10^7 cfu/ml. A control group was included where fish were injected with the same volume of sterile phosphate buffered saline (PBS). Clinical signs and morbidity were recorded daily for one week and the experiment was terminated when 100% morbidity or mortality occurred among the challenged groups. Newly dead or moribund shrimp were examined and tissues were inoculated onto Thiosulfate-Citrate-Bile Salt-Sucrose (TCBS) agar plate supplemented with 2% NaCl.

Identification of *Vibrio parahaemolyticus*

The green or bluish green colour colonies measuring about 3–5 mm was isolated from TCBS plate, and was inoculated into sterile sucrose medium supplemented with NaCl (3% w/v). Only sucrose non-fermenting colonies were streaked onto sterile tryptone soy agar slants supplemented with NaCl (3% w/v; TSAS) and maintained at room temperature for further identification. The isolates were confirmed to be *V. parahaemolyticus* based upon the ability to give typical biochemical reactions as listed in the USFDA (2001) viz., motile, no acid from sucrose, Gram (-), no H2S was produced on triple sugar iron agar, acetoin was not produced and grows in 3–8% NaCl but unable to grow in >10% NaCl. Each bacterium was further confirmed by RAPID Hi-Vibrio TM identification kit (KB007, HiMedia, India) and finally 16S rRNA sequencing could be performed from genomic DNA following BLAST search [56].

Few other sea food contaminations could be differentiated biochemically and many medium available from Himedia. The use of 6% NaCl in medium and biochemical tests for arginine dihydrolase and l-histidine decarboxylase can be useful to differentiate the growth of *P. shigelloides* from *Vibrio* species. Similarly, lysine decarboxylase and ornithine decarboxylase assays differentiate *P. shigelloides* from *Aeromonas* species and the cytochrome oxidase test differentiates *P. shigelloides* from other *Enterobacteriaceae* [57]. Other biochemical identification tests used include indole, inositol, and glucose fermentation, production of *β*-hemolysis, sensitivity to vibriostatic O/129 or a variety of commercial kits, such as API 20E, the Vitek 2 system, or the BD Phoenix
(https://store.pda.org/TableOfContents/ERMM_V2_Ch01.pdf).

DNA extraction, PCR amplification and sequencing

Pure colonies were grown overnight in TSB medium at a concentration of 10^9 CFU/ml. Then 1.5 ml each culture was transferred into a microcentrifuge tube and centrifuged at 5000 rpm for 10 min. The pellet was re-suspended in 100µl Solution-I, 200µl Solution-II and 150µl Solution-III as described by Maniatis et al. 1989 [58]. The pellet removed by centrifugation at 10,000 rpm/10min and 1ml ethanol was added. The pellet dried and suspended in TE buffer and four tubes combined into one tube and extracted with phenol-chloroform and treated with RNaseA and ethanol precipitated. The extracted gDNA was used to amplify the 16S rRNA genes using the universal primer set for prokaryotes [59]. The PCR assay (30 µl) contained a final concentration of 10x PCR buffer (XTPs 2mM), 0.5 mM of each primer, 2.5 U/µl of Taq DNA polymerase, 2µl of DNA sample and nuclease free water was added to achieve the total volume of PCR mixture. Then amplifications were carried out in a thermal cycler with an initial denaturation of 95°C for 3min, followed by 30 cycles of 94°C for 30 sec, 52°C for 90 sec, 72°C for 1.5min, and an additional final extension of 72°C for 7min. The expected PCR product of ~1,500bp was detected by electrophoresis in 1% agarose stained with ethidium bromide and photographed under UV light. Sequencing was done by automated dideoxy sequencer. Then the sequenced was BLAST searched to identify bacteria. The *V. parahaemolyticus* 16S rRNA gene (accession no. MZ015567) could be compared by Blast-2 search.

The pirAB genes PCR assay was developed for diagnosis of AHPND disease in shrimp [60]. The Vp_PirAB-F (5'- GTG GAA ATG GTG AAC TTG CG-3') and Vp_PirAB-R primer sequences (5'- GGC GTT GCA ATC TAA GAC AT-3') were used for

amplification of V. parahaemolyticus plasmid-derived PirAB genes (accession no. AB972427) the *toxR*-based PCR assay was preformed to identify *V. parahaemolyticus* from all the presumptive isolates. Detection of *toxR* gene was carried out using primer *toxR*-F (5′-ATA CGA GTG GTT GCT GTC ATG-3′) and *toxR*-R (5′-GTC TTC TGA CGC AAT CGT TG-3′) with the expected amplicon size of 368 bp (accession no. ABADIT010000001, nt. 466250-467128) [61]. The detection of the genes *tdh* (Thermostable Direct Haemolysin) and *trh* (Thermostable direct-haemolysin Related Haemolysin) was done using the primer pairs TDHF (5-GTA AAG GTC TCT GAC TTT TGG AC-3′) and TDHR (5- TGG AAT AGA ACC TTC ATC TTC ACC-3′) for tdh and TRHF (5-TTG GCT TCG ATA TTT TCA GTA TCT-3′) and TRHR (5-CAT AAC AAA CAT ATG CCC ATT TCC G-3′) for trh [62]. The *tlh* gene PCR was performed to confirm the identity of *V. parahaemolyticus* strains. The primers *tlh*-F (5' AAA GCG GAT TAT GCA GAA GCA CTG 3') and *tlh*-R (5' GCT ACT TTC TAG CAT TTT CTC TGC 3') were used to amplify a 450-bp fragment of the thermolabile haemolysin gene [63].

Results

Plasmid-mediated mdr genes in shrimp-contaminated bacteria

Mdr genes in *Vibrio parahaemolyticus* plasmid pVPH1 (183730bp) was isolated from Shrimp fish (Hong Kong, 2015). The Dhfr protein is dihydro folate reductase gives trimethoprim resistance, Sul1 protein is dihydropteroate synthase, Mph protein is macrolide 2'-phosphotransferase gives aminoglycoside antibiotic resistance whereas blaPER is extended spectrum beta-lactamase that cleaves the beta-lactam ring of penicillin drugs and very much prominent in diverse bacterial species like *Pseudomonas*, *Escherichia* and *Acinetobacter* species (accession nos. EU022369, JAHJKU010000050, DADBKW010000136) (Figure 3).

Mdr genes was located in *Vibrio alginolyticus* plasmid pC1394 (167140bp; see, Figure 2C for plasmid structure). The bacterium was isolated from shrimp fish in China on 1st August, 2016 The Dhfr enzyme reduces 7,8-dihydrofolate to 5,6,7,8-tetrahydrofolate with NADPH as a cofactor. This is an essential step in the biosynthesis of deoxythymidine phosphate and gave resistant to trimethoprim antibiotic. The dihydropteroate synthase (Sul1) produces sulphonamide antibiotic resistance (Figure 4). The QnrA1 gene is quinolone resistant pentapeptide protein. The *sul1* gene was also detected in *V. cholerae* strain CNRVC190247 chromosome-2 (accession number: OW443151) as well as in plasmid 3 (accession number: OW443149). But similar Blast search did not find *sul1* gene in *V. parahaemolyticus* chromosomes (accession numbers: CP034305, CP043421, CP034295, and CP068648) and plasmids (KP324996, MH890610 and CP020036). The blaNDM-1 gene was first detected in a New Delhi patient in 2009 being a deadly *mdr* gene and could cleave ampicillin, oxacillin, cefotaxime as well as imipenem antibiotics (Figure 4). The blaNDM1 gene also detected in *V. alginolyticus* plasmids pC1394, pVb1762 and pVb2134 (accession nos. MH457126, OK146920 and OK085530) an also found in chromosome-1 of *V. alginolyticus* strain AUSMDU00064140 (accession no. CP110670).

