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Research Article

Characterization of Chromosomal VmeAB, MacAB and EmrD Multidrug-Efflux Genes of *Vibrio parahaemolyticus* to Design Diagnostic PCR Primers to Improve Associated Problems in Shrimp Aquaculture

Asit Kumar Chakraborty*, Uttam Maity and Chandan Halder

Department of Biotechnology and Biochemistry, Oriental Institute of Science and Technology-West Bengal, Vidyasagar University, India

***Corresponding Author:** MAsit Kumar Chakraborty, Department of Biotechnology and Biochemistry, Oriental Institute of Science and Technology-West Bengal, Vidyasagar University, India. Email: chakraakc@gmail.com.

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Abstract

Vibrio species contamination in fish is a serious threat to human population worldwide. Aquaculture of shrimp has increased in India due to high demand in the Europe and America. Previously, we reported the presence of unique chromosomal blaCARB, pbp1B and CatC1 *mdr* genes involved in multi-resistance of *Vibrio parahaemolyticus* (Vp) and useful for diagnostic primers design. *Escherichia coli* AcrA-AcrB-TolC tripartite multidrug efflux genes were related to MexA-MexB-OprM genes of *Pseudomonas aeruginosa* and OqxAB-TolC genes of *Klebsiella pneumoniae*. Here, we showed that VmeAB and VmeYZ related to acrAB or mexAB as well as MacAB drug-efflux genes might be also responsible for multidrug resistance in Vp. Many heterogeneous Vme-isomers like vmeAB, vmeCD, vmeEF, vmeHI, vmeJK located in Ch-1 whereas vmeHI and vmeCD located in the complement strand (accession no. CP034294). The Ch-2 of Vp (accession no. CP020428) contained vmeYZ, vmePQ, vmeUV (RND-type) as well as macAB (ABC-type), EmrD (MFS-type) and mdtL (MATE-type) drug efflux genes. BLAST-2 analysis suggested only vmeB and vmeZ had similarities over 40% to acrB, mexB or oqxB types popular bacterial RND permease subunits. The macB protein of Vp has 56% similarity to *E. coli* macB protein and within the related *Vibrio* species (*V. harveyi* and *V. alginolyticus*) the similarity found to be round 98%. The Vp EmrD protein has 41.49% homology to *E. coli* while *V. cholerae* protein has only 29% homology. The MdtL protein of Vp has 61% similarity to *E. coli* and not suitable for primer design. We designed the primers using NCBI Primer Design Software and oligonucleotides were validated by Oligo Analyzer Software 3.2. Multi-alignment of vme-related sequences done by CLUSTAL-Omega software to confirm heterogeneities and BLASTN search of primers confirmed specificities to Vp Ch-1 or Ch-2. The vmeBF2/vmeBR2 primers for VmeB gene and macBF2'/macBR2 primers for MacB gene should be useful with good species specificity. Such primers will be useful to detect *V. parahaemolyticus* contamination in fish aquaculture.

Keywords: Multidrug Efflux Genes; Diagnostic PCR Primers; *Vibrio parahaemolyticus*; Shrimp Aquaculture; Multidrug Resistance; acrAB and mexAB, macAB and emrCD

Abbreviations

Vp: *Vibrio parahaemolyticus*; Vc: *Vibrio cholerae*; Ec: *Escherichia coli*; RND: Resistance Nodulation Cell Division Family Protein; MFS: Major Facilitator Superfamily Transporters; MATE: Multidrug And Toxic Compound Extrusion; SMR: Small Multidrug Resistance Proteins; AcrAB: Acriflavine Resistant Transporters

Introduction

MDR genes were isolated from thousand of plasmids and genomes of *Escherichia coli, Klebsiella pneumoniae, Pseudomonas aeruginosa, Salmonella enterica* and *Acinetobacter baumannii* bacteria and related species [1-3]. However, drug efflux genes like acrAB/CD, MacAB, MexAB/CD/EF were well known drug efflux genes to give multi-resistance in those bacteria. Five major groups of drug efflux transporter families that have currently been identified as follows: the RND (Resistance Nodulation cell Division) family, MFS (Major Facilitator Superfamily) family, MATE (Multidrug And Toxic-compound Extrusion) family, SMR (Small Multidrug Resistance) family, and ABC (ATP Binding Cassette) family [4-8]. The AcrA-AcrB-TolC system in *Escherichia coli* belongs to the RND (resistance-nodulation-cell division)-type transporter family and is composed of an inner membrane transporter (AcrB), a periplasmic membrane fusion protein (acrA) and an adaptor outer protein (TolC) [9-13]. Among the three proteins in *Pseudomonas aeruginosa*, the inner membrane transporter protein called mexB or mexD, periplasmic membrane fusion protein called mexA or mexC and outer membrane protein called OprM or OprJ and those proteins were well characterized [14-20]. The macAB genes were MFS-type and very specifically gave resistance to macrolide antibiotics [21-24].

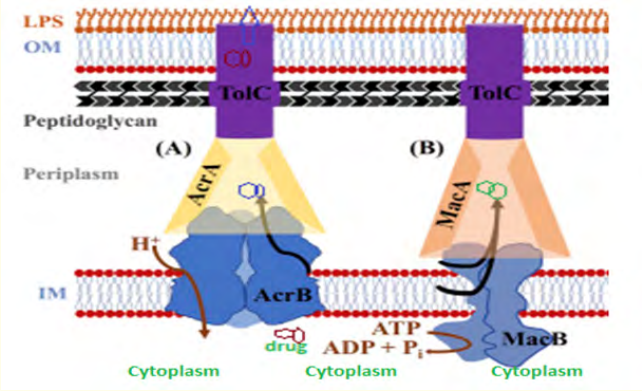


Figure 1: Mechanism of drug efflux from bacterial cytoplasm to outside by acrA-acrB-TolC proteins as well as macA-macB-TolC proteins located in the membrane of *E. coli* (www. google.com). The hypothesis suggested trimeric AcrB bound to hexameric AcrA which in turn bound to trimeric TolC making a channel to remove drugs from bacterial cytoplasm pertaining drug resistance.

The genome sequences of *V. parahaemolyticus* RIMD2210633 have been completely determined in 2003 [25]. The genome consisted of two circular chromosomes of 3288558 bp and 1877212 bp; it contained 4832 genes [26-28]. Extensive genome sequencing identified many multidrug resistant RND-type genes since then including vmeAB, vmeCD, vmeEF and vmeIJ in Ch-1 and vmePQ, vmeUV and vmeYZ were located in Ch-2 [29-31]. Two RND-type drugefflux transporters of *Vibrio cholerae*, vexAB and vexCD, have previously been reported and vexB has only 25% similarities to vexD protein [32]. The vexB in Ch-1 of *V. cholerae* O1 biovar El Tor (protein id. KAE8432050; 1036 AA; Accession no. JPLT02000019, nt. 3399-6509 as well as protein id. KAE8425006; 1036 AA; Accession no. LUCN02000011, nt. 159963-163073) and vexD RND permease drug transporter protein (protein id. BAF66267; 1016 AA; Accession no. AB306973, nt. 1104-4154 and protein id. EMP97711; 1016 AA; Accession no. APFM01000059, nt. 154133-157183) were identified. There were few amino acids changes between El Tor vs. non-El Tor V. chalerae vexD genes (R122C, A128T, A241V, E420D, T487I, V577I, T585A, I781V, M805T and A940E). VexA of Vc El Tor has 30.36% homology to vmeB of Vp, 88.42% strong homology to vmeD, 35.25% to vmeF and 22.56% to vmeK whereas VexD of Vc has only 24% similarity to vmeB of Vp, 25.38% to vmeD, 23.39% to vmeF and 23.65% to vmeK (table 1). However, there were much divergent between *V. parahaemolyticus* with *V. cholerae* chromosomal sequences than *V. alginolyticus* or *V. harveyi* [25].

The vmeAB, vmeCD, vmeEF, vmeHI, vmeJK genes located in Ch-1 of *V. parahaemolyticus*. The Vp Ch-1 (accession no. CP068627; 3422475bp) contained vmeK (protein id. UJW94790; nt. 659964-663095), vmeI (protein id. UJW93156; nt. 2138965-2142066 complement), vmeB (protein id. UJW93237; nt. 2225301-2228462 complement) and vmeF (protein id. UJW93380; nt. 2407557-2410670 complement) including their vmeJ, vmeH, vmeA and vmeE acrA-like partners. The TolC adapter for the tripartite acrA-acrB-TolC-like complex was located in Ch-1 (protein id. UJW95635; nt. 1554301-1555716 complement) but a different TolC-like protein located also in Ch-2 (Figure-2A/B). The details BLAST-2 similarities were presented in table 1. Three penicillin-binding proteins in Ch-1 of Vp were reported: (1) pbp2 (protein id. UJW93576, nt. 2632970-2634895) (2) pbp1B (protein id. UJW94765, nt. 621291-623663) and (3) pbp1A (protein id. UJW94527, nt. 332602-335172 complement) as well as tet(35) tetracycline resistant protein

(protein id. UJW93198, nt.2184146-2185747 complement). Although there is a <30% similarity between pbp1A and pbp1b, very little homology was detected between pbp1A/B and pbp2. Previously, we reported the diagnostic primers for Vp using pbp1B located in Ch-1 whereas blaVERB and CatC1 genes also used for diagnostic primers located in the Ch-2 (Chakraborty., *et al*. 2023, Suntext Review in Biotechnology, in press). Such search was conducted because plasmids bearing pirAB and trh toxin genes did not contained *mdr* genes and only few plasmids were isolated from *V. parahaemolyticus* and *mdr* genes of such plasmids had 100% similarities to Gamaproteobacteria plasmids being unsuitable for diagnostic primer design. We elegantly characterized VmeAB-related genes (RND-type), macAB (ABC-type) and EmrD (MFS-type) as well as mdtL (MATE-Type) multidrug efflux genes in *V. parahaemolyticus* Ch-1 and Ch-2. We designed two sets of primers of those drug efflux genes and demonstrated their specificity to Vp genome. However, as the vme-related genes diverged significantly, many more primers could be designed easily (Table 1).

