



Antibiotic resistance pattern and its correlation to the presence of *tdh* gene and CRISPR-Cas system in *Vibrio parahaemolyticus* strains isolated from seafood

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ABSTRACT

Vibrio parahaemolyticus is a pathogen native to the aquatic environment. In this study, 46 environmental isolates of *V. parahaemolyticus* were subjected to a correlational analysis to find the association between their antimicrobial susceptibility pattern, prevalence of CRISPR-Cas system and thermostable direct hemolysin (*tdh*) gene. Antibiotic resistance profiling against eleven antibiotics revealed the isolates to be multidrug resistant. Isolates exhibited highest resistance to vancomycin (97.8%) followed by ampicillin (91.3%), cefotaxime (69.6%), ceftazidime/clavulanic acid (54.4%), ceftazidime (45.7%) and gentamicin (39.1%). CRISPR loci and *tdh* gene were detected in 47.83 and 58.7% of strains respectively. No significant correlation was observed between antibiotic resistance to presence of CRISPR, except in the case of gentamicin wherein, a negative correlation was seen ($r=-0.272$, $p<0.10$). Similarly, *tdh* did not correlate to antibiotic resistance. Seventeen strains in this study harboured the CRISPR loci as well as *tdh* gene, the association of which was found to be statistically significant.

Keywords: Antimicrobial resistance, CRISPR-Cas, MAR index, Thermostable direct hemolysin (*tdh*), *Vibrio parahaemolyticus*

Introduction

Vibrio parahaemolyticus is a Gram negative halophilic bacterium that is the leading cause of acute gastroenteritis resulting from the consumption of contaminated seafood (Letchumanan *et al.*, 2014). Occasionally, it can cause wound infections and septicemia (Hiyoshi *et al.*, 2010). Recently, certain strains of *V. parahaemolyticus* have been associated with an emerging disease, acute hepatopancreatic necrosis disease (AHPND) that has caused significant damage and huge economic losses to the global shrimp industry (Xiao *et al.*, 2017).

In *V. parahaemolyticus*, the thermostable direct hemolysin encoded by the *tdh* gene is a major virulence factor, hence considered a marker for its pathogenicity (Honda *et al.*, 1992). Our preliminary examination for the location of *tdh* gene in the genome of *V. parahaemolyticus* showed it to be located in chromosome II and lying in close proximity to the CRISPR (Clustered Regularly-Interspaced Short Palindromic Repeats) - Cas (CRISPR associated proteins) operon (Fig. 1). The CRISPR-Cas operon encompass a cluster of identical repetitive sequences separated by non-identical spacer sequences and multiple cas genes that confer immunity against foreign DNA (Jore *et al.*, 2012; Gophna *et al.*, 2015; Li *et al.*, 2016). CRISPR-Cas systems have been

hypothesised to be involved in replicon partitioning, DNA repair, regulation and chromosomal rearrangement (Kunin *et al.*, 2007). Additionally, they have also been implicated to resist horizontal gene transfer and in acquisition of virulence genes (Bikard *et al.*, 2012; Hullahalli *et al.*, 2017). The indiscriminate use of antibiotics in treating *V. parahaemolyticus* infections have led to the emergence of antibiotic resistant strains of this pathogen globally (Letchumanan *et al.*, 2015; Elmahdi *et al.*, 2016). Therefore, the present study was undertaken to assess whether a correlation exists between the antibiotic resistance phenotypes to the presence of CRISPR-Cas as well as to virulence gene *tdh* in environmental strains of *V. parahaemolyticus*.

Materials and methods

Bacterial strains

In this study, forty six *V. parahaemolyticus* stock cultures maintained at -80°C in tryptic soya broth containing 30% glycerol were used. The strains comprised of isolates from clams (n=11), oysters (n=12), shrimp (n=15), fish (n=7) and an ATCC reference strain AQ4037. The test cultures were grown overnight in 5 ml of Luria Bertani broth (HiMedia Laboratories Pvt. Ltd, Mumbai, India) at 37°C in a shaker incubator. A loopful of culture was streaked onto TCBS (Thiosulfate-citrate-bile salts-