Table 1: *Design of primers for V. parahaemolyticus genomic mdr genes specific PCR.*

Primers	Sequence	Tm	Size	GC%	Location
blaCARBf1	5'-CAAAACGTTAGCGTGTGCCA-3'	59.97	453bp	50	Ch-2
blaCARBr1	5'-GAGCGCATGAGAGAATCCGA-3'	59.97		55	Ch-2
CatBf1	5'-GCTTCTGGAGCCACCTTCAT-3'	60.03	338bp	55	Ch-2
CatBr1	5'-GGCCAGTTCCACCATTGGAT-3'	60.32		55	Ch-2
Pbp1Bf2	5'-CGTCGTGACCAATTCCCTGA-3'	60.04	461bp	55	Ch-1
Pbp1Br2	5'-TTAGTGCGCTCAGGATAGCG-3'	59.97		55	Ch-1

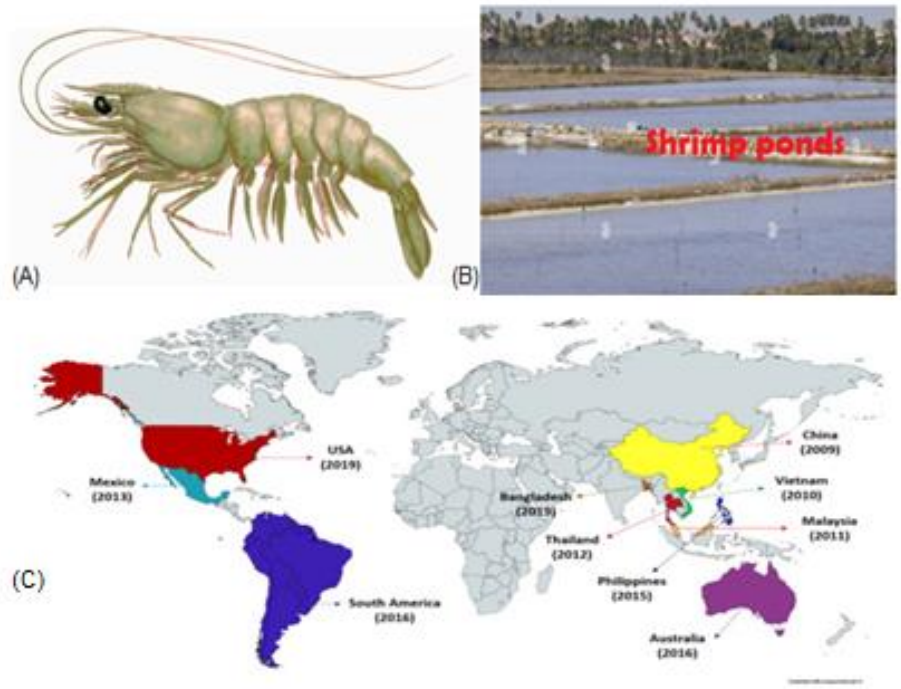

Figure 1: *(A) A white leg shrimp fish (14-18cm), (B) shrimp ponds and (C) map of worldwide.*

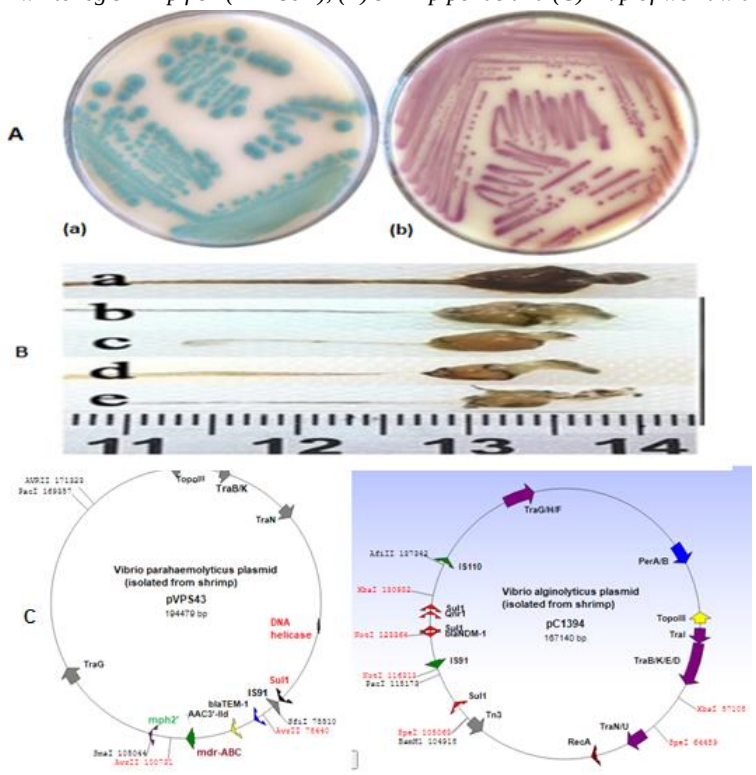

Figure 2: *(A) Colony structure of Vibrio species in chromogenic agar plates: (a) V. parahaemolyticus and (b) V. cholerae (Yeung M and Thorsen T. Journal of Visualized Experiments, 2016; 117: 54493). (B) Intestinal track of shrimp: Normal fish (a) vs. V. parahaemolyticus infected shrimps with AHPND-disease (b-e). (C) Plasmid pVPS43 and pC1394 structures of V. parahaemolyticus and V. alginolyticus both isolated from shrimp. Bacteria normally present in the skin (10^2–10^7 CFU/cm^2) and in gills and intestines*

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>KP688397-dhfr
        1 mrnrratlsp fmaaltqrsi vkislmaaka rngvigcgsd ipwnakgeql lfkaitynqw
       61 llvgrktfea mgalpnrkya vvsrsgsvat nddvvvfpsi eaamrelktl tnhvvvsggg
      121 eiyksliaha dtlhistids epegnvffpe ipkefnvvfe qefhsninyr yqiwqrg
>KP688397-sull
        1 mvtvfgilnl tedsffdesr rldpagavta aiemlrvgsd vvdvgpaash pdarpvspad
       61 eirriaplld alsdqmhrvs idsfqpetqr yalkrgvgyl ndiqgfpdpa lypdiaeadc
      121 rlvvmhsaqr dgiatrtghl rpedaldeiv rffearvsal rrsgvaadrl ildpgmgffl
      181 spapetslhv lsnlqklksa lglpllvsvs rksflgatvg lpvkdlgpas laaelhaign
      241 gadyvrthap gdlrsaitfs etlakfrsrd ardrgldha
>KP688397-mph
        1 mtvvttadts qlyalaarhg lklhgpltvn elgldyrivi atvddgrrwv lriprraevs
       61 akvepearvl amlknrlpfa vpdwrvanae lvaypmleds tamviqpgss tpdwvvpqds
      121 evfaesfata laalhavpis aavdagmlir tptqarqkva ddvdrvrref vvndkrlhrw
      181 qrwldddssw pdfsvvvhgd lyvghvlidn tervsgmidw searvddpai dmaahlmvfg
      241 eeglaklllt yeaaggrvwp rlahhiaerl afgavtyalf aldsgneeyl aaakaqlaaa
      301 e
>KP688397-blaPER-1
        1 mnviikavvt astllmvsfs sfetsaqspl lkeqiesivi gkkatvgvav wgpddlepll
       61 inpfekfpmq svfklhlaml vlhqvdqgkl dlnqtvivnr akvlqntwap imkayqgdef
      121 svpvqqllqy svshsdnvac dllfelvggp aalhdyiqsm giketavvan eaqmhaddqv
      181 qyqnwtsmkg aaeilkkfeq ktqlsetsqa llwkwmvett tgperlkgll pagtvvahkt
      241 gtsgikagkt aatndlgiil lpdgrpllva vfvkdsaess rtneaiiaqv aqtayqfelk
      301 klsalspn
```

Figure 3: *Mdr genes in Vibrio parahaemolyticus plasmid pVPH1/2 (accession no. KP688397, 183730bp; KP791968, 198487bp) isolated from Shrimp fish (Hong Kong, 2015). Dhfr protein is dihydro folate reductase, sul1 protein is dihydropteroate synthase, mph protein is macrolide 2'-phosphotransferase and blaPER-1(protein id. AKD43563) is extended spectrum beta-lactamase. The same blaPER-1 gene was located in many plasmids of Acinetobacter, Pseudomonas, Escherichia, Aeromonas species and thus such gene could not be used as marker for V. parahaemolyticus.*

```
>NZ_MH457126-dhfr
        1 mklslmaaks kngiignqpd ipwsakgeql lfkaitynqw llvgrktfes mgalpnrkya
       61 vvtrsnfstn degvmvfssi qdalinleei tdhvivsggg eiyksliskv dtlhistvdi
      121 erdgdivfpe ipdtfklvfe qdfesninyc yqiwqks
>NZ_MH457126-sull
        1 mvtvfgilnl tedsffdesr rldpagavta aiemlrvgsd vvdvgpaash pdarpvspad
       61 eirriaplld alsdqmhrvs idsfqpetqr yalkrgvgyl ndiqgfpdpa lypdiaeadc
      121 rlvvmhsaqr dgiatrtghl rpedaldeiv rffearvsal rrsgvaadrl ildpgmgffl
      181 spapetslhv lsnlqklksa lglpllvsvs rksflgatvg lpvkdlgpas laaelhaign
      241 gadyvrthap gdlrsaitfs etlakfrsrd ardrgldha
>NZ_MH457126-qnrA1
        1 mdiidkvfqq edfsrqdlsd srfrrcrfyq cdfshcqlqd asfedcsfie sgavegchfs
       61 yadlrdasfk acrlslanfs gancfgiefr ecdlkganfs rarfynqvsh kmyfcsayis
      121 gcnlaytnls gqclekcelf ennwsnanls gaslmgsdls rgtfsrdcwq qvnlrgcdlt
      181 fadldgldpr rvnlegvkic awqqeqllep lgvivlpd
>NZ_MH457126-blaNDM-1
        1 melpnimhpv aklstalaaa lmlsgcmpge irptigqqme tgdqrfgdlv frqlapnvwq
       61 htsyldmpgf gavasngliv rdggrvlvvd tawtddqtaq ilnwikqein lpvalavvth
      121 ahqdkmggmd alhaagiaty analsnqlap qegmvaaqhs ltfaangwve patapnfgpl
      181 kvfypgpght sdnitvgidg tdiafggcli kdskakslgn lgdadtehya asarafgaaf
      241 pkasmivmsh sapdsraait htarmadklr
```

Figure 4: *Mdr genes isolated in Vibrio alginolyticus plasmid pC1394 (167140bp). The bacterium was isolated from shrimp fish in China on 1.8.2016. The dhfr enzyme reduces 7,8-dihydrofolate to 5,6,7,8-tetrahydrofolate with NADPH as a cofactor. This is an essential step in the biosynthesis of deoxythymidine phosphate and gave resistant totrimethoprim antibiotic. The dihydropteroate synthase (sul1) produces sulphonamide antibiotic resistance. The QnrA1 gene is quinolone resistant pentapeptide protein. The blaNDM-1 gene was first detected in a New Delhi patient in 2009 and it could cleave ampicillin, oxacillin, cefotaxime as well as imipenem antibiotics.*