Vme-Vp	acrB-Ec	mexB-Pa	mexD-Pa	mexF-Pa	vexB-Vc	oqxB-Kp
vmeB	63.72/98	61.03/100	46.43/98	43.53/99	30.63/96	40.93/99
vmeD	31.57/99	31.84/97	29.71/99	33.27/99	88.42/100	31.08/99
vmeF	29.87/97	30.03/98	29.76/99	31.36/97	35.27/99	29.68/98
vmeK	23.66/97	22.39/96	22.48/97	23.06/96	22.56/94	21.81/98
vmeI	23.45/97	23.50/97	22.13/97	22.90/97	24.27/96	22.31/98
vmeV	24.17/99	24.22/98	23.49/99	23.28/99	23.37/96	23.74/98
vmeZ	40.49/96	41.41/98	41.02/98	44.08/99	23.37/96	42.45/98
Vp=*V. parahaemolyticus*; Ec=*E. coli*; Pa=*P. aerugi-nosa*; Vc=*V. cholerae*; Kp=*K. pneumoniae*; Vme=*Vibrio Multidrug Efflux*						

Table 1: BLAST-2 homologies of different RND drug efflux proteins with *Vibrio parahaemolyticus* vmeB/D/F/K/I proteins of Ch-1 and vmeQ/V/Z proteins of Ch-2 (% of Similarity/% of Cover).

Methods

V. parahaemolyticus cultivation and characterization

Vibrio parahaemolyticus was grown in TCBS medium. In TCBS-Agar plate *V. parahaemolyticus* caused green colonies, *V. cholerae* caused red colonies and *V. owensii* caused yellow colonies. 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 [34]. Genomic DNA was isolated using Proteinase-Phenol-chloroform method and used for PCR amplification according to Manufacturer's protocol [35]. The Vme drug efflux genes PCR amplification and sequencing were described previously (Matsuo T., *et al*. 2013) [29]. The primers were made as follows: VmeCDf 5'-CAC CAG GAT CCA ATT ATC AAA CAC TAA CTT G-3'; VmeCDr 5'-AAA GGA TCC TCG CCA TTT AGA TGG TAA AA-3'; VmeEFf 5'- CAG GGG ATC CAG TTT AAT GAC ATA AGT TT-3'; VmeEFr 5'- CCG AGG ATC CTA GAA ATA TAA AAA AAC GCC-3'; VmeJKf 5'- GAG AGG ATC CAG GAG AGA ATA ATA AAA AGG-3'; VmeJKr 5'-AGA GGG ATC CAA TGA GAT AAA CGG AAA AGT-3' [29].

The acrAB, mexAB, macAB genes were selected from GenBank. *Vibrio parahaemolyticus* vmeAB/CD/EF genes were also selected from NCBI Database (www.ncbi.nlm.nih.gov/nucleotide). The WGS of *V. parahaemolyticus* also selected from NCBI database including genomic fragments which were easy to investigate. The related species like *Vibrio cholerae* multidrug efflux genes designated as vex-related genes but we also found acrAB-related genes. Other *Vibrio* species (*V. herveyi* and *V. algenolyticus*) there were vme-

related knowmwnclature but also acrAB-types nomenclature and we have to confirm always by BLAST-2 search with acrB and mexB to confirm such classification as reported in table 1. The map of the two chromosomes of Vp for drug efflux genes were given in figure 2A and figure 2B. Vp specific PirAB toxin genes PCR assays were described previously [36]. Similarly, tdh/trh toxin genes PCR assays were developed to detect Vp in shrimp fish [37].

Ethidium drug efflux assay

V. parahaemolyticus cells were grown in LB broth supplemented with 40 mmol/L potassium lactate, and washed twice with Medium S (50 mmol/L Tris-HCl, 200 mmol/L NaCl, 25 mmol/L $MgSO_4$, 10 mmol/L $(NH_4)_2SO_4$, 10 mmol/L KCl, 1 mmol/L $CaCl_2$, 0.33 mmol/L K_2HPO_4, 10 µmol/L $FeSO_4$, pH 7.5). Cells were incubated in Medium S including 10 µmol/L ethidium bromide and 40 µmol/L CCCP (carbonylcyanide *m*-chlorophenylhydrazone) at 37°C for 1 hr [29]. Cells were harvested and washed twice with the same buffer containing 10 µmol/L of ethidium bromide and Potassium lactate (40 mmol/L) for energization. *E. coli* KAM43 cells reference standard harboring both pBVT3 and either pSVP201 (carrying vmeCD) or pSVP202 (carrying vmeEF) were grown in LB broth supplemented with 100 µg mL^{-1} of ampicillin and 20 µg mL^{-1} of chloramphenicol were also compared [29]. Cells were harvested and washed twice with the same buffer containing 10 µmol/L of ethidium bromide and 2 mmol/L MgSO and Potassium lactate (40 mmol/L). The fluorescence intensity of ethidium was measured at an excitation wavelength of 500 nm and emission wavelength of 580 nm, respectively, using an F-2000 Fluorescence Spectrophotometer (Hitachi, Ltd., Chiyoda-ku, Tokyo, Japan). How the three proteins (AcrA, AcrB, TolC) kick off drugs from bacterial cytoplasm was demonstrated in figure 1. It was postulated that 3 molecules acrB, 6 molecules of acrA and 3 molecules of TolC were involved in drug efflux process [38-40].

Result

During our initial search of *V. parahaemolyticus* genomic fragments, we got few multidrug efflux genes like VmeAB (accession no. AB251606), MacB (protein id. HCG8624795; 654AA; Accession no. DAHSNT010000001.1, nt. 88014-89978), EmrD (protein id. EGR2894425; Accession no. AAXOBG010000023.1, nt. 48642-49847), MATE (protein id. MQP53919; 460AA; accession no. WHOI01000001.1, nt. 30760-32142). When we BLAST-P

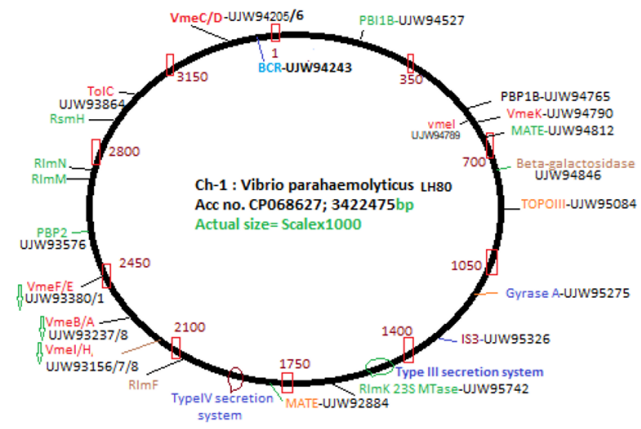


Figure 2A: Map of *V. parahaemolyticus* chromosome-I with respect to multidrug efflux genes and few penicillin binding proteins and 23S/16S rRNA methyltransferases. VmeG has no similarity to TolC. Thus, VmeA-VmeB-TolC might be involved but VmeH-VmeI-VmeG types tripartite drug efflux system similar to AcrA-AcrB-TolC system in E. coli was not established. MATE proteins were also heterogeneous (protein ids: UJW94364, UJW92884, UJW93112). Many heterogeneous MFS and ABC transporters were located in the Ch-1 with diverse functions.