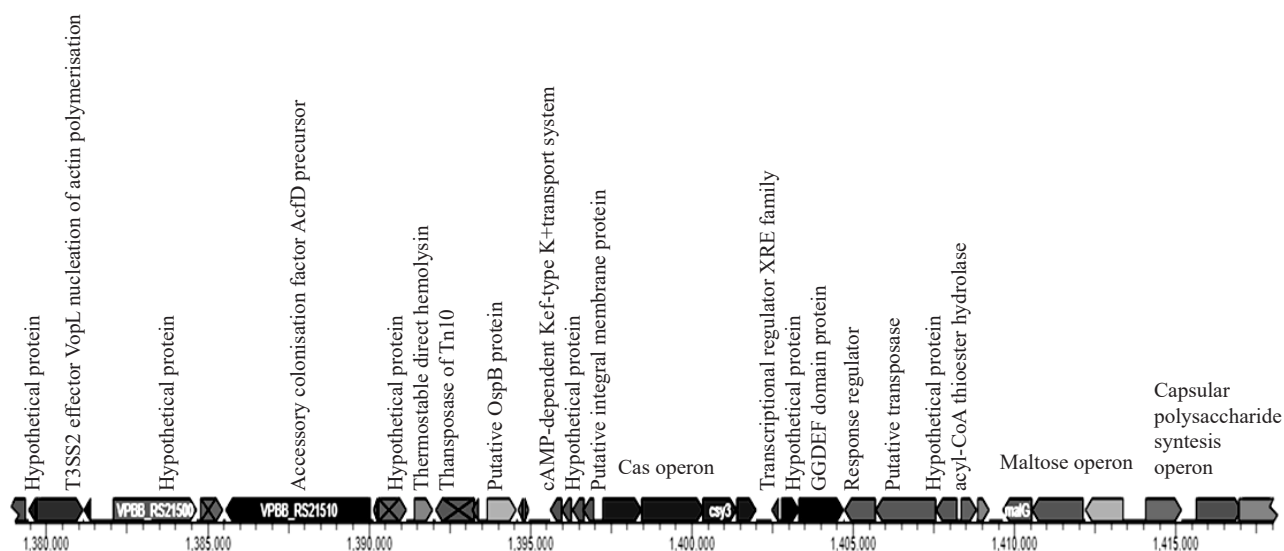


Fig. 1. Genomic context for the location of thermostable hemolysin gene and CRISPR-Cas operon in an environmental *V. parahaemolyticus* strain (BB22OP, acc.No.:NC019971)

sucrose) agar (HiMedia Laboratories Pvt. Ltd, Mumbai, India), to confirm the presence of colonies typical of *V. parahaemolyticus*

Determination of antibiotic resistance

The antimicrobial susceptibilities of the isolates were determined by the Kirby-Bauer's disc diffusion method (Bauer *et al.*, 1966) on Mueller-Hinton agar (MHA) plates. The strains were tested for susceptibility against eleven antibiotics, which included nalidixic acid (NA, 30 µg), tetracycline (TE, 30 µg), cotrimoxazole (COT, 25 µg), ciprofloxacin (CIP, 5 µg), chloramphenicol (C, 30 µg), ampicillin (AMP, 10 µg), gentamicin (GEN, 10 µg), vancomycin (VA, 30 µg), cefotaxime (CTX, 30 µg), ceftazidime (CAZ, 10 µg) and ceftazidime/clavulanic acid (CAC, 30/10 µg). The antimicrobial discs (HiMedia Laboratories Pvt. Ltd, India), each containing a known amount of an antibiotic, was laid in a bacterial lawn on MHA plates and incubated at 30°C for 18-24 h. The zone of inhibition was recorded and the results interpreted according to the CLSI guidelines (CLSI, 2017). However, since these guidelines did not include the interpretive criteria for *V. parahaemolyticus*, the breakpoints employed for *Escherichia coli* were adopted. For strains showing intermediate results, if the diameter of the clear zone was closer to the breakpoint of «sensitive», it was considered sensitive, else considered resistant. The multiple antibiotic resistance (MAR) index of isolates was calculated based on the formula x/y , where x represents the number of antimicrobials to which a particular isolate was resistant and y represents the total number of antimicrobials to which the isolate was exposed (Krumperman *et al.*, 1983).