```
>KY399740-blaOXA-1
   1 mkntihinfa ifliianiiy ssasastdis tvasplfegt egcfllydas tnaeiaqfnk
  61 akcatqmapd stfkialslm afdaeiidqk tifkwdktpk gmeiwnsnht pktwmqfsvv
 121 wvsqeitqki glnkiknylk dfdygnqdfs gdkernnglt eawlesslki speeqiqflr
 181 kiinhnlpvk nsaientien mylqdldnst klygktgagf tanrtlqngw fegfiisksg
 241 hkyvfvsalt gnlgsnltss ikakknaiti lntlnl
>KY399740-CatB3
   1 mtnyfdspfk gkllseqvkn pnikvgrysy ysgyyhghsf ddcarylfpd rddvdkliig
  61 sfcsigsgas fimagnqghr ydwassfpff ymqeepafss aldafqkagn tvignqvwig
 121 seamvmpgik ighgavigsr slvtkdvepy aivggnpakk ikkrftdeei slllemewwn
 181 wslekikaam pmlcssnivg lhkywlefav
>KY399740-Arr3
   1 mvkdwipish dnykqvqgpf yhgtkanlai gdllttgfis hfedgrilkh iyfsalmepa
  61 vwgaelamsl sglegrgyiy iveptgpfed dpnltnkrfp gnptqsyrtc eplrivgvve
 121 dweghpveli rgmldsledl krrglhvied
```

Figure 5: *The Salmonella enterica small plasmid (2788bp; acc. no. KY399740) borne mdr genes. The bacterium was isolated from shrimp fish in China. The blaOXA gene cleaves oxacillin more efficiently than ampicillin. The catB3 gene acetylates chloramphenicol and acetylated drug could not able to bind ribosome to inhibit bacterial protein synthesis. The arr3 gene ribosylates rifampicin and ribosylated rifampicin could not able to bind RNA polymerase to inhibit transcription.*

```
>blaCARB
ATGAAAAAGTTATTCCTGTTGGTTGGGCTGATGGTTTGCTCAACTGTTAGTTACGCCTCC
AAATTAAACGAAGACATCTCCCTCATCGAGAAACAAACATCTGGGCGAATTGGAGTGTCA
GTCTGGGATACACAAACGGACGAGCGTTGGGATTATCGCGGAGACGAACGTTTCCCATTA
ATGAGCACATTCAAAACGTTAGCGTGTGCCACCATGCTAAGCGACATGGACAGCGGCAAA
CTCAACAAAAATGCCACAGCGAAAATCGATGAACGCAATATTGTGGTTTGGTCTCCGGTG
ATGGATAAACTGGCTGGACAAAGCACACGTGTCGAACACGCTTGTGAGGCTGCCATGTTG
ATGAGCGACAACACCGCCGCGAACTTAGTGCTAAATGAAATTGGTGGTCCTAAAGCGGTC
ACGCTGTTTTTGCGATCTATTGGCGACAAAGCAACGCGACTTGACCGATTGGAACCCCGT
TTGAATGAAGCAAAACCGGGCGATAAGCGAGACACCACAACGCCTAACGCCATGGTAAAC
ACCCTACATACCTTGATGGAAGATAACGCCCTATCTTACGAGTCACGCACACAGCTGAAA
ATCTGGATGCAAGACAACAAAGTATCGGATTCTCTCATGCGCTCTGTTCTGCCAAAAGGC
TGGTCGATTGCAGACCGCTCTGGCGCAGGTAACTACGGTTCACGCGGCATTAGCGCGATG
ATCTGGAAAGACAACTACAAGCCGGTTTACATCAGTATTTACGTCACAGACACCGACCTT
TCGCTTCAAGCTCGCGATCAACTGATCGCGCAAATCAGCCAACTGATTTTAGAGCACTAC
AAAGAAAGTTAA