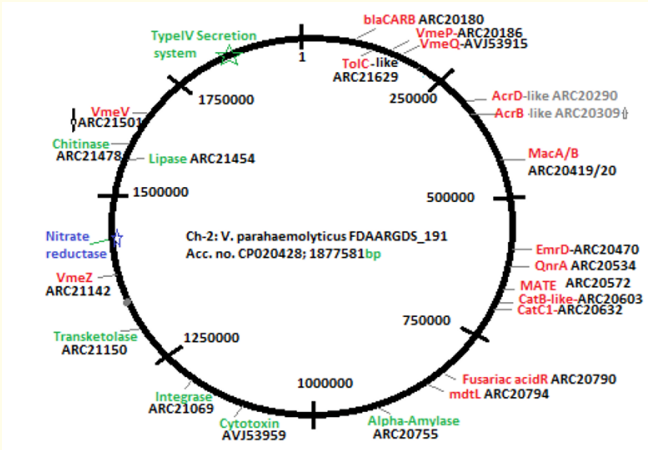


Figure 2B: Genetic map of Ch-2 of *V. parahaemolyticus* drug efflux genes (VmePQ, MacAB, AcrD-like, VmeZ, EmrD, mdtL) and mdr genes (blaCARB, CatC, QnrA) including few house keeping genes (Amylase, transketolase, Alpha Amylase, Nitrate reductase).

searched with those individual protein, we detected many similar genes with 99-100% similarities suggesting such genes were located in *V. parahaemolyticus* genomes. But nucleotide search with VmeB drug efflux gene predicted many vme-related genes (vmeD, vmeC, vmeL, vmeV, vmeZ etc) of *V. parahaemolyticus* isolated by Matsuo T., *et al*. 2013 [29]. Multi-alignment of acrB, mexB, vmeB, vmeD, vmeV, vmeL, vmeZ etc. indicated that only vmeB and vmeZ have good similarities to acrB or mexB genes (Figure 3). In truth, acrB/mexB-related drug efflux genes were located in other Vibrio species like *V. alginolyticus*, *V. harveyi* and *V. cholerae* in the database and essentially such genes named as Vme or Vibrio Multidrug Efflux but such nomenclature also extended to vexB/D genes or acrD/F genes. The acrB gene had 72% homology at certain point with vmeB whereas mexB gene had also similar 71% homology at certain point but higher % homologies at other point suggesting VmeB was more related to mexB (nt. 1525-1547; 96%) of *P. aeruginosa* (Figure 4). But vmeD, vmeF and vmeI had very little homologies with acrB and mexB genes (~22%). Such study also indicated that there was no great homology between vmeB and vmeD as well as other vme-isomers (Table 1). We also found an acrB gene in *V. parahaemolyticus* genome (accession no. AOOY02000001.1, nt. 1889-5002) with essentially no homology to acrB gene of *E. coli* as well as mexB of *P. aeruginosa* implying such gene was not really acrB acridine efflux gene and we designated as acrB-like or acrD-like genes (~22% homology). However, acrB-type gene from *V. harveyi* has 64% homology to acrB protein of *E. coli* (protein id. CAD6014546) suggesting real acrB-related gene and same was true for *V. cholerae* with 48% homology (protein id. SPM18221) and 63% homology with *V. alginolyticus* acrB-related protein (protein id. ARP02750) located in Ch-1. Such analysis clearly indicated that acrB-related gene yet to be identified in the genome search of *V. parahaemolyticus* and analysis found the acrB protein was actually related to vmeF protein (protein id. WP_017634756) reported in the database. Finally, we understood that vmeB was actually acrB of *E. coli* and vmeAB locus located in 674151bp genomic fragment (accession no. DAHRPD010000003) at nucleotides 151668-155971 position and other two distantly related vmeEF was located at nucleotides 288-4495 and vmeHI at nucleotides 237410-241593. Interestingly, the same Ch-1 genomic fragment contained tetracycline resistant tet(35) gene (protein id. HCG5247465) at nt. 194813-196414 position and a MATE multidrug resistant protein (protein id. HCG5247774) at nt. 553649-555019 position. The Tet(34) gene was also located in genomic fragment with accession JABCCN010000005 (protein id. MBE5158622) which also contained a penicillin-binding protein at nt. 175341-177266 (protein id. MBE5158670) and likely we knew that such fragment generated fron Ch-2.

Then, we made maps of Ch-1 (accession no. CP068627) and Ch-2 (accession no. CP020428) (figure-2A and figure-2B) of *V. haemolyticus* to pinpoint the localization of multidrug efflux genes related to VmeAB as well as VmeYZ and MacAB. We had given the protein identification numbers in the map so that one could get the respective proteins for their analysis. There was a problem as all genomes data described partial nomenclature of the multidrug efflux genes. As for example, we reported acrD-like and acrB-like proteins (protein ids. ARC20290, ARC20309; accession no. CP020428) with overall ~22% homologies to vexB, vexD, mexD, vmeZ and acrB genes. However, database reported as AcrB/AcrD/AcrF like gene or sometime wrongly described as Acriflavine resistant protein. In some other sequence acrA-like gene postulated as mexE (protein id.ARC21141) in the adjacent to VmeZ which was reported as hydrophobe efflux-1 family RND transporter. To confirm the vmeZ gene, we had first obtained the gene for vmeZ from database and then Blast-2 searched the Ch-2 sequence to align with >99% homology. The vmeZ had 41% homology to acrB gene of *E. coli* (table 1). Thus, mexE was written as vmeY in few sequences in the database and it had 37% amino acid homology to *P. aeruginosa* mexE protein (protein id. PWU35054) while mexF protein had 44% similarity to VmeZ suggesting VmeZ was very related to mexF of *P. aeruginosa* (table 1). Similarly, TolC-like protein reported in Ch-2 (protein id. ARC21629) with 24% similarity to TolC of *E. coli* while a TolC protein (protein id. UJW95635) was located in Ch-1 of Vp which had 22% sequence similarity. More interestingly both TolC-like proteins had no overall similarities. At the end, we identified a real TolC protein in Vp (protein id. TXM47846) with great homology (47%) to *E. coli* TolC protein. We detected the abundance of such protein in the Vp database (protein ids. WP_161608117, EID0697214, MBE4260963, EHH1171644, HCG8601058) and defined in Ch-1 at 2988025-2989329 (protein id. UJW93864; accession no. CP068627).

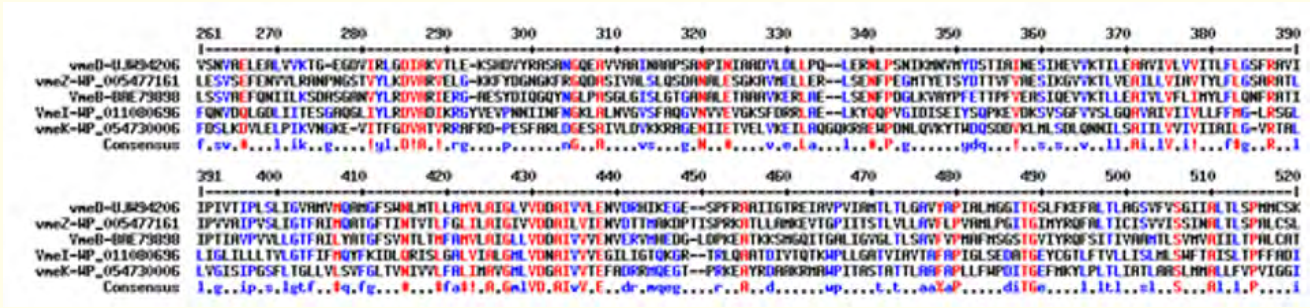


Figure 3: Multi-alignment of different VmeB-related proteins of *V. parahaemolyticus*. The vmeAB was located in Ch-1 as well as vmeCD, vmeEF and vmeHI (accession no. DAHRPD010000003). The vmeZ was located in Ch-2 of Vp. The homology portion was only shown here with red colour.

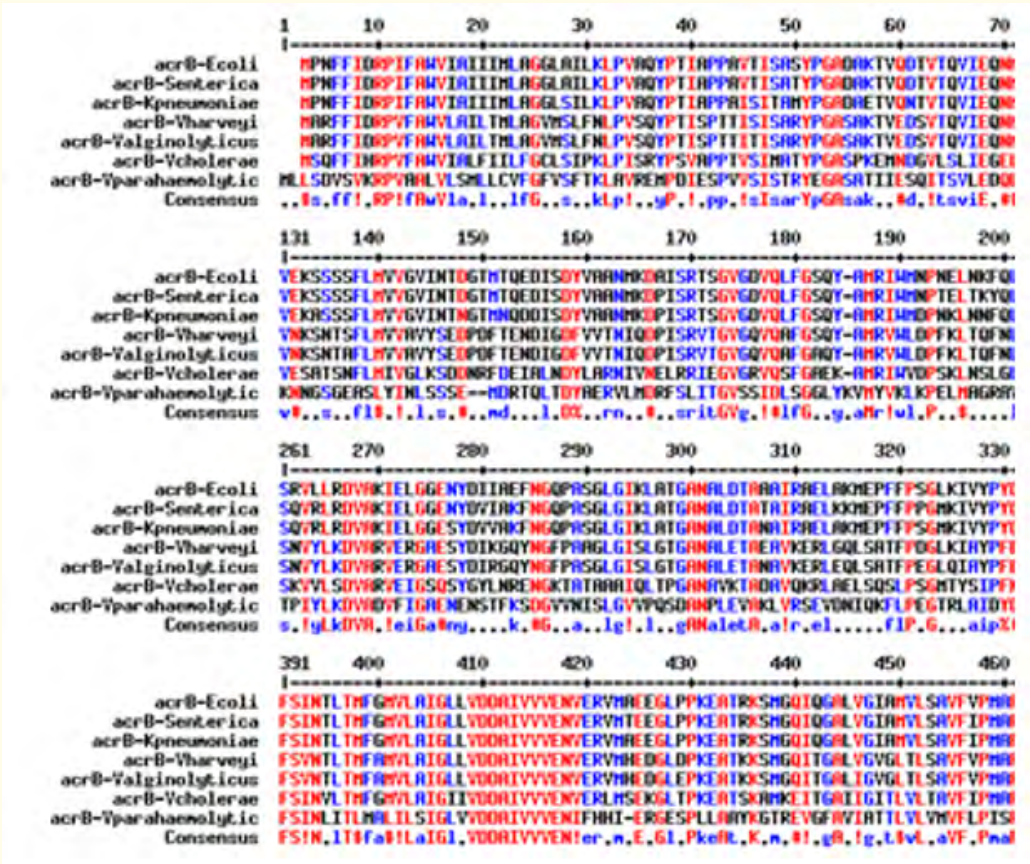


Figure 4: Multi-alignment of acrB and vmeB-related different bacterial drug-efflux proteins showing similarities. Parts of the alignment was shown with highest similarities (red colour).