PCR for the identification of *tdh* gene and CRISPR region

The isolates were checked for presence of *tdh* gene as well as CRISPR loci by PCR. The DNA templates were prepared by heating and snap chilling method using 18 h old culture grown in LB broth. The *tdh* gene was detected using previously designed primer sequences (Tada *et al.*, 1992) CCACTACCCTCATATGC (forward) and GGTACTAAATGGCTGACATC (reverse). However, for the detection of CRISPR loci, novel primers were designed using the primer sequences: GGGTAAATTCGTAGAAAAACCA (CIF-F) and AATGCCAAAGCAAACAGC (CIF-R) to yield an amplicon size of 260 bp. PCR was carried out in a 30 µl mixture consisting of 3 µl of 10X buffer (GeneiTM, Merck Bioscience, Bangalore), 50 µM each of the four deoxynucleotide triphosphates (dNTPs), 10 p mol of each primer and 1.0 U of *Taq* DNA polymerase. Two microliters of crude lysate were used as DNA template. The PCR assay was performed in a programmable thermocycler (PTC 200; Bio-Rad, CA). The PCR conditions were programmed as follows: initial denaturation at 94°C for 5 min, 30 cycles of 94°C for 1min, 52°C for 1 min, 72°C for 1 min and a final extension at 72°C for 10 min. Same cycling conditions were used for the detection of *tdh* except for the annealing temperature, which was set at 55°C for 1 min. Samples were loaded on 1.5% agarose gel and the size of amplicons was determined by comparison with a 100 bp DNA ladder (GeneiTM, Merck Bioscience, Bangalore). The *V. parahaemolyticus* strain VP49 known to harbour CRISPR was used as a positive control, while the ATCC strain AQ4037 was used as a negative control. The bands were visualised under UV transilluminator

(Biorad, Gel Doc™ XR + with Image lab software). The CRISPR positive PCR products were sequenced (Bioserve Biotechnologies Ltd., Hyderabad) in both forward and reverse directions. The CRISPR sequences obtained were analysed to find the number and type of direct repeats and spacers.

Statistical analysis

The association between antibiotic resistance to the presence of *tdh* gene and CRISPR loci in *V. parahaemolyticus* strains was statistically determined using the non-parametric Pearson's correlation coefficient test. To interpret the results of correlation, we used the correlation coefficient (r values) and levels of significance (p-values). Mann-Whitney U test was applied to compare the strain groups with CRISPR and without CRISPR and also groups with *tdh* and without *tdh*. Differences were considered significant when $p < 0.05$. A separate analysis for the co-occurrence of CRISPR loci, *tdh* gene and antibiotic resistance was performed using the one-tailed Fisher's exact test with a 2x2 contingency table.

Results

Determination of antibiotic susceptibility

The antibiotic susceptibility results for the 46 *V. parahaemolyticus* isolates tested is presented in Table 1. As seen from Table 1, the highest resistance was seen for vancomycin (97.8%) followed by ampicillin (91.3%), cefotaxime (69.6%), ceftazidime/clavulanic acid (54.4%), ceftazidime (45.7%) and gentamicin (39.1%). Similarly, the highest antibiotic susceptibility was seen for nalidixic acid (97.8%) followed by cotrimoxazole (91.3%), tetracycline (89.1%), chloramphenicol and ciprofloxacin (87%). Most of the strains in this study exhibited multi drug resistance (MDR) wherein, resistance was seen for three or more antibiotics and with a MAR index ranging between 0.27 - 0.64 (Table 2). Significant associations were observed between resistance to antibiotics. While a negative correlation was associated for the co-occurrence of resistance to C/CTX ($r = -0.330$ $p > 0.05$) and CIP/VA ($r = -0.39$ $p > 0.01$) a positive correlation was observed for CAC/CAZ, CAC/CTX, CAZ/COT and CAZ/GEN (Table 3).

Table 1. Percentage of *V. parahemolyticus* strains showing antibiotic susceptibility, presence of CRISPR loci and *tdh* gene

Antibiotic	Antibiotic susceptibility	No. and % (in parentheses) of isolates	No. and % (in parentheses) of isolates with	
			CRISPR	<i>tdh</i>
Ampicillin (AMP)	R	42(91.3)	20 (47.6)	24 (57.1)
	S	4 (8.7)	2 (40.0)	3(75.0)
Chloramphenicol (C)	R	6 (13.0)	4 (66.6)	4 (66.6)
	S	40 (87.0)	18(45.0)	23 (57.5)
Ceftazidime/clavulanic acid (CAC)	R	25(54.4)	12(48.0)	15 (60.0)
	S	21(45.7)	10 (47.6)	12 (57.1)
Ceftazidime (CAZ)	R	21(45.7)	9 (42.9)	10 (47.6)
	S	25 (54.4)	13 (52.0)	17 (68.0)
Ciprofloxacin (CIP)	R	6 (13.0)	4 (66.6)	6(100)
	S	40 (87.0)	18 (45.0)	21(52.5)
Cotrimoxazole (COT)	R	4 (8.7)	2 (50.0)	1(25.0)
	S	42 (91.3)	20 (47.6)	26 (61.9)
Cefotaxime (CTX)	R	33 (71.7)	17 (51.5)	18(54.5)
	S	13 (28.3)	5 (38.5)	9(69.2)
Gentamicin (GEN)	R	19(41.3)	6(31.6)	10(52.6)
	S	27(58.7)	16(59.3)	17(63)
Nalidixic acid (NA)	R	1 (2.2)	1(100)	1(100)
	S	45(97.8)	21(46.7)	26 (57.7)
Tetracycline (TE)	R	5(10.9)	2 (40.0)	3(60.0)
	S	41(89.1)	20(48.8)	24 (58.5)
Vancomycin (VA)	R	45(97.8)	21 (46.7)	26 (57.8)
	S	1(2.17)	1 (100.0)	1(100.0)