>pbp1B protein
   1 mtdskkpsak kapakkstas kgttkrprrt pskkptnekr swlkvlwsfs wkagvalaav
  61 llfvgiylds vvkerfdgql felptvvyar ilnlnpgeni tiqelrneld vlnyrkvsqp
 121 rypgeyssss trvelirrpf efadgpepdr hvmlhfsdsg lqriqslesk gdlgylrlep
 181 kmlgmlekdr deqrlflrrd qfpeilvdal latedrdfyq hdgvsplaia ralvanikag
 241 rtvqggstlt qqlaknlflt rdktlwrkvr eayialildy ryskdrilea ylnevylgqs
 301 ggeaihgfgl asryyfgqpi qelridqlam lvgmvkgpsy ynpvrypert kerrdlvlrl
 361 lmqqnmltsq qyeqavsrpl dtqskprias rqpayfqqln ielkekvgdr fkaetglrvf
 421 tsldpvsqsk meqaiakkip dlakrggkel eaaavavdrh sgeiramvgg krvgyegfnr
 481 alnasrpigs lvkpaiylta leqpdkynlg ttlhdtplsl kgskgsvwtp rnydrkyrgd
 541 vplylalaks lnvptvrlgm elgipevsgt lerlgvnkde irpvpsmflg sfsltpfeva
 601 qmyqtltnsg krakltalrs vidmdgsvly qslprssrav deqaawltty amkqgvaqgt
 661 grylqsqfaw aalagktgts ndtrdswfvg idgrevttiw lgrddnkpin ltgssgalrv
 721 yaeylaqrip erldlpwpke vttlgfkpts ngglemncrs dyklpiwdkt gqikqqcekk
 781 snwlnslfdw
```

Figure 6: *(A) The novel blaCARB gene sequence located Ch-2 of Vibrio parahaemolyticus. The primer sequences were underlined and very specific for V. parahaemolyticus. (B) Sequence of Vibrio parahaemolyticus Ch-1 penicillin-binding protein (pbp1B).*

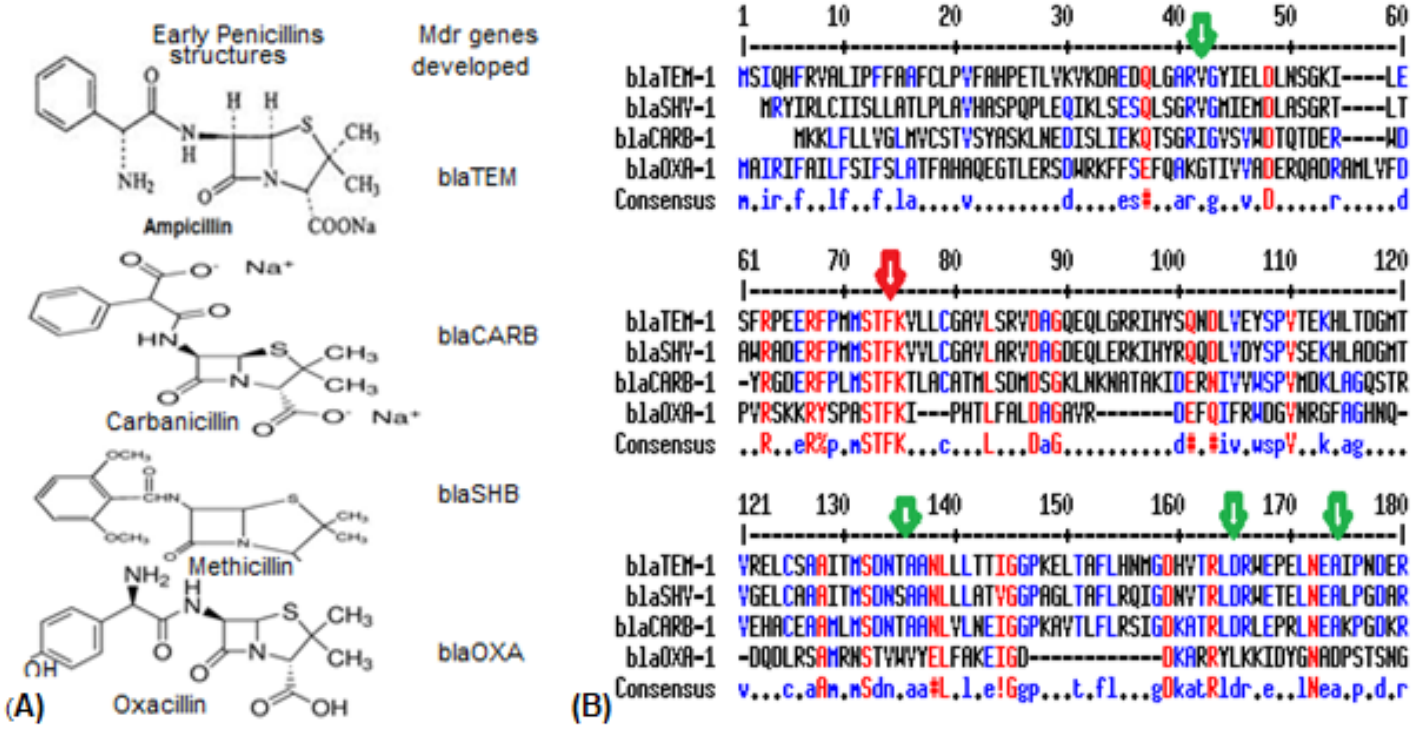


Figure 7: *(A) Structures of early penicillin drugs and the corresponding beta-lactamase genes were developed naturally to cleave the drugs. (B) Sequence heterogeneity of blaCARB-1 gene with other bla-isomers to be useful for primer design. Part of the multi-alignment was shown.*

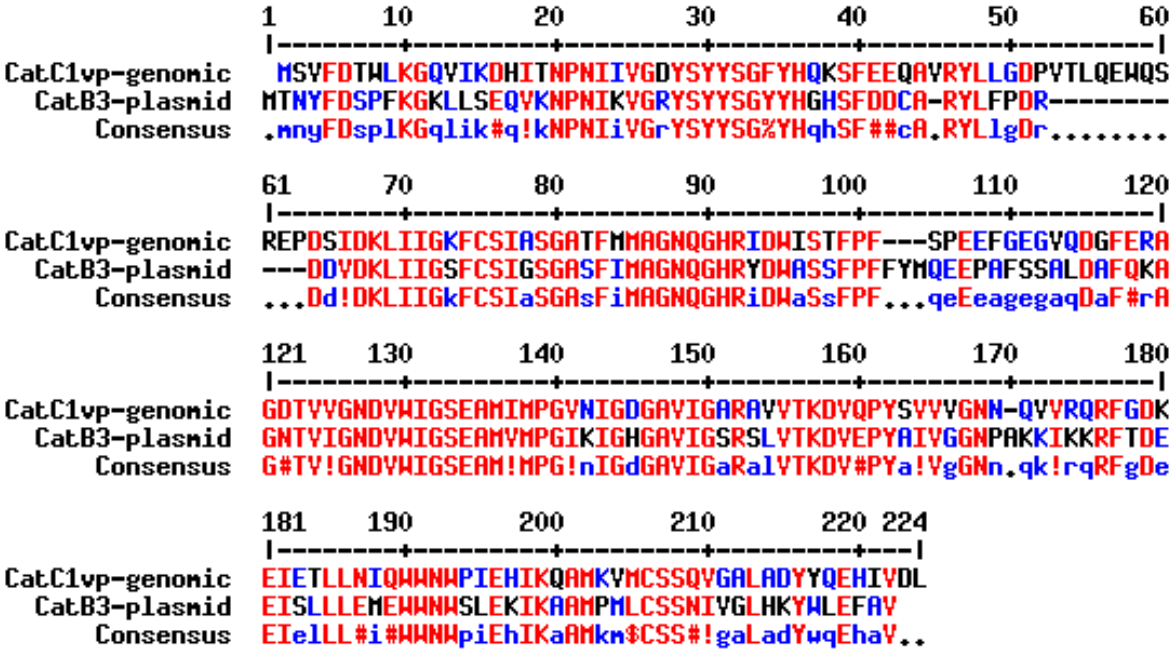


Figure 8: *Difference in amino acid sequence of plasmid-mediated catB3 enzyme and chromosomal catC1 enzyme (protein id. MBE4483988) of Vibrio parahaemolyticus. Such differences were useful for primer design for PCR diagnostics.*

```
>PirA-toxin
       1 msnnikhetd yshdwtvepn ggvtevdskh tpiipevgrs vdientgrge ltiqyqwgap
      61 fmaggwkvak shvvqrdety hlqrpdnafy hqrivvinng asrgfctiyy h
>PirB toxin
       1 mtneyvvtms sltefnpnna rksylfdnye vdpnyafkam vsfglsnipy aggflstlwn
      61 ifwpntpnep dieniweqlr driqdlvdes iidaingild skiketrdki qdinetienf
     121 gyaaakddyi glvthyligl eenfkreldg dewlgyailp llattvslqi tymacgldyk
     181 defgftdsdv hkltrnidkl yddvssyite laawadndsy nnanqdnvyd evmgarswct
     241 vhgfehmliw qkikelkkvd vfvhsnlisy spavgfpsgn fnyiatgted eipqplkpnm
     301 fgerrnrivk ieswnsieih yynrvgrlkl tyengevvel gkahkydehy qsielngayi
     361 kyvdviangp eaidrivfhf sddrtfvvge nsgkpsvrlq leghficgml adqegsdkva
     421 afsvayelfh pdefgtek
```

Figure 9: *PirA and PirB toxin proteins from V. parahaemolyticus and V. campbelli plasmids isolated in 2021 from Penaeus vannamei (white leg shrimp) in Asia (accession number: MH890610, KP324996). However, similar plasmids pLA16-2 (accession no. CP021148) and pVPA3-1 (accession no. NC_025152) had no PirAB toxin genes.*

```
>tdh protein-GU971653
       1 mkhqyfakks flfismlaaf ktsafelpsv pfpapgsdei lfvvrdttfn tqapvnvkvs
      61 dfwtnrnvkr kpyedvygqs vfttsgtkwl tsymtvnind kdytmaavsg yksghsavfv
     121 ksdqvqlqhs ynsvanfvge degsipskmy ldetpeyfvn veayesgsgn ilvmcisnke
     181 sffeykhqq
>tdh-GU971653
       1 atgaaacacc aatattttgc aaaaaaatca tttttattta tatccatgtt ggctgcattc
      61 aaaacatctg cttttgagct tccatctgtc ccttttcctg cccccggttc tgatgagata
     121 ttgtttgttg ttcgagatac aacttttaat acccaagctc cggtcaatgt aaaggtctct
     181 gacttttgga caaaccgtaa tgtaaaaaga aaaccgtacg aagatgttta tggtcaatca
     241 gtattcacaa cgtcaggtac taaatggttg acatcctaca tgactgtgaa cattaatgat
     301 aaagactata caatggcagc ggtgtctggc tataagagcg gtcattctgc tgtgttcgta
     361 aaatcagatc aagtacaact tcaacattcc tataattctg tagctaactt tgttggtgaa
     421 gatgaaggtt ctattccaag taaaatgtat ttggatgaaa ctccagaata ttttgttaat
     481 gtagaagcat atgagagtgg tagtggtaat atattggtaa tgtgtatatc caacaaagaa
     541 tcgtttttg aatataaaca tcaacaataa
```

Figure 10: *The TDH protein and tdh gene sequence from V. parahaemolyticus (Nakagawa H. et al. Method for Detecting and Quantitatively Determining Hemolysin-producing Bacteria by Totally Detecting and Quantitatively Determining Thermostable Direct Hemolysin-related Gene (tdh-related toxin gene) of Food Poisoning Bacteria. Patent: WO-2003033702-A6; 24-04-2003).*

```
>TRH protein
       1 mklrlyfafs lllvsifsis ksfaidlpsi pfpspgsdel lfvvrnttik tespvkaive
      61 dywtnrtikr kpykdvygqs vfttagskwl saymtvning hnytmaalsg ykhgtstvft
     121 ksektslnqd fysvksfvdd seesipsiny ldetpeyfvt veayesgngh mfvmcisnkl
     181 sfgecksqi
>trh gene-LM993807
       1 atgaaactaa gactctactt tgcattcagt ttgctattgg tttcaatatt ttcaatatct
      61 aaatcattcg cgattgatct gccatcaata ccttttcctt ctcctggttc cgatgagcta
     121 ttatttgttg ttagaaatac aacaatcaaa actgaatccc cagttaaggc aattgtggag
     181 gactattgga caaaccgaac cataaaaaga aaaccataca aagatgtata cggtcaatcg
     241 gttttcacaa ctgcgggttc aaagtggtta agcgcctata tgacagtcaa catcaatggt
     301 cacaactata cgatggctgc tctttctggt tataaacatg gtacttctac ggtcttcaca
     361 aaatcagaga aaacaagcct aaaccaagac ttttattcgg taaaatcttt tgttgatgat
     421 agcgaagaat caataccaag tataaattat ttagatgaaa caccagaata ctttgttact
     481 gtcgaggcat atgagagcgg caatggacat atgtttgtta tgtgcatctc caacaaatta
     541 tcatttggcg aatgtaaatc acaaatttaa
```

Figure 11: *The TRH protein and trh gene of V. parahaemolyticus (accession number: LM993807).*