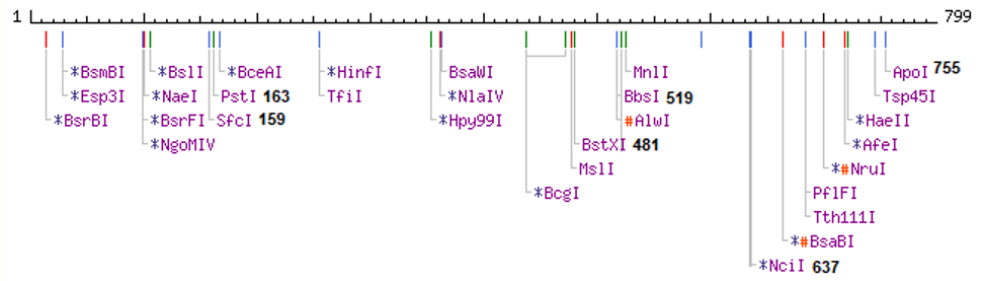
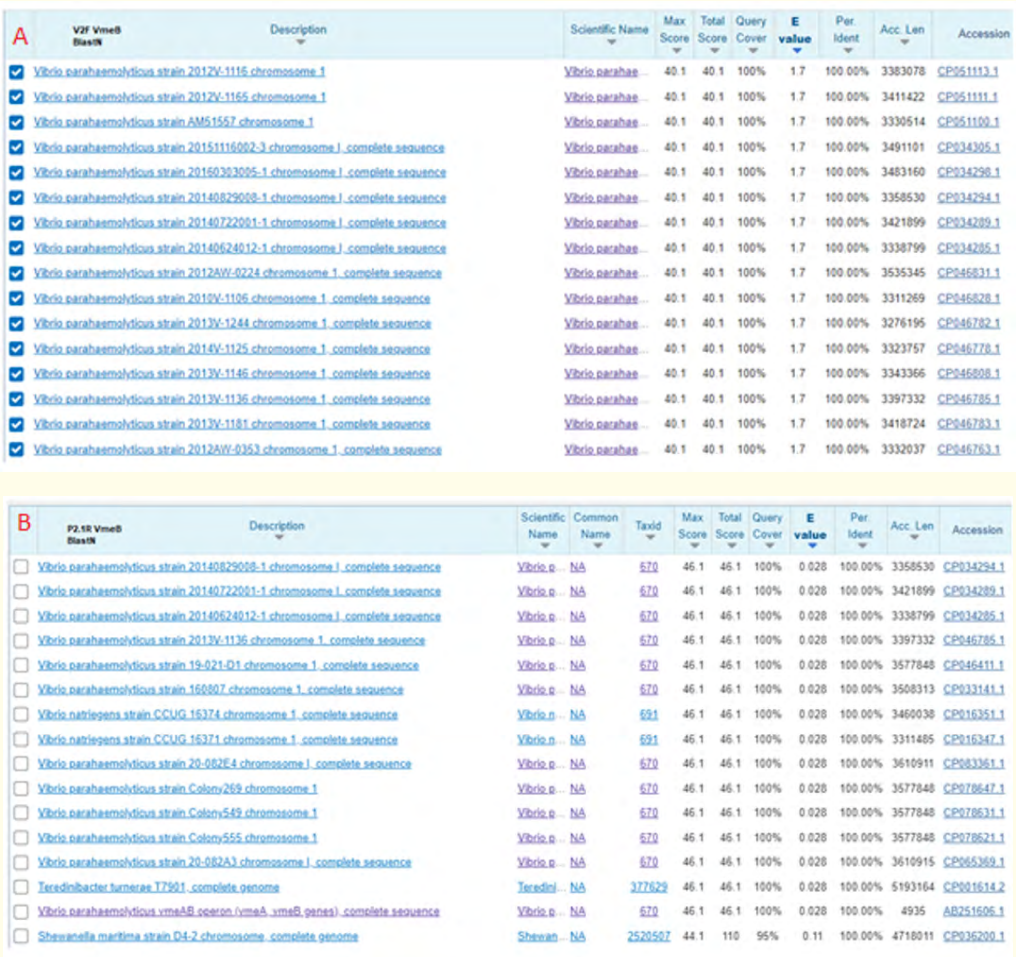


Figure 5: Restrition digeation pattern of PCR fragment of *V. parahaemolyticus* VmeB gene using P2F-vmeB and P2.1R-vmeB primers. Pst1 fragments=163bp, 635bp; BbS1 fragments=279bp, 519bp and NciI fragments=161bp, 637bp.



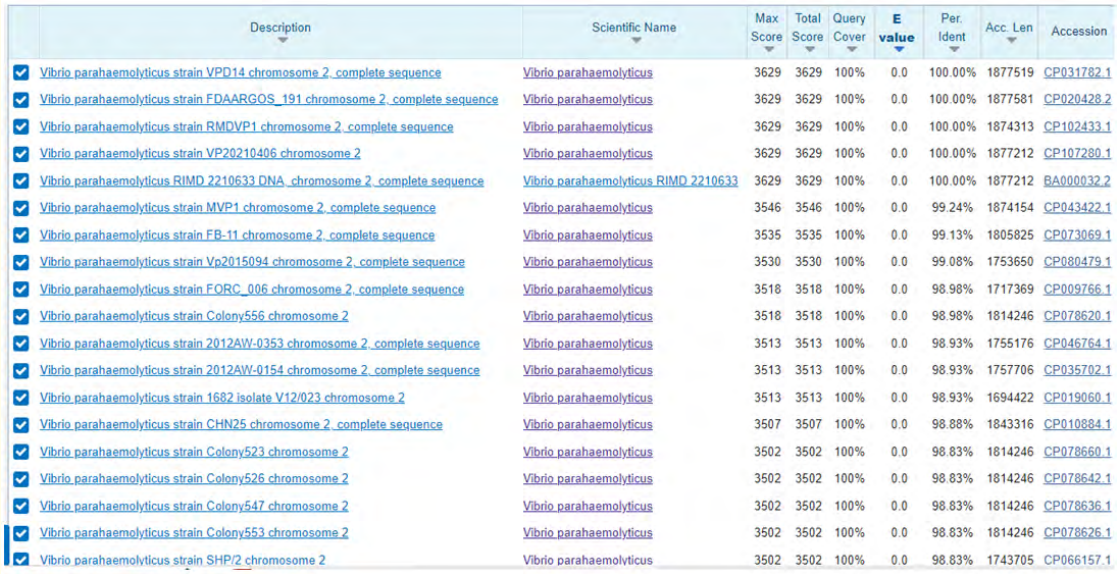
A

V2F VmeB BlastN Description	Scientific Name	Max Score	Total Score	Query Cover	E value	Per Ident	Acc. Len	Accession
Vibrio parahaemolyticus strain 2012V-1116 chromosome 1	Vibrio parahae...	40.1	40.1	100%	1.7	100.00%	3383078	CP051113.1
Vibrio parahaemolyticus strain 2012V-1165 chromosome 1	Vibrio parahae...	40.1	40.1	100%	1.7	100.00%	3411422	CP051111.1
Vibrio parahaemolyticus strain AM51557 chromosome 1	Vibrio parahae...	40.1	40.1	100%	1.7	100.00%	3330514	CP051100.1
Vibrio parahaemolyticus strain 20151116002-3 chromosome I, complete sequence	Vibrio parahae...	40.1	40.1	100%	1.7	100.00%	3491101	CP034305.1
Vibrio parahaemolyticus strain 20160303005-1 chromosome I, complete sequence	Vibrio parahae...	40.1	40.1	100%	1.7	100.00%	3483160	CP034298.1
Vibrio parahaemolyticus strain 20140829008-1 chromosome I, complete sequence	Vibrio parahae...	40.1	40.1	100%	1.7	100.00%	3358530	CP034294.1
Vibrio parahaemolyticus strain 20140722001-1 chromosome I, complete sequence	Vibrio parahae...	40.1	40.1	100%	1.7	100.00%	3421899	CP034289.1
Vibrio parahaemolyticus strain 20140624012-1 chromosome I, complete sequence	Vibrio parahae...	40.1	40.1	100%	1.7	100.00%	3338799	CP034285.1
Vibrio parahaemolyticus strain 2012AW-0224 chromosome 1, complete sequence	Vibrio parahae...	40.1	40.1	100%	1.7	100.00%	3535345	CP046831.1
Vibrio parahaemolyticus strain 2010V-1106 chromosome 1, complete sequence	Vibrio parahae...	40.1	40.1	100%	1.7	100.00%	3311269	CP046828.1
Vibrio parahaemolyticus strain 2013V-1244 chromosome 1, complete sequence	Vibrio parahae...	40.1	40.1	100%	1.7	100.00%	3276195	CP046782.1
Vibrio parahaemolyticus strain 2014V-1125 chromosome 1, complete sequence	Vibrio parahae...	40.1	40.1	100%	1.7	100.00%	3323757	CP046778.1
Vibrio parahaemolyticus strain 2013V-1146 chromosome 1, complete sequence	Vibrio parahae...	40.1	40.1	100%	1.7	100.00%	3343366	CP046808.1
Vibrio parahaemolyticus strain 2013V-1136 chromosome 1, complete sequence	Vibrio parahae...	40.1	40.1	100%	1.7	100.00%	3397332	CP046785.1
Vibrio parahaemolyticus strain 2013V-1181 chromosome 1, complete sequence	Vibrio parahae...	40.1	40.1	100%	1.7	100.00%	3418724	CP046783.1
Vibrio parahaemolyticus strain 2012AW-0353 chromosome 1, complete sequence	Vibrio parahae...	40.1	40.1	100%	1.7	100.00%	3332037	CP046763.1