Table 2. Antibiotic resistance profiles, MAR index, presence of CRISPR loci and *tdh* gene in *V. parahaemolyticus* isolates

Antimicrobial resistance profile	No. of isolates showing profile	CRISPR	<i>tdh</i>	MAR index
AMP-C-CAC-CAZ-CTX-GEN-VA	1	1	–	0.64
AMP-C-CAC-CIP-GEN-VA	1	1	1	0.55
AMP-C-CAC-GEN-VA	1	–	1	0.45
AMP-C-CAC-VA	1	1	1	0.36
AMP-C-GEN-TE-VA	1	–	–	0.45
AMP-CAC-CAZ-CIP-GEN-VA	1	0	1	0.55
AMP-CAC-CAZ-COT-CTX-GEN-VA	2	1	0	0.64
AMP-CAC-CAZ-COT-CTX-TE-VA	1	0	1	0.64
AMP-CAC-CAZ-COT-CTX-VA	1	1	0	0.55
AMP-CAC-CAZ-CTX-GEN-VA	5	1	2	0.55
AMP-CAC-CAZ-CTX-TE-VA	1	1	1	0.55
AMP-CAC-CAZ-CTX-VA	3	1	1	0.45
AMP-CAC-CIP-CTX-VA	1	1	1	0.45
AMP-CAC-CTX-NA-VA	1	1	1	0.45
AMP-CAC-CTX-VA	2	1	1	0.36
AMP-CAC-VA	1	–	1	0.27
AMP-CAZ-CTX-GEN-VA	3	2	2	0.45
AMP-CAZ-CTX-VA	1	–	–	0.36
AMP-CIP-CTX	1	1	1	0.27
AMP-CIP-CTX-VA	1	1	1	0.36
AMP-CTX-GEN-VA	1	–	–	0.36
AMP-CTX-VA	4	3	2	0.27
AMP-NA-VA	1	0	1	0.27
AMP-TE-VA	1	0	–	0.27
AMP-VA	4	2	3	0.18
C-CAC-CAZ-CTX-TE-VA	1	1	1	0.55
CTX-GEN-VA	1	–	1	0.27
VA	2	1	1	0.09

Table 3. Statistical significant values for the association between resistance to different antibiotics for *V. parahaemolyticus* isolates

Antibiotic	AMP		C		CAC		CAZ		CIP		COT		CTX		GEN		NA		TE		VA	
	r	p	r	p	r	p	r	p	r	p	r	p	r	p	r	p	r	p	r	p	r	p
AMP	-	-																				
C	-0.11	NS	-	-																		
CAC	0.18	NS	0.23	NS	-	-																
CAZ	0.13	NS	-0.10	NS	0.49	1%	-	-														
CIP	0.12	NS	0.04	NS	0.10	NS	-0.1	NS	-	-												
COT	0.10	NS	-0.12	NS	0.28	10%	0.34	5%	-0.12	NS	-	-										
CTX	0.15	NS	-0.33	5%	0.30	5%	0.58	1%	0.10	NS	0.19	NS	-	-								
GEN	0.10	NS	0.20	NS	0.15	NS	0.38	1%	0.07	NS	0.05	NS	0.13	NS	-	-						
NA	0.05	NS	-0.06	NS	0.14	NS	-0.14	NS	-0.06	NS	-0.05	NS	0.09	NS	-0.13	NS	-	-				
TE	-0.14	NS	0.28	10%	0.04	NS	0.10	NS	-0.14	NS	0.14	NS	-0.09	NS	-0.15	NS	-0.05	NS	-	-		
VA	-0.05	NS	0.06	NS	0.16	NS	0.14	NS	-0.38	1%	0.05	NS	-0.09	NS	0.13	NS	0.02	NS	0.05	NS	-	-