```
TRH   1    MKLRLYFAFSLLLVSIFSISKSFAIDLPSIPFPSPGSDELLFVVRNTTIKTESPVKAIVE  60
           MK + +   S L +S+ +  K+ A +LPS+PFP+PGSDE+LFVVR+TT  T++PV   V
TDH   1    MKHQYFAKKSFLFISMLAAFKTSAFELPSVPFPAPGSDEILFVVRDTTFNTQAPVNVKVS  60

TRH   61   DYWTNRTIKRKPYKDVYGQSVFTTAGSKWLSAYMTVNINGHNYTMAALSGYKHGTSTVFT  120
           D+WTNR +KRKPY+DVYGQSVFTT+G+KWL++YMTVNIN  +YTMAA+SGYK G S VF
TDH   61   DFWTNRNVKRKPYEDVYGQSVFTTSGTKWLTSYMTVNINDKDYTMAAVSGYKSGHSAVFV  120

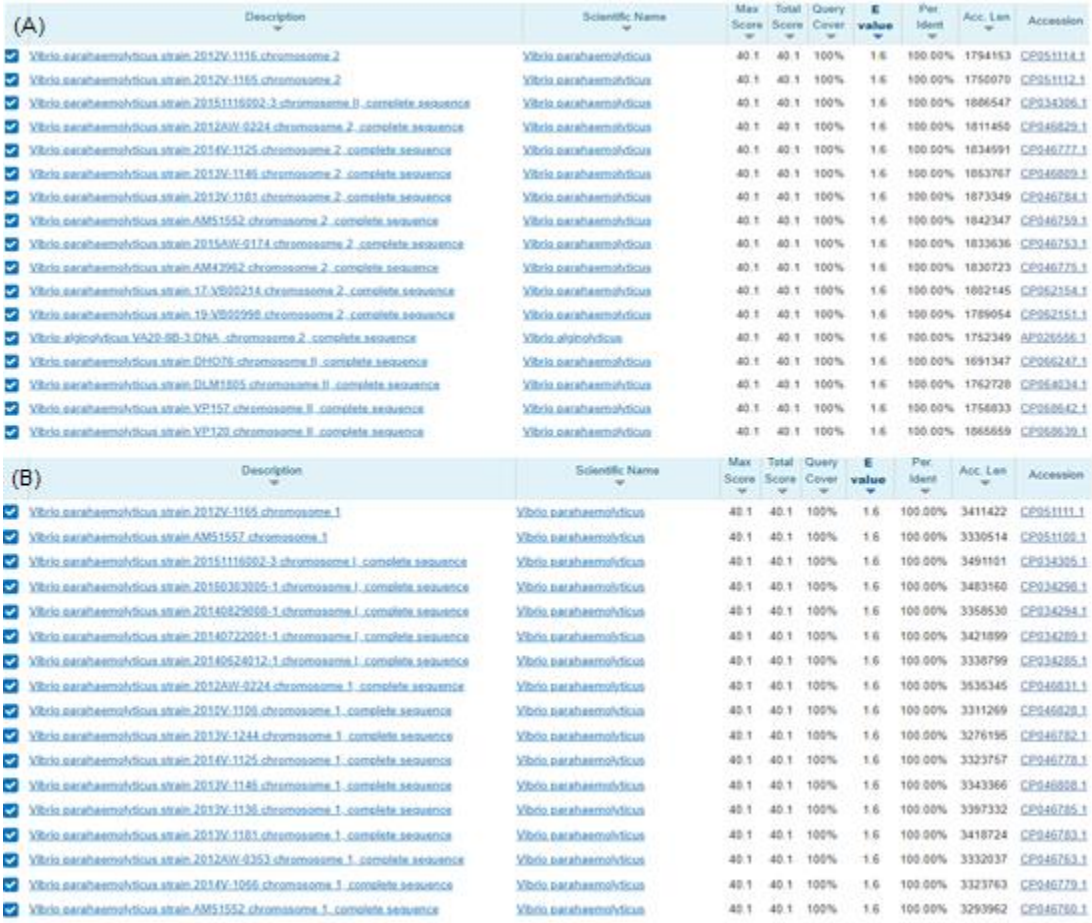
TRH   121  KSEKTSLNQDFYSVKSFVDDSEESIPSINYLDETPEYFVTVEAYESGNGHMFVMCISNKL  180
           KS++  L   + SV +FV + E SIPS  YLDETPEYFV VEAYESG+G++ VMCISNK
TDH   121  KSDQVQLQHSYNSVANFVGEDEGSIPSKMYLDETPEYFVNVEAYESGSGNILVMCISNKE  180

TRH   181  SFGECKSQ  188
           SF E K Q
TDH   181  SFFEYKHQ  188
```

Figure 12: *Sequence similarity (61%) between TRH and TDH two related haemolysin proteins of V. parahaemolyticus. However, 10% clinical V. parahaemolyticus did not contain the both genes.*