B

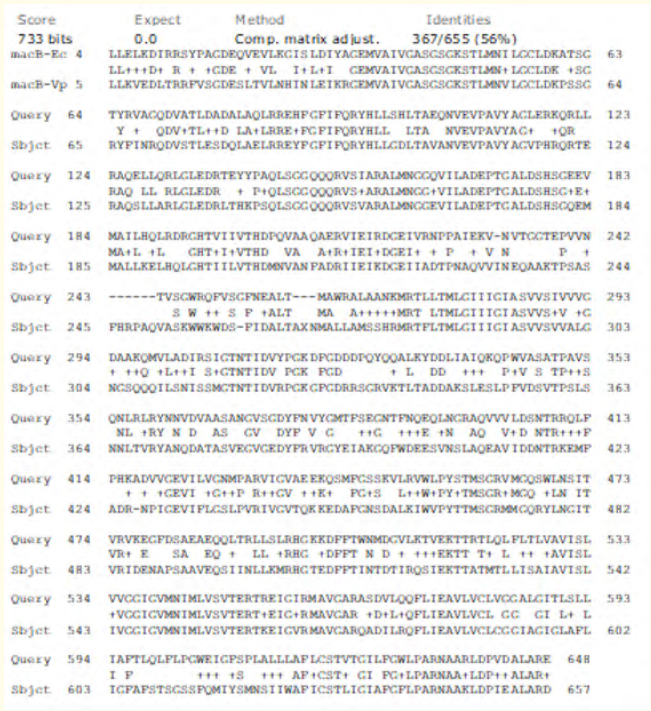
P2.1R VmeB BlastN Description	Scientific Name	Common Name	Taxid	Max Score	Total Score	Query Cover	E value	Per Ident	Acc. Len	Accession
Vibrio parahaemolyticus strain 20140829008-1 chromosome I, complete sequence	Vibrio p...	NA	670	46.1	46.1	100%	0.028	100.00%	3358530	CP034294.1
Vibrio parahaemolyticus strain 20140722001-1 chromosome I, complete sequence	Vibrio p...	NA	670	46.1	46.1	100%	0.028	100.00%	3421899	CP034289.1
Vibrio parahaemolyticus strain 20140624012-1 chromosome I, complete sequence	Vibrio p...	NA	670	46.1	46.1	100%	0.028	100.00%	3338799	CP034285.1
Vibrio parahaemolyticus strain 2013V-1136 chromosome 1, complete sequence	Vibrio p...	NA	670	46.1	46.1	100%	0.028	100.00%	3397332	CP046785.1
Vibrio parahaemolyticus strain 19-021-D1 chromosome 1, complete sequence	Vibrio p...	NA	670	46.1	46.1	100%	0.028	100.00%	3577848	CP046411.1
Vibrio parahaemolyticus strain 160807 chromosome 1, complete sequence	Vibrio p...	NA	670	46.1	46.1	100%	0.028	100.00%	3508313	CP033141.1
Vibrio natriegens strain CCUG 16374 chromosome 1, complete sequence	Vibrio n...	NA	691	46.1	46.1	100%	0.028	100.00%	3460038	CP016351.1
Vibrio natriegens strain CCUG 16371 chromosome 1, complete sequence	Vibrio n...	NA	691	46.1	46.1	100%	0.028	100.00%	3311485	CP016347.1
Vibrio parahaemolyticus strain 20-082E4 chromosome I, complete sequence	Vibrio p...	NA	670	46.1	46.1	100%	0.028	100.00%	3610911	CP083361.1
Vibrio parahaemolyticus strain Colony269 chromosome 1	Vibrio p...	NA	670	46.1	46.1	100%	0.028	100.00%	3577848	CP078647.1
Vibrio parahaemolyticus strain Colony549 chromosome 1	Vibrio p...	NA	670	46.1	46.1	100%	0.028	100.00%	3577848	CP078631.1
Vibrio parahaemolyticus strain Colony555 chromosome 1	Vibrio p...	NA	670	46.1	46.1	100%	0.028	100.00%	3577848	CP078621.1
Vibrio parahaemolyticus strain 20-082A3 chromosome I, complete sequence	Vibrio p...	NA	670	46.1	46.1	100%	0.028	100.00%	3610915	CP065369.1
Teredinibacter turnerae T7901, complete genome	Teredini...	NA	377629	46.1	46.1	100%	0.028	100.00%	5193164	CP001614.2
Vibrio parahaemolyticus vmeAB operon (vmeA, vmeB genes), complete sequence	Vibrio p...	NA	670	46.1	46.1	100%	0.028	100.00%	4935	AB251606.1
Shewanella maritima strain D4-2 chromosome, complete genome	Shewan...	NA	2520507	44.1	110	95%	0.11	100.00%	4718011	CP036200.1

Figure 6: BlastN-search of Forward (A) and reverse (B) VmeB primers showing *V. parahaemolyticus* specificities.



Description	Scientific Name	Max Score	Total Score	Query Cover	E value	Per. Ident	Acc. Len	Accession
Vibrio parahaemolyticus strain VPD14 chromosome 2, complete sequence	Vibrio parahaemolyticus	3629	3629	100%	0.0	100.00%	1877519	CP031782.1
Vibrio parahaemolyticus strain FDAARGOS_191 chromosome 2, complete sequence	Vibrio parahaemolyticus	3629	3629	100%	0.0	100.00%	1877581	CP020428.2
Vibrio parahaemolyticus strain RMDVP1 chromosome 2, complete sequence	Vibrio parahaemolyticus	3629	3629	100%	0.0	100.00%	1874313	CP102433.1
Vibrio parahaemolyticus strain VP20210406 chromosome 2	Vibrio parahaemolyticus	3629	3629	100%	0.0	100.00%	1877212	CP107280.1
Vibrio parahaemolyticus RIMD 2210633 DNA, chromosome 2, complete sequence	Vibrio parahaemolyticus RIMD 2210633	3629	3629	100%	0.0	100.00%	1877212	BA000032.2
Vibrio parahaemolyticus strain MVP1 chromosome 2, complete sequence	Vibrio parahaemolyticus	3546	3546	100%	0.0	99.24%	1874154	CP043422.1
Vibrio parahaemolyticus strain FB-11 chromosome 2, complete sequence	Vibrio parahaemolyticus	3535	3535	100%	0.0	99.13%	1805825	CP073069.1
Vibrio parahaemolyticus strain Vp2015094 chromosome 2, complete sequence	Vibrio parahaemolyticus	3530	3530	100%	0.0	99.08%	1753650	CP080479.1
Vibrio parahaemolyticus strain FORC_006 chromosome 2, complete sequence	Vibrio parahaemolyticus	3518	3518	100%	0.0	98.98%	1717369	CP009766.1
Vibrio parahaemolyticus strain Colony556 chromosome 2	Vibrio parahaemolyticus	3518	3518	100%	0.0	98.98%	1814246	CP078620.1
Vibrio parahaemolyticus strain 2012AW-0353 chromosome 2, complete sequence	Vibrio parahaemolyticus	3513	3513	100%	0.0	98.93%	1755176	CP046764.1
Vibrio parahaemolyticus strain 2012AW-0154 chromosome 2, complete sequence	Vibrio parahaemolyticus	3513	3513	100%	0.0	98.93%	1757706	CP035702.1
Vibrio parahaemolyticus strain 1682 isolate V12/023 chromosome 2	Vibrio parahaemolyticus	3513	3513	100%	0.0	98.93%	1694422	CP019060.1
Vibrio parahaemolyticus strain CHN25 chromosome 2, complete sequence	Vibrio parahaemolyticus	3507	3507	100%	0.0	98.88%	1843316	CP010884.1
Vibrio parahaemolyticus strain Colony523 chromosome 2	Vibrio parahaemolyticus	3502	3502	100%	0.0	98.83%	1814246	CP078660.1
Vibrio parahaemolyticus strain Colony526 chromosome 2	Vibrio parahaemolyticus	3502	3502	100%	0.0	98.83%	1814246	CP078642.1
Vibrio parahaemolyticus strain Colony547 chromosome 2	Vibrio parahaemolyticus	3502	3502	100%	0.0	98.83%	1814246	CP078636.1
Vibrio parahaemolyticus strain Colony553 chromosome 2	Vibrio parahaemolyticus	3502	3502	100%	0.0	98.83%	1814246	CP078626.1
Vibrio parahaemolyticus strain SHP/2 chromosome 2	Vibrio parahaemolyticus	3502	3502	100%	0.0	98.83%	1743705	CP066157.1

Figure 7: BlastN search of genomic macB gene sequence of *V. parahaemolyticus* strain FDAARGOS_191 (accession no. CP020428.2, nt. 464328-462364) showing very related macB gene sequences. Such macB gene sequence was used for primer design.



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 Score          Expect          Method                  Identities
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macB-Ec 4    LLELKDIRRSYPAGDEQVEVLKGISLDIYACEMVAIVGASGSGKSTLMNILGCLDKATSG  63
             LL+++D+ R + +GDE + VL  I+L+I  CEMVAIVGASGSGKSTLMN+LGCLDK +SG
macB-Vp 5    LLKVEDLTRRFVSGDESLTVLNHINLEIKRGCEMVAIVGASGSGKSTLMNVLGCLDKPSSG  64

Query  64    TYRVAGQDVATLDADALAQLRREHFGFIFQRYHLLSHLTAEQNVEVPAVYACLERKQRLL  123
              Y +  QDV+TL++D LA+LRRE+FGFIFQRYHLL  LTA  NVEVPAVYAG+  +QR
Sbjct  65    RYPINRQDVSTLESDQLAELRREYFGFIFQRYHLLGDLTAVANVEVPAVYAGVPHRQRTE  124

Query  124   RAQELLQRLGLEDRTEYYPAQLSGGQQQRVSIARALMNGGQVILADEPTGALDSHSGEEV  183
             RAQ LL RLGLEDR  + P+QLSGGQQQRVS+ARALMNGG+VILADEPTGALDSHSG+E+
Sbjct  125   RAQSLLARLGLEDRLTHKPSQLSGGQQQRVSVARALMNGGEVILADEPTGALDSHSGQEM  184

Query  184   MAILHQLRDRGHTVIIVTHDPQVAAQAERVIEIRDGEIVRNPPAIEKV-NVTGGTEPVVN  242
             MA+L +L   GHT+I+VTHD  VA  A+R+IEI+DGEI+ + P  + V N      P  +
Sbjct  185   MALLKELHQLGHTIILVTHDMNVANFADRIIEIKDGEIIADTPNAQVVINEQAAKTPSAS  244

Query  243   ------TVSGWRQFVSGFNEALT---MAWRALAANKMRTLLTMLGIIIGIASVVSIVVVG  293
                    S W ++ S F +ALT    MA  A+++++MRT LTMLGIIIGIASVVS+V +G
Sbjct  245   FHRPAQVASKWWKWDS-FIDALTAXNMALLAMSSHRMRTFLTMLGIIIGIASVVSVVALG  303

Query  294   DAAKQMVLADIRSIGTNTIDVYPGKDPGDDDPQYQQALKYDDLIAIQKQPWVASATPAVS  353
             + ++Q +L++I S+GTNTIDV PGK PGD       + L  DD  +++   P+V S TP++S
Sbjct  304   NCSQQQILSNISSMGTNTIDVRPGKGPGDRRSGRVKTLTADDAKSLESLPFVDSVTPSLS  363

Query  354   QNLRLRYNNVDVAASANGVSGDYFNVYGMTFSEGNTFNQEQLNGRAQVVVLDSNTRRQLF  413
               NL +RY N D  AS  GV  DYF V G    ++G   +++E +N  AQ  V+D NTR+++F
Sbjct  364   NNLTVRYANQDATASVEGVGEDYFRVRGYEIAKGQFWDEESVNSLAQEAVIDDNTRKEMF  423

Query  414   PHKADVVGEVILVCNMPARVICVAEEKQSMPGSSKVLRVWLPYSTMSCRVMCQSWLNSIT  473
              + + +GEVI +G++P R++GV  ++E+  PG+S   L++W+PY+TMSCR+MCQ +LN IT
Sbjct  424   ADR-NPIGEVIFLGSLPVRIVGVTQKEDAPGNSDALKIWVPYTTMSCRMMCQRYLNGIT  482

Query  474   VRVKEGFDSAEAEQQLTRLLSLRHGKKDFFTWNMDGVLKTVEKTTRTLQLFLTLVAVISL  533
             VR+ E   SA  EQ +  LL +RHG +DFFT N D + +++++EKTT T+ L ++ +AVISL
Sbjct  483   VRIDENAPSAAVEQSIINLLKMRHGTEDFFTINTDTIRQSIEKTTATMTLLISAIAVISL  542

Query  534   VVCGICVMNIMLVSVTERTREIGIRMAVGARASDVLQQFLIEAVLVCLVGGALGITLSLL  593
             +VCGICVMNIMLVSVTERT+EIG+RMAVGAR +D+L+QFLIEAVLVCL GG  GI L+ L
Sbjct  543   IVCGICVMNIMLVSVTERTKEIGVRMAVGARQADILRQFLIEAVLVCLCGGIAGICLAFL  602

Query  594   IAFTLQLFLPCWEIGPSPLALLLAFLCSTVTGILPGWLPARNAARLDPVDALARE  648
             I F         +++ +S  +++ AF+CST+ GI PG+LPARNAA+LDP++ALAR+
Sbjct  603   ICFAFSTSGSSFQMIYSMNSIIWAFICSTLIGIAPGFLPARNAAKLDPIEALARD  657
```

Figure 8: BlastP homology between macB macrolide drug transporter of *E coli* (protein id. AAC73966) and *V. parahaemolyticus* (protein id. KIT26103).

```
 Score                Expect               Method              Identities
 295 bits             2e-101               Comp.l matrix adjust.  161/388(41%)
emrD-Ec 8   NLLLMLVLLVAVGQMAQTIYIPAIADMARDLNVREGAVQSVMGAYLLTYGVSQLFYGPIS  67
             L  ++ +L AVGQM QT+Y+P+I  MA +  V   ++Q+VM  YL+ YG+SQ  YG +S
emrD-Vp  9   KLTFLIAILTAVGQMTQTMYVPSIGHMAGEFLVSASSLQAVMACYLIPYGLSQFAYGTLS  68

Query  68   DRVGRRPVILVGMSIFMLATLVAVTTSSLTVLIAASAMQGMGTGVGGVMARTLPRDLYER  127
            DR+GR+P+I+VG+ I++L TLVA+         +A S +QG+G G GG M+RTL RD +E
Sbjct  69   DRLGRKPIIIVGLLIYILGTLVALFAHQFEWFLAGSFIQGLGIGCGGAMSRTLTRDCFEG  128

Query  128  TQLRHANSLLNMGILVSPLLAPLIGGLLDTMWNWRACYLFLLVLCAGVTFSMARWMPETR  187
             +L  ANSL++M ++ SPL+AP++GG L   + WR+ YLFL +    V  +M   M ET
Sbjct  129  AELHRANSLISMCVIFSPLMAPVLGGYLTEAFGWRSSYLFLALFGIAVVITMMTSMMETL  188

Query  188  PVDAPRTR-LLTSYKTLFGNSGFNCYLLMLIGGLAGIAAFEACSGVLMGAVLGLSSMTVS  246
            P +  +   + SYK +  +  F  +L+ L+   AG+  FEA +GVL+G VL L + TVS
Sbjct  189  PKEKRKFEPVAKSYKHVLSDRRFQGFLICLVATFAGVGVFEAAAGVLLGGVLALPATTVS  248

Query  247  ILFILPIPAAFFGAWFAGRPNKRFSTLMWQSV--ICCLLAGLLMWIPDWFGVMNVWTLLV  304
            +LFILPIP    GA  +    +R S     +V  +   +   ++ IP  FGV    TL+
Sbjct  249  LLFILPIPGYLVGAGLSSYIAQRRSERRALNVGLVSIFIGSAVVLIPGLFGVTTALTLIG  308

Query  305  PAALFFFGAGMLFPLATSGAMEPFPFLAGTAGALVGGLQNIGSGVLASLSAMLPQTGQGS  364
             A ++F GAG+LFP AT+GA+ PFP+ AGTAGA +GG+QN+G+G+   L+++ P   Q
Sbjct  309  GATIYFLGAGILFPAATTGAIAPFPYHAGTAGATLGGMQNLGAGIATLLTSLFPAHNQMP  368