```
   1 msnrspssql fehhsaslek mdytspmdia astfssffqq crgclsksea kqvyreaksy
  61 rqqlqaktrk vlthsnpqlk hvqhlaidsl qqesldyeal fgqradayvs essvasmfsp
 121 aayltelyre gkqlhtsdqp qyldkrrpdl kslslsqssl dtevttlals neillntiqn
 181 rtdknanqvy dslastaypf dlpyhkpftn ietaltrqns sfealaqala psqsvgafpi
 241 ntrtayanhl spglrkllle pipseseeae lnsallrhfg tadieqlsdv eyfcqrvdit
 301 raeldaylal pgfvqslntf sshrfttepi pvnpetygas yvndtsiegn fiaviddslr
 361 wdtstlmrtg gatsecnllp nvdgdkirfi ykftkhytst gwdsltvrvn rkvvetravy
 421 dsyqnwleff vplagnmsgk ieiehhpews tltytkshqg lsatalirls kvirytqktg
 481 lspaaldsli alsqgsaatv sditentlll saraleyqqr yslseddalv lagadinaya
 541 pagelsqfdr lfnnpplndv afttndandt isfdpddtay vqeravlkra lgvddaglat
 601 laaivdhddf awarslanls tlyrvglwar mhdltpqelq rllqlngkke dllfasntll
 661 adyldaiynt sqwlvaqqls vaelnvmttn vypetmtsev dgfirtlyqa lkannvasis
 721 eidslhqlla phiaatfgld nvdsaitqqv wveqiavdqg lsltdmasfc daivsyceid
 781 tptsesaqlv tfsqalgqla fiiqywklna aalklavnkp stlgsnltdk lalnlntqqa
 841 lnryktlqlc ageaiseclt lldtnqldta vlarwldmpe sevaeaakcv gadsanlnas
 901 qavfmmewld qsvslglsvq algdllnsei dqpysewqnl tgalvagmtd sskrnqmnil
 961 lgeslstalc ayfintvaps lpssisiknr delfdyllid nqvsgqittt rlaeaissvq
1021 lyinrclqgl eesvdrnqlv eafftqwnsy nkrystwagv sklayypeny idptlrynqs
1081 glqgellnel nqsqlnkdsv etaysnylng fegianlkvl cgyhhaaeln kglsyfvgrs
1141 tatpyryywr slnhdsgdgv ggylasawtd weeiqgqitp indevrpvlf nnrlyiawvs
1201 qqmvsdegap sgepsgepnl kaqyilqlsh rkingswapa msfmlpdipe sffdheqpkf
1261 nlylsyhslq dailfmlydp slvpnnydgi gvegpayggf iynnmeneli tgeawssiys
1321 lyshnlnnry kpnkliryin avsyavnvtp stpsdttepe wlvvnnvdiv degvdnlnlp
1381 ielsytaqav iqitdlssep davleyeeie edvgtefnvd slevtvtkvd hsfrfrlrtv
1441 ittypdafgn npgsitiyfe gaariipagp arthvidgle lsipwsntgg hftryarasv
1501 vadytggpqq swkqhfrlgh tqyipgreyt yswgeeeitl rndattdisk dysfdihgdg
1561 leqtrtltvr qdkvlvyekd fkinvtktda vitnptdniv ivngdnqasy lennthpkrt
1621 rlntlfahel vmraqsgldn vlswdtqqlp epklgdgtyv klvfepydpq ihgnskefti
1681 ynievfkgld afpvasgtls kdqktevqff lsrhpdaygd kdhlyvkaqy qsgwtgrilf
1741 drtedtddla pkgwylkgel fegldsaqal qdktepmdfn ganglyfwel fyytpmmvae
1801 kllqsqnfee aerwlkyvfs pegylegkfp erhhvdrqwn srpleedtaw detqadstdp
1861 dvvaqadpmh ykvttfmkll dlliargdma yrqlerdtla eakmwylsal qllgpqpdlp
1921 eqgnwsnptl sqaasdttmq qslmlmerlv rgetselttl eartanslta lflpsqndkl
1981 kgywqtlelr lfnlrhnlsi dgqpltlplf aapadpkalq naaaasaggs dalpnnaais
2041 iqrfpvmles arnlvgqliq ygstlssvle rkdsealnal lqtqaqelmq ysqqlqdkti
2101 eqlqaeqkvl sasldaattr rdsyqellne gistierqsi neriasssms vaanamrsag
2161 avldmapnvf gmavggsrwg avtsaiasgm disaiglata adahsmteqy rrrqqewsiq
2221 rdsaahecaq leaqqhslav qleaaqlqrd yvaaqqaqtq tqldflktkf snvelyswmq
2281 grlsavfyqf ydltvarcmr aelgyqwetq dpsffiqpga wdgnhaglls gealllnlaq
2341 mesaylewdg ralevnrtvs makemgvdsa gfnaevnqvl ndavsslqph tlemveimgt
2401 eskvftasid lnalaiaddy pdamlsnsgs svrrikqisv slpallgpye diqavlgysg
2461 ngngihqsct htaishgind sgqfqldfnd skylpfeglp ingdgsaklt lsfphagdtg
2521 kqrsilqsln divlhiryti ltd
```

Figure 13: *Nuraminidase toxin Tc-A in plasmid pva1 of V. parahaemolyticus GL601.*



(A)

Description	Scientific Name	Max Score	Total Score	Query Cover	E value	Per. Ident	Acc. Len	Accession
Vibrio parahaemolyticus strain 2012V-1116 chromosome 2	Vibrio parahaemolyticus	40.1	40.1	100%	1.6	100.00%	1794153	CP051114.1
Vibrio parahaemolyticus strain 2012V-1165 chromosome 2	Vibrio parahaemolyticus	40.1	40.1	100%	1.6	100.00%	1750070	CP051112.1
Vibrio parahaemolyticus strain 20151116002-3 chromosome II, complete sequence	Vibrio parahaemolyticus	40.1	40.1	100%	1.6	100.00%	1886547	CP034306.1
Vibrio parahaemolyticus strain 2012AW-0224 chromosome 2, complete sequence	Vibrio parahaemolyticus	40.1	40.1	100%	1.6	100.00%	1811450	CP046829.1
Vibrio parahaemolyticus strain 2014V-1125 chromosome 2, complete sequence	Vibrio parahaemolyticus	40.1	40.1	100%	1.6	100.00%	1834591	CP046777.1
Vibrio parahaemolyticus strain 2012V-1146 chromosome 2, complete sequence	Vibrio parahaemolyticus	40.1	40.1	100%	1.6	100.00%	1863767	CP046809.1
Vibrio parahaemolyticus strain 2012V-1181 chromosome 2, complete sequence	Vibrio parahaemolyticus	40.1	40.1	100%	1.6	100.00%	1873349	CP046784.1
Vibrio parahaemolyticus strain AM51552 chromosome 2, complete sequence	Vibrio parahaemolyticus	40.1	40.1	100%	1.6	100.00%	1842347	CP046759.1
Vibrio parahaemolyticus strain 2015AW-0174 chromosome 2, complete sequence	Vibrio parahaemolyticus	40.1	40.1	100%	1.6	100.00%	1833636	CP046753.1
Vibrio parahaemolyticus strain AM42962 chromosome 2, complete sequence	Vibrio parahaemolyticus	40.1	40.1	100%	1.6	100.00%	1830723	CP046775.1
Vibrio parahaemolyticus strain 17-VB00214 chromosome 2, complete sequence	Vibrio parahaemolyticus	40.1	40.1	100%	1.6	100.00%	1802145	CP062154.1
Vibrio parahaemolyticus strain 19-VB00998 chromosome 2, complete sequence	Vibrio parahaemolyticus	40.1	40.1	100%	1.6	100.00%	1789054	CP062151.1
Vibrio alginolyticus VA20-8B-3 DNA, chromosome 2, complete sequence	Vibrio alginolyticus	40.1	40.1	100%	1.6	100.00%	1752349	AP026556.1
Vibrio parahaemolyticus strain DH076 chromosome II, complete sequence	Vibrio parahaemolyticus	40.1	40.1	100%	1.6	100.00%	1691347	CP066247.1
Vibrio parahaemolyticus strain DLM1805 chromosome II, complete sequence	Vibrio parahaemolyticus	40.1	40.1	100%	1.6	100.00%	1762728	CP064034.1
Vibrio parahaemolyticus strain VP157 chromosome II, complete sequence	Vibrio parahaemolyticus	40.1	40.1	100%	1.6	100.00%	1756833	CP060642.1
Vibrio parahaemolyticus strain VP120 chromosome II, complete sequence	Vibrio parahaemolyticus	40.1	40.1	100%	1.6	100.00%	1865659	CP060639.1

(B)

Description	Scientific Name	Max Score	Total Score	Query Cover	E value	Per. Ident	Acc. Len	Accession
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Vibrio parahaemolyticus strain AM51557 chromosome 1	Vibrio parahaemolyticus	40.1	40.1	100%	1.6	100.00%	3330514	CP051100.1
Vibrio parahaemolyticus strain 20151116002-3 chromosome I, complete sequence	Vibrio parahaemolyticus	40.1	40.1	100%	1.6	100.00%	3491101	CP034305.1
Vibrio parahaemolyticus strain 20150303005-1 chromosome I, complete sequence	Vibrio parahaemolyticus	40.1	40.1	100%	1.6	100.00%	3483160	CP034296.1
Vibrio parahaemolyticus strain 20140829008-1 chromosome I, complete sequence	Vibrio parahaemolyticus	40.1	40.1	100%	1.6	100.00%	3358530	CP034294.1
Vibrio parahaemolyticus strain 20140722001-1 chromosome I, complete sequence	Vibrio parahaemolyticus	40.1	40.1	100%	1.6	100.00%	3421899	CP034289.1
Vibrio parahaemolyticus strain 20140624012-1 chromosome I, complete sequence	Vibrio parahaemolyticus	40.1	40.1	100%	1.6	100.00%	3338799	CP034285.1
Vibrio parahaemolyticus strain 2012AW-0224 chromosome 1, complete sequence	Vibrio parahaemolyticus	40.1	40.1	100%	1.6	100.00%	3535345	CP046831.1
Vibrio parahaemolyticus strain 2012V-1106 chromosome 1, complete sequence	Vibrio parahaemolyticus	40.1	40.1	100%	1.6	100.00%	3311269	CP046828.1
Vibrio parahaemolyticus strain 2013V-1244 chromosome 1, complete sequence	Vibrio parahaemolyticus	40.1	40.1	100%	1.6	100.00%	3276195	CP046782.1
Vibrio parahaemolyticus strain 2014V-1125 chromosome 1, complete sequence	Vibrio parahaemolyticus	40.1	40.1	100%	1.6	100.00%	3323757	CP046778.1
Vibrio parahaemolyticus strain 2012V-1146 chromosome 1, complete sequence	Vibrio parahaemolyticus	40.1	40.1	100%	1.6	100.00%	3343366	CP046808.1
Vibrio parahaemolyticus strain 2013V-1136 chromosome 1, complete sequence	Vibrio parahaemolyticus	40.1	40.1	100%	1.6	100.00%	3397332	CP046785.1
Vibrio parahaemolyticus strain 2013V-1181 chromosome 1, complete sequence	Vibrio parahaemolyticus	40.1	40.1	100%	1.6	100.00%	3418724	CP046783.1
Vibrio parahaemolyticus strain 2012AW-0353 chromosome 1, complete sequence	Vibrio parahaemolyticus	40.1	40.1	100%	1.6	100.00%	3332037	CP046763.1
Vibrio parahaemolyticus strain 2014V-1056 chromosome 1, complete sequence	Vibrio parahaemolyticus	40.1	40.1	100%	1.6	100.00%	3323763	CP046779.1
Vibrio parahaemolyticus strain AM51552 chromosome 1, complete sequence	Vibrio parahaemolyticus	40.1	40.1	100%	1.6	100.00%	3293962	CP046760.1

Figure 14: *BLAST-N search with blaCARBf1 oligo (A) and Pbp1Bf2 oligo (B) to demonstrate the 100% similarity with 100% cover of Ch-2 (A) and Ch-1(B) of V. parahaemolyticus. The result was similar with blaCARBr1 and Pbp1Br2 oligos (data not shown). Part of the Blast data was shown in both cases.*