Query  365  LGLLMTLMGLLIV--LCWLPLATRMSHQ  390
            LG+LM  M ++ +  L W+  A   S++
Sbjct  369  LGVLMMAMSIVAMLGLLWVHRAHDHSNE  396
```

Figure 9: Amino acid homology between emrD MFS drug efflux proteins of *E. coli* (protein id. EFK3440626; accession no. AATEUH010000019.1; nt. 63846-65030 complement) and *V. parahaemolyticus* (protein id. EGR2894425; Accession no. AAXOBG010000023.1, nt. 48642-49847).

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 Score                Expect               Method              Identities
 473 bits             1e-171               Comp. matrix adjust.  240/392(61%)
mdtL-Ec 1   MSRFLICSFALVLLYPAGIDMYLVGLPRIAADLNASEAQLHIAFSVYLAGMAAAMLFAGK  60
            MS FL+CSFALVLLYP  ID+YLVGLP+IA+DLNASE+QLHIAFS+YLAGMA  MLFAGK
mdtL-Vp 1   MSIFLLCSFALVLLYPTAIDLYLVGLPQIASDLNASESQLHIAFSIYLAGMATTMLFAGK  60

Query  61   VADRSGRKPVAIPGAALFIIASVFCSLAETSTLFLAGRFLQGLGAGCCYVVAFAILRDTL  120
            +AD  GRKPVA+ GA +F++AS    +AE    FL  RF QG+GAG CYVVAFAILRDTL
Sbjct  61   IADSVGRKPVAVVGAMIFVLASFLGGMAEQPNAFLIARFCQGIGAGSCYVVAFAILRDTL  120

Query  121  DDRRRAKVLSLLNGITCIIPVLAPVLGHLIMLKFPWQSLFWAMAMMGIAVIMLSLFILKE  180
            DD RRAKVLS+LNGITCIIPV+APV+GHLIMLKFPW SLF  MA MGI  V +L++F+LKE
Sbjct  121  DDERRAKVLSMLNGITCIIPVIAPVIGHLIMLKFPWPSLFTTMAGMGILVSVLAIFVLKE  180

Query  181  TRPAAPAASD-KPRENSESLLNRFFLSRVVITTLSVSVILTFVNTSPVLLMEIMGFERGE  239
            + P+        P  + E+   RFF+SR++IT L V+ ILTFVN SP+++M ++GF+RG
Sbjct  181  SLPSQQGEEQTTPESHQETFFERFFISRLIITALGVTTILTFVNASPIVVMSMLGFDRGG  240

Query  240  YATIMALTAGVSMTVSFSTPFALGIFKPRTLMITSQVLFLAAGITLAVSPSHA----VSL  295
            Y++IMA TA +SM +SFS P ALGIFK RTLM+TSQVL   A I L+ +  H       +
Sbjct  241  YSSIMAGTATISMLISFSAPLALGIFKQRTLMMTSQVLLACAAIVLSAAHFHDGQSLYYV  300

Query  296  FGITLICAGFSVGFGVAMSQALGPFSLRAGVASSTLGIAQVCGSSLWIWLAAVVGIGAWN  355
            FG+ LICAGF+ GFGVAMSQAL PFS +AGVASS LGIAQVC S+ +IW    +G+ A N
Sbjct  301  FGLGLICAGFACGFGVAMSQALSPFSQQAGVASSLLGIAQVCSSAFYIWFMGFIGVSALN  360

Query  356  MLIGILIACSIVSLLLIMFVAPGRPVAAHEEI  387
            ML+ IL+  S++SL LI+ +        +EEI
Sbjct  361  MLVFILVLGSVISLALILLIPKPVHDTHYEEI  392
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Figure 10: BlastP homology between mdtL of *E. coli* (protein id. CDJ73164) and *V. parahaemolyticus* (protein id. TBT29980).

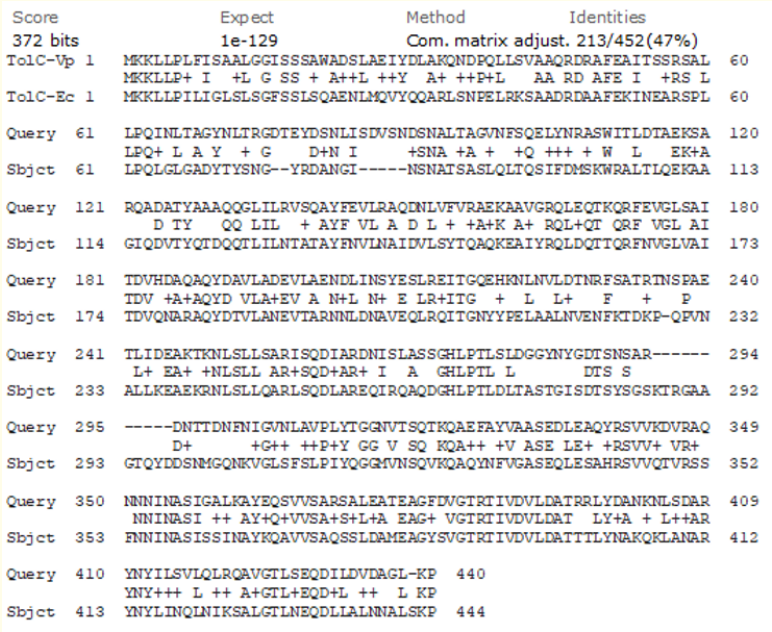

Figure 11: Amino acid homology between TolC outer protein of *E. coli* and *V. parahaemolyticus* (protein id. TXM47846).

We then made primers for vmeB of *V. parahaemolyticus* using NCBI software (www.ncbi.nlm.nih.gov/tools/primer-blast/primertool.cgi). As vmeV vs. vmeD gave some similarity at nt. 1178-1254 and also vmeB vs. acrB gene of *P. aeruginosa* gave some similarities (nt. 10-314 = 66%; 838-908 = 69%, 1003-1484 = 71%; 1525-1547 = 96%; and 2356-3064 = 67%), we choose primers design between nt. 350-800 for forward and nt. 1500-2300 for reverse in the NCBI primer design portal. We selected two primers as shown in table 2. The BlastN search indicated that primer vmeBF1 and vmeBF2 were good and located in Ch-1 but reverse primers were non-specific as has 100% homology to chromosomes of *E. coli* (accession nos. CP117717, CP117714) or Klebsiella pneumoniae (accession no. CP117745) for vmeBR1 primer and also chromosomes of *Providentia stuarti* (accession nos. CP048621, AP022374) or *Kluvera intermedia* (accession nos. CP045843, CO045845) for vmeBR2 primer. When we added 3 nt at the 5'-end of P2R VmeBR2-primer (table 2), the specificity to *V. parahaemolyticus* was obtained but not when we added three nucleotides at the 3'-end. Thus, we coined vmeBR2 primer as 5'-TTT ACG GTT AAA CCA GCC GAA GA-3' which should be good with vmeBF2 primer pair although self hairpin and dimer formation probability were increased. However, we determined the restriction pattern of the 798bp PCR fragment as shown in figure 5. Thus, digestion with PstI gave 163bp and 635bp two fragments and similarly, digestion with BbS1 gave 279bp and 519bp two fragments and such analysis would give confirmatory data regarding *V. parahaemolyticus* strain. Both vmeBF1 and vmeBR2 primers gave good specificities on BlastN homology search (Figure 6).

The homology between vmeB and vmeD was rare and only 1178-1254 nucleotides of vmeB has 82% similarity to the nucleotides 1157-1233 of vmeD gene and Clustal-Omega search indicated poor similarities throughout the vmeB/vmeD genes and primers design was possible for *V. parahaemolyticus* diagnosis. Further, mexB and acrB genes were 66-71% similarity in some points to vmeB gene of *V. parahaemolyticus* and Blast-2 analysis suggested many points for primers design consideration and multi-alignment confirmed such idea.

We initially detected macB gene in Ch-2 of *V. parahaemolyticus* in different strains. As for example, strain *1682* macB gene located

between nt. 225504-227468 (accession no. CP019060) but BlastN search of sequence gave no new 100% similarity sequence in the database but <98.8% similarity (must be >99.9% similarity). Such analysis also true for strains *AM46865* (accession no. CP046762), *PB1937* (accession no. CP068634), *FB-11* (accession no. CP073068), *RMPVP1* (accession no. CP102433) and strain *BTXS2* (accession no. CP063526). Finally, we selected macB gene of a popular-type strain FDAARGOS_191 which gave good BlastN search result as shown in figure 7. Such macB gene was used for macB gene specific primers for *V. parahaemolyticus* as demonstrated in table 3. The Blast-2 homology between *E. coli* macB gene and *V. parahaemolyticus* macB gene demonstrated 56% homology (Figure 8). The macBF1/R1 primers have similarities to V. alginolyticus and so we attempted macBF2/R2 primers. It was found by BlastN homology search that P2F primer was very specific to Ch-2 of *V. parahaemolyticus* but in some strains a T>C polymorphism were present at nucleotide 5 position of P2F primer of strain *colony127* (accession no. CP078730), strain *AM46865* (accession no. CP046762) and strain *2014V-1125* (accession no. CP046777). Hence, the desired macBF2' primer sequence would be, 5'-GCC G(T/C)C AGT AAG TGG TGG AA-3' and P2R-macB should be, 5'- AAT CAC TGC TTC TTG GGC GA-3'. Both primers gave good specificities to *V. parahaemolyticus* on BlastN homology search similar to vmeB gene primers (data not shown).

The vmeA protein complexed with vmeB and macB complexed with macA proteins as well as another accessory outer protein (TolC/oprM) similar to tripartite complex of acrA-acrB-TolC or mexA-mexB-oprM that extruded drugs from bacterial cytoplasm to outside cells. So, we checked vmeA and macA genes which usually located adjacent to vmeB and macB genes similar to well established for acrAB and mexAB/CD/EF types drug efflux genes. The Ch-1 of Vp (accession no. CP047990; 3354580bp) indeed contained vmeAB locus (nt. 1131694-1132833 and 1132836-1135997) as well as vmeHI (nt. 1218751-1219836; 1219833-1222934) and vmeEF (nt. 969080-970174; 970174-973287). The Ch-1 also contained MATE family transporters (protein ids. WCZ06201, MCZ06319 and MCZ07149). The vmeA of Vp has no similarity to *E. coli* acrA gene except short nucleotide streaches between nt. 616-642 and nt. 853-878 and thus could be useful for primers design. On the other hand, among the related vmeA, vmeE and vmeH acrA-related protein of *V. parahaemolyticus*, no great homology detected (<25%). The primer designed for vmeA gene of Vp showed great Vp Ch-1 specificities as described for other primers (table 4). The Ch-1 (accession no. CP034294) contained vmeAB (protein ids. QHH04753 and QHH04752), vmeHI (protein ids. QHH04668 and QHH04667) and vmeEF (protein ids. QHH04891 and QHH04890) in one strand whether vmeCD (QHH05738 and QHH05739) and vmeJK (protein id. QHH03428 and QHH03429) located in the complement strand. We disclosed three penicillin-binding proteins in Ch-1 of Vp: pbp1A (protein id. QHH03133), pbp1b and pbp2 whereas previously we made diagnostic primer from pbp1B. There was no homology between pbp1 and pbp2 but pbp1A and pbp1b have 30% similarities through out the gene.

An EmrD MFS-type multidrug efflux gene was located in Ch-2 of Vp strain FDAARGOS_667 (accession no. CP044063, nt. 1117347-1118552) and had >99.9% similarities to EmrD gene of strain LVP2 (accession no.CP040102), strain HA2 (accession no. CP023709) and strain D3112 (accession no. CP034566). The emrD protein of *E. coli* had 41% similarities to emrD gene of *V. parahaemolyticus* (figure 9). We complement reversed the emrD gene using DNA editing software (http://genome2d.molgenrug.nl/g2d_tools_conversions.html). EmrD primers were also very specific to *V. parahaemolyticus* (Table 5).

A mdtL MFS-type multidrug efflux protein was located in Ch-2 (protein id. ARC20794; 396AA; accession no. CP020428) and has 61% similarity to the *E. coli* mdtL enzyme (CDJ73164) (Figure 10). It is very similar to Vibrio diabolicus (protein id. WP_182033202) and was not considered for primer design. Two types of MATE-related transporter were located in Vp Ch-1 with less than 25% similarities. MATE-1 located between nt. 1579702-1581072 and MATE-2 located between nt. 1273424-1274800 and we have not tried to make any diagnostic primer from such sequence because a new member of MATE-3 located in a Vp genomic fragment (protein id. MQP53919; accession no. WHOI01000001.1, nt. 30760-32142).

The TolC outer protein in the tripartite drug efflux complex (acrA-acrB-TolC) is ubiquitious and it has located in Ch-1 of Vp (protein id. OAR17803). We found 47% homology between Ec TolC and Vp TolC protein (Figure 11) whereas only 23% overall homology between *E. coli* TolC protein and *P. aeruginosa* OprM protein. But the other outer protein in *P. aeruginosa* OprJ had

45% homology to OprM protein. Whereas both OprJ and OprM of Pa had 27% overall similarity to the out protein of Vp located near an acrA-like protein in Ch-2 (protein id. ARC20291) but no significant similarity to TolC of *E. coli*. Thus, multiple outer protein in the tripartite complexes like acrAB-TolC, mexAB-OprM, mexEF-OprJ, macAB-TolC etc might be located in Vp chromosomes (Ch-1 and Ch-2).

Primer name	Sequence of the primers	Position	Tm	Size
VmeBF1	5'-TGAAATCAGATGCGAGCGGT-3'	752-1541	60.11	790bp
vmeBR1	5'-AACCAGCCGAAGAAGCCTTT-3'	1541-1522	60.18	
vmeBF2	5'-GAAATCAGATGCGAGCGGTG-3'	753-772	59.70	788bp
vmeBR2	5'-ACGGTTAAACCAGCCGAAGA-3'	1541-1529	59.61	
vmeBR2'	5'- TTTACGGTTAAACCAGCCGAAGA-3'	1541-1526	59.71	

Table 2: Primer design for vmeB acrB-like gene of *Vivrio parahaemolyticus*.

Primer name	Sequence of the primers	Position	Tm	Size
macBF1	5'-CGCTGAAAATTTGGGTGCCA-3'	1364-1383	59.97	581bp
macBR1	5'-TATCGGATCGAGTTTGGCGG-3'	1944-1925	59.97	
macBF2	5'-GCCGTCAGTAAGTGGTGGAA-3'	751-770	59.97	483bp
macBF2'	5'-GCCG(T/C)CAGTAAGTGGTGGAA-3'	751-770	59.97	
macBR2	5'-AATCACTGCTTCTTGGGCGA-3'	1233-1214	59.96	

Table 3: Primer design for *V. parahaemolyticus* specific macB macrolide drug efflux gene.

Primer name	Sequence of the primers	Position	Tm	Size
vmeAF1	5'-CGTAGAACCACGCTATCGCT-3'	7-26	59.97	548bp
vmeAR1	5'-TTGCTGATCACGCCACTGAT-3'	554-535	60.04	
vmeAF2	5'-TTGCTTAGTGCAGAAGCGGA-3'	310-329	59.97	524bp
vmeAR2	5'-AATTCCGCACGCAAAGTCAC-3'	833-814	60.04	

Table 4: Primer design for vmeA gene of *V. parahaemolyticus*.

Primer name	Sequence of the primers	Location	Tm	Size
emrDF1	5'-CGCAATATTGACTGCCGTGG-3'	42-61	59.97	455bp
emrDR1	5'-AGCTAGAGCGCCAACCAAAT-3'	496-477	60.04	
emrDF2	5'-TCTGCCTAGTTGCGACGTTT-3'	647-666	59.97	473bp
emrDR2	5'-CATCAAAACACCAAGCGGCA-3'	1119-1100	59.97	

Table 5: Primer design for emrD MFS-type multidrug efflux gene of *V. parahaemolyticus*.

Discussion

Multidrug-resistance is a central problem as diverse bacterial population in the environment were gradually acquiring different mdr genes and integrons in large plasmids [41]. More than 6500 unique enzymes capable of degrading β-lactam compounds (penicillin drugs) have been identified to date. We have to opt for new target development and new drug design. Vibrio contamination in shrimp fish is a serious threat to human. Such contamination is serious for shrimp trade to Europe and America [42,43]. More use

of different antibiotics in aquculture was also inhibited as residue antibiotic might cause many fatal diseases like cancers. We firmly showed that phyto-drugs from few Indian medicinal plant ethanol extracts (*Suregada multiflora, Cassia fistula, Jatropha gossipifolia*) greatly inhibited MDR-bacteria isolated from Ganga River water, milk, chicken meat and human hair [44,45]. Drug-efflux genes may be chromosomal but may be plasmid mediated also [46]. Evaluation of such genes in bacterial contamination may be thus useful. We suggested that PCR diagnostics was possible for *V. parahaemolyticus* if there were sequence divergence of such genes with other Vibrio species [47,48]. Similarly, sequence divergence with other bacteria like *E. coli* was also observed [7,10]. Furniss., *et al*. showed that chemical inhibition of DsbA enzyme sensitized multidrug-resistant clinical isolates to existing antibiotics. Thus, by targeting the primary disulfide bond formation enzyme DsbA with an inhibitor 4,5-dichloro-2-(2-chlorobenzyl) pyridazin-3-one, a group of bacteria with activated MDR enzymes like β-lactamases, MCR enzymes, and RND drug transporters could be inhibited. Thus, characterization and purification of drug-efflux proteins is an urgent task to tackle multi-drug related problems [49].

Mdr genes were located in many *Vibrio* species both in chromosomes and plasmids (Elmahdi S., *et al*. 2016). A blaCMY-2 gene was shown to be located in an ~150-kb IncA/C-type conjugative plasmid of *V. parahaemolyticus* with a genetic structure consisting of traB-traV-traA-ISEcp1-blaCMY-2-blc-sugE-encR-orf1-orf2-orf3-orf4-dsbC-traC giving resistance to penicillin drug [50]. Plasmid pVp94-1 of *V. parahaemolyticus* strain Vp2015094 carried tetracycline resistance genes (tetB/M), aminoglycoside resistance genes (aph3''-Ib, aph6-Id), sulphonamide resistance genes (sul2), diaminopyrimidine resistance gene (dhfrA6), fluoroquinolone resistance gene (qnrVC6), phenicol resistance gene (floR) and penam resistance gene ($bla_{CARB-19}$) [51].

Never-the-less, although Matsuo T., *et al*. 2013 have designed some primers for Vp drug-efflux genes, we found some problems during blast-2 search [29]. However, our cross-analysis of genomes was very clear to give the optimal PCR primers for those genes. Given to the fact that biochemical analysis vmeA-vmeB-TolC tripartite proteins in Vp or Vc has not done carefully. Thus, characterization of such genes will give many helpful hind for their further characterization and drug design. *V. herveyi* and *V. alginolyticus* are very closely related to Vp and significantly differs from Vc. We thought, the vmeR2 primer 5'- ACG GTT AAA CCA GCC GAA GA-3' and vmeR2' primer 5'- TTT ACG GTT AAA CCA GCC GAA GA-3' would be great species specificity. Similarly, the macBF2' primer 5'-GCC G(T/C)C AGT AAG TGG TGG AA-3' and macBR2 primer 5'- AAT CAC TGC TTC TTG GGC GA-3' would be useful for the identification of *V. parahaemolyticus* contamination in shrimp fish. An ABC-type efflux pump, SmdAB, similar to VcaM from *Vibrio cholerae*, protects *S. marcescens* from fluoroquinolones and tetracycline. We have not discussed many unrelated ABC and MFS transporters in our study here due to unknown functions. Surely, careful primers design depends on how to identify *V. parahaemolyticus* contamination in shrimp fish aquaculture. The PirAB genes would cause devastating AHPND disease in shrimp whereas tdh/trh genes cause membrane pore formation which is also very fatal to shrimp growth. Thus, primers designed from multidrug efflux genes would be beneficial. Taken together, previously reported blaCARB and PBP1B genes diagnostic primers (Chakraborty., *et al*. Suntext Review in Biotechnology, 4(1): 146, 2023) and present multidrug efflux genes primers may be a new milestone for *V. parahaemolyticus* research for their identification, characterization and drug design.

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Competent Interest

The authors declare no conflict of interest.

Ethical Issues

The data provided was computer generated using GenBank Database and no human or animal was used.

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