The *V. cholerae* strain 116-17a plasmids pNDM-116-17 and pNDM-116-14 (accession nos. LN831185 and LN831184) also contained the blaNDM-1 gene to inactivate the all penicillin drugs. The blaNDM-1 gene was found in most bacterial plasmids and also in *E. coli, P. mirabilis* and *Providencia sp*ecies chromosomes (accession nos. CP053614, CP042861 and CP013483). A blaPSE4-type beta-lactamase gene located in shrimp-derived *V. parahaemolyticus* (protein id. NMT93259) and similar gene was found in Oyster-derived *V. parahaemolyticus* genomic fragments (Accession numbers: DACQME010000048, ABFJXO0000001 and AAXOFK010000086) with similar protein sequence (protein ids. HAS686330, EIZ0308401 and EGR348697) and isolated from United States in 2008, 2021 and 2013 respectively. The gene gave resistant to penicillin-G drug (see, accession no. SRKW010000001, nt. 140681-141532). However, blaTEM-1 beta-lactamase-containing plasmid pVSP43 was found in *V. parahaemolyticus* which was also isolated from shrimp (see, Figure 2C for plasmid structure).

The *Salmonella enterica* small plasmid-borne (2788bp; acc. no. KY399740) *mdr* genes also investigated (Figure 5). The bacterium was isolated from shrimp fish in China. The blaOXA gene cleaves oxacillin more efficiently than ampicillin. The catB3 gene acetylates chloramphenicol and acetylated drug does not able to bind ribosome to inhibit bacterial protein synthesis. The arr3 gene ribosylates rifampicin and ribosylated rifampicin does not able to bind RNA polymerase to inhibit transcription. The shrimp fish isolated *V. parahaemolyticus* showed resistant to as high as 5-12 antibiotics. The blaOXA-1 was found very similar to *Klebsiella pneumoniae*, *Salmonella enterica* and *Shigella flexneri* chromosomal genes (accession nos. DAGOGD010000073, CP034250 and ABGERN010000356) and could be used for *V. parahaemolyticus* diagnostic PCR [64]. We compared the CatB3 protein of *Salmonella* plasmid with chromosomal catC1 protein of *V. parahaemolyticus* showing divergence and useful for primers design and diagnostics PCR (Table 1).

We looked *V. parahaemolyticus* genomic fragments and found blaCARB gene (Figure 6A) and penicillin binding protein gene

(accession no. SRKW01000006, nucleotides 109851-112223; protein id. WP_0220835404) (Figure 6B). To access the heterogeneities among the conventional blaTEM-1, blaSHV-1 and blaOXA-1 enzymes as compared to blaCARB-1, we multialigneg the corresponding proteins and found strong differences that would be useful for primer design for *V. parahaemolyticus* (see, Figure 7A for antibiotics and corresponding genes developed naturally in bacterial plasmids and see, Figure 7B for multi-alignment data of beta-lactamases). So, blaCARB-1 was distinct and generated due to chromosomal rearrangement of *V. parahaemolyticus*. We also found few MDR transporters (protein ids. WP_025788558, WP_011106254) as well as MacB transporter (protein id. WP_025594350) pinpointing the multidrug resistance for penicillin (ampicillin) and macrolide (erythromycin) antibiotics in *V. parahaemolyticus*. Further, we detected few rRNA methyl transferasees in *V. parahaemolyticus* and *V. Cholerae*. As for example, 23S rRNA ^{2552}Uridine 2'-O methyltransferase (RlmE; WP_015297227), 23S rRNA ^{1939}Uridine C_5-methyltransferase (WP_025789428) as well as 16S rRNA 16S rRNA 1207-Guanosine N_2-methyl transferase (RsmC; WP_005479074), 16S rRNA ^{1518}Adenine-^{1519}Adenine-N_6-di-methyltransferase (RsmA; WP_005459622) and23S rRNA ^{2498}Cytidine 2'-O-methyltransferase (RlmM; MBE5158644). These rRNA methyltransferases may give resistance to drugs that binds ribosome (composed of 50 ribosomal proteins plus 23S, 16S and 5S RNAs) inhibiting protein synthesis of bacteria. Thus, we pinpointed the mechanism of multi-drug resistance in *Vibrio* species which seriously infected shrimp and other fishes in aquaculture and located in chromosome. Such mechanism appeared primitive as very few plasmids so far detected in *V. parahaemolyticus* (Figure 8).

Toxin genes in shrimp-contaminated bacteria

The PirA and PirB toxin genes located in many *Vibrio parahaemolyticus* conjugative plasmids and cause acute hepatopancreatic necrosis disease (AHPND) in shrimp [65,66]. As for examples, pirA (QHH18415) and pirB (QHH18416) located in plasmid pVPGX2015-2; pirA (QHH13410) and pirB (QHH13411) in plasmid pVPSD2016-5; pirA (AWG82359) and pirB (AWG82360) in plasmid pVpR14_74Kb; pirA (UJX11662) and pirB (UJX11663) in plasmid pVP17-1; pirA (QHH02797) and pirB (QHH02798) in plasmid pVPCZ2014-3 as well as pirA (QGT94608) and pirB (QGT94609) in plasmid pVP_Kor-D1-2 were well documented in NCBI GenBank Database. The similarly *Vibrio owensii* plasmid pVHvo has pirA (QGH51089) and pirB (QGH51090) proteins as well as plasmid pVa1 (accession number: CP097860) of *V. parahaemolyticus* (Figure 9). *Vibrio campbellii* strain LMB29 also has PirAB toxin genes in plasmid pVCON1 (accession number: MH890610) as well as in plasmid pVCGX1 (accession number: CP020078) but no *tdh* and *trh* virulence genes [67]. Interestingly, no *mdr* genes located in pVA1, pVPE619, pVa, pVp_kor-D-1-2, pVHvo, pVPGD2014-1/2/3 and pVPGX2015-2 PirAB gene containing conjugative plasmids (accession numbers: KP324996, CP043423, CP034288, CP034293, CP034297, CP034308, AP014860 and KX268305). The *Vibrio parahaemolyticus* chromosomes were fully sequenced. As for examples, The PirAB genes located in Ch-1 from nt. 1276780-3063548 (accession number: CP034294, length 3358530bp). Such genome wide data suggested nt. 2500951-3163071 contained pirAB genes in accession number CP046831 and nt.2605940-2871943 in accession number CP028342 and also nt. 2605305-2871943 in accession number CP028341 of different *V. parahaemolyticus* strains [68,69].

Virulence genes in aquaculture fish ponds

The virulence genes *tdh* and *trh* were detected in two *V. parahaemolyticus* shrimp isolates from the Cochin estuary by multiplex PCR. Using 16S rRNA sequence analysis, one isolate exhibited 100 % similarity to the *V. parahaemolyticus* O3:K6 pandemic clone. TDH is a pore forming toxin and forms pores of ~2nm in diameter on erythrocyte membrane. These thermostable haemolysin-like proteins exert a variety of biological activities like haemolytic activity, enterotoxicity, cardiotoxicity and cytotoxicity. The *trh* and *tdh* genes share 70% homology and both proteins activate chloride channel. The TDH virulence factor is composed of four soluble monomers, in which a central pore is formed to allow the diffusion of small molecules, known as Kanagawa phenomenon (KP) (Figure 10). The Tdh^+ strains of *V. parahaemolyticus* exhibit β-haemolytic activity when plated on blood-agar media known as Wagatsuma agar. Purified TDH is heat stable at 100°C for 10 min [70]. The TDH and TRH proteins cause haemolysis, enterotoxicity, cytotoxicity and cardiotoxicity in experimental animals. The TRH protein also causes haemolytic activity similar to that of TDH on blood cells (Figure 11). Moreover, TRH activates Cl^- channels and causes altered ion influx, in a manner analogous to TDH [71,72]. Thus, many disease-related reports available due to sea food contamination of *Vibrio* species. An Indian study reported that 178 *V. parahaemolyticus* strains were isolated from 13,607 diarrheal patients admitted in Infectious Diseases Hospital in Kolkata (India) since 2001-2012 [73]. TDH and TRH proteins have overall 60% sequence similarities although perform very similar cellular tropism (Figure 12).

The *tdh* gene was located in chromosome-2 of *V. parahaemolyticus* (accession number: CP003973). It appeared that two copies of *tdh* gene in ch-2 (nt.1391390-1391959 and nt.1334189-1334758) with 97-99% similarities. A copy of *tdh* gene also found in Ch-1 of *V. haemolytica* (accession no. CP046785; nt. 1945873-1946442with 94% similarity). The *tdh* gene was also located in pFPPDNB1-3 plasmid of *Photobacterium damselae* strain KC-Na-NB1 with 89%

similarity (accession number: CP03546). However, all *V. parahaemolyticus* chromosomes had no *tdh* or *trh* virulence genes as described above for chromosomes with PirAB genes (accession numbers: CP028342, CP046831 and CP034294).

The *trh* gene was located in Ch-2 of *V. parahaemolyticus* (nt. 1271013-1271582; accession number: CP066247) and also located in Ch-1 (nt.2504989-2505558; accession number: CP035701) and appeared a single copy gene found in both chromosomes. Such gene also located in plasmid pTJ187-3 (86kb) of *V. parahaemolyticus* strain TJ-187 with 94% similarity (nt. 3037-3606; accession number: CP068651). BLAST-2 sequence analysis showed about 73% homology between *tdh* and *trh* genes and a divergence was found at the 5'-terminal first 60 nucleotides. We also located large Nuraminidase toxin Tc-A in plasmid pva1 of *V. parahaemolyticus* GL601 (Figure 13).

Primer design for blaCARB-1, pbp1B and catC1 genes of V. parahaemolyticus

After the characterization of all described genes to find similarities with other bacterial genes, we came to conclusion that blaCARB-1, pbp1B and catC1 genes were very specific for *Vibrio parahaemolyticus*. We used NCBI Primers design software to make PCR primers for three chromosomal genes as described in (Table 1). There were ten primers selected each and we analysed the hairpin structure, dimer formation to choose one only that gave at least 100-200 *V. parahaemolyticus* genomic sequences with 100% similarity and 100% cover (Figure 14). The primers for *pirAB, tdh, trh* and *tlh* genes were already reported (see, Material and Methods). No other plasmid or genomic sequence had higher than 75% similarity and cover. Thus, we described many *mdr* genes, *toxin* genes and *virulence* genes in plasmids and chromosomes of *Vibrio parahaemolyticus* that caused acute destruction in shrimp aquaculture. We also found few genomic primers for identification purposes. Such article was lacking in the PubMed Database.

Discussion

We clearly described the occurrence of *mdr* genes in *Vibrio* species in aqua shrimp fish culture (Figure 3 and Figure 4). The blaCARB-1 gene was distinctly located in only *V. parahaemolyticus* genome and had profound difference with blaTEM-1, blaSHV-1 as well as blaOXA-1 genes located in bacterial plasmids of *E. coli, K. pneumoniae* and *A. baumannii* etc. (Figure 7). Das et al have isolated many *Vibrio spp*. Including *V. alginolyticus, V. parahaemolyticus, V. cholerae, V. mimicus,* and *V. fluvialis* along with *Aeromonas hydrophila*, *Aeromonas salmonicida* and *Salmonella enterica* from the shrimp cultures on TCBS medium. The *V. alginolyticus* was found to be the most resistant isolate by showing multiple antibiotic resistance (MAR) index of 0.60 followed by *V. mimicus* (0.54) and *V. parahaemolyticus* (0.42) [74]. The *V. alginolyticus* plasmid pVAS19 contained many *mdr* genes like blaPER-1, sul1, catB3, aac6'-Ia, tetB, Qnr1, dhfr and ANT3" (accession no. KX957968) [75].

Tendencia et al. described that most of the bacteria isolated from pond were *Vibrio harveyi* and resistant to at least two antimicrobials like oxolinic acid (24%) and penicillin G (19%) and rest by varying percentages to chlorotetracycline, ciprofloxacin, erythtromycin, gentamycin, neomycin, nitrofurazole, ofloxacin, oxytetracycline, polymyxin B, rifampicin, streptomycin, sulphamethezole, and sulphafurazole [76,77]. *Pan J et al described that Vibrio vulnificus* gram-negative bacterium found in scrimp ponds of China that were resistant or intermediate resistant to cefepime (3.03%), tetracycline (6%), aztreonam (24%), streptomycin (45%), gentamicin (94%), tobramycin (100%), and cefazolin (100%) [78,79].

Babu et al. described that in East Indian shrimp ponds mainly contaminated Enterocytozoon hepatopenaei (EHP) and *V. parahaemolyticus*. In this study, *V. parahaemolyticus* isolated from *L. vannamei* was sensitive to chloramphenicol and oxytetracycline but resistant to erythromycin and nalidixic acid. Interestingly, White Spot Syndrome Virus (WSSV) was also frequently observed with trace amount of Infectious Hematopoietic Necrosis Virus (IHNV) and Monodon type baculovirus [80]. *Yano et al described that isolated V. parahaemolyticus* had higher affinity for non-native white-leg scrimp fish than for native black-tiger shrimp. Such bacteria were resistance to ampicillin, oxytetracycline and nalidixic acid [81]. The bacteria like *Acinetobacter, Achromobacter* and *Alcaligenes* were also isolated from ponds, those were currently using Oxacillin antibiotic in aquaculture indicating such bacteria acquired large conjugative MDR plasmids [70]. Such bacterial contamination and virus infections are serious threat in shrimp aquaculture. Based on baseline and unusual mortality in tilapia fishes in Bangladesh, a total loss of 875.7 million USD annually was occurred recently [82].

Haifa-Haryani et al. isolated many Vibrios from cultured shrimp in Peninsular Malaysia and plasmids (<10kb) were detected. These bacteria were characterized based on *pyrH* gene [83]. The populations of different Vibrios were detected as follows: *V. parahaemolyticus* (55%), *V. communis* (9%), *V. campbellii* (8%), *V. owensii* (7%), *V. rotiferianus* (5%), *V. cholerae* (4%), *V. alginolyticus* (3%), *V. brasiliensis* (2%), *V. natriegens* (2%), *V. xuii* (1%), *V. harveyi* (1%) and *V. hepatarius* (0.4%). Antibiotic susceptibility profiles revealed that all isolates were resistant to penicillin G (100%), but susceptible to norfloxacin (96%). The *V. haemolyticus* strain V22G1 was resistant to twelve antibiotics comprising ampicillin, chloramphenicol, gentamycin, kanamycin, cefotaxime, ceftazidime, cephalothin, nitrofurantoin, sulfometioozone-trimethoprim, erythromycin, vancomycin and

penicillin G with MAR index as high as 0.75. The MAR index of the isolates from the Cochin estuary ranged from 0.31 to 0.75 and that from the shrimp farm ranged from 0.19 to 0.5 (see, plasmids pVAS19 and pVPS43) (Figure 3). Mercury reductase and other *mer* genes located in *V. parahaemolyticus* plasmids [84].

Viral diseases were also hampered the shrimp aquaculture. In India, Penaeus monodon, black tiger shrimp was previously the foremost-cultivated shrimp species but the American white leg shrimp *Litopenaeus vannamei* has effectively replaced it. The White spot syndrome virus (WSSV), Hepatopancreatic parvovirus (HPV), Monodon baculovirus (MBV) and Infectious hypodermal and hematopoietic necrosis virus (IHHNV) are the other significant infectious agents of *P. monodon* and *L. vannamei*. A more recent disease of *L. vannamei* in India is monodon slow growth syndrome (MSGS), a component of which seems to be Laem-Singh virus (LSNV) [85,86].

The *pirAB* toxin genes as well as *tdh*, *trh* and *tlh* virulence genes primers were used to detect *V. parahaemolyticus* in shrimp fish. We added few chromosomal *mdr* genes primers for the detection of *V. parahaemolyticus* (table-1) [87]. Drug sensitivity test will be performed carefully to address the spread of *mdr* genes in fish aquaculture. There is hope that phyto-antibiotics may be utilized to avert the multi-resistance [88]. We have to be careful to add excessive antibiotics into aquaculture as scientists predicted that such process accelerating mdr genes spraed in the environment and as high as 10million people may die due to AMR in 2050.

Conclusion

Vibrio species are a group of bacteria naturally found in freshwater, estuaries and marine environments and are responsible for numerous human diseases attributed to the natural microbiota of aquatic environments and seafood. Globally, about 59.51 million people were associated with fishing or aquaculture. Fish is an important and significant source of animal protein for 4.5 billion people who rely on them. The *V. parahaemolyticus, was* serious food-borne pathogens and highly found in shrimp aquaculture with high molecular weight plasmids giving multi-drugs resistance. We designed chromosomal genes (blaCARB-1, pbp1B and catC1) specific primers for the detection of *V. parahaemolyticus*. The international aquaculture expansion and expanding global trade of shrimp have been accompanied by long distance geographical redistribution spreading many bacteria and animal viruses. The shrimp fish marketed to Europe and America from West Bengal. Thus, spread of *mdr* genes must be studied in fish aquaculture. We foresee new plant-based remedies for shrimp mortality control and to increase shrimp fish trade.

Acknowledgement

We thank Prof Bidhuyt Bandhopadhyay, Principal of OIST for his interest in aquaculture research. We also thank NCBI, NIH for free Database and BLAST search engine. Uttam Maity is QC Biotechnologist at Jana Brothers Seafood LLP, Digha (Email: uttam.uv703@gmail.com).

Competing interest

The authors declared no conflict of interest to any agency.

Ethical issues

No animal and no human was used in this study.

Funding

No funding was obtained from any Government agency.

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