AMP: ampicillin; C: chloramphenicol; CAC: ceftazidime/clavulanic acid; CAZ: Cefotaxime; CIP: Ciprofloxacin; COT: Cotrimoxazole; CTX: Cefotaxime; GEN: Gentamicin; NA: Nalidixic acid; TE: Tetracycline; VA: Vancomycin

r = Correlation coefficient value: -1 (perfect negative relationship); +1 (perfect positive relationship); 0 (absence of linear relationship)
p = significance level; Bold = significant correlation between resistance to antibiotics

Distribution of *tdh* in *V. parahaemolyticus* isolates

PCR amplification of the *tdh* gene yielded an amplicon of 251 bp (Fig. 2b). Among the 46 *V. parahaemolyticus* strains, *tdh* gene was detected in 27 (58.7%) of the isolates. Presence of *tdh* gene in relation to antibiotic resistance is presented in Table 1. Although, the resistance to antibiotics did not differ significantly ($p>0.05$) in presence or absence of *tdh* gene, a significant correlation was seen in the case of ciprofloxacin resistance and the presence of *tdh* gene in strains ($r=0.3249$, $p=0.05$) (Table 2). Fisher's exact test also demonstrated CIP resistance to be significantly associated to *tdh* gene at $p<0.05$.

Distribution of CRISPR in *V. parahaemolyticus* isolates

PCR amplification of the CRISPR loci yielded amplicons ranging from 250-270 bp (Fig. 2a). To characterise the CRISPR, few representative PCR products were sequenced and submitted to GenBank (Accession Nos. MG765517, MG765518, MG765519, MG765520 and MG76552).

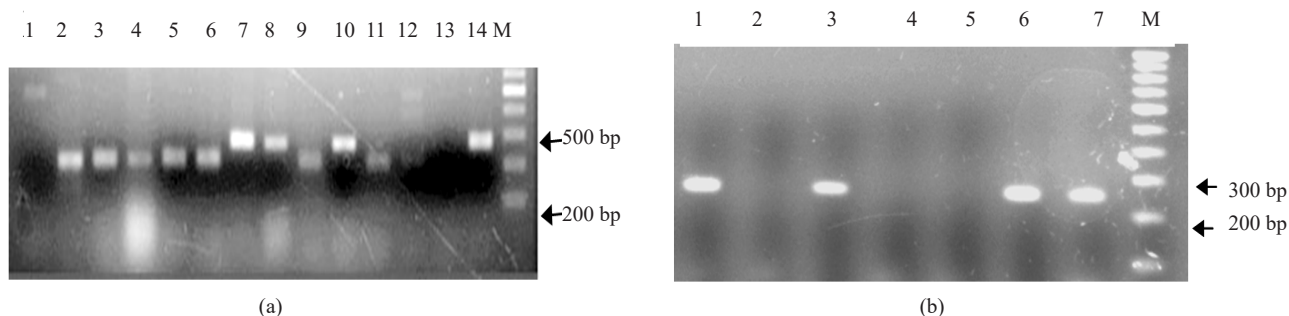


Fig. 2. PCR amplification of CRISPR loci and *tdh* gene

(a) PCR amplification of CRISPR loci. Lane 1: negative control (AQ4037); Lane 2: Positive control (VP49); Lanes 3-14: *V. parahaemolyticus* strains positive for CRISPR; Lane M: 100 bp ladder

(b) PCR amplification of *tdh* gene. Lanes 1,3,6,7: *V. parahaemolyticus* strains positive for *tdh*. Lane M:100 bp ladder

The CRISPR loci was detected in 22 (47.8%) of the 46 *V. parahaemolyticus* strains tested in this study (Table 2). No significant correlation was observed between antibiotic resistance and presence/absence of CRISPR, except for gentamicin resistance which was observed to be significant and negatively correlated ($r=-0.272$, $p<0.10$). Significant association for gentamicin resistance and CRISPR was also observed in Fisher's exact test ($p<0.10$). Seventeen of the 22 CRISPR positive isolates also were detected to harbour the *tdh* gene (Table 2), the association of which was found to be statistically significant at $p<0.05$.

Discussion

V. parahaemolyticus an important human pathogen that displays high genetic variability due to high rates of

recombination and mutation (Li *et al.*, 2017). While the horizontally acquired antibiotic resistance and virulence traits could confer a fitness advantage (Bondy-Denomy and Davidson, 2014), they could also prove to be a burden on host bacteria, triggering the need for their removal via CRISPR mediated immunity (Jiang *et al.*, 2013). Our analysis showed that resistance to multiple antibiotics is widespread among the environmental isolates of *V. parahaemolyticus*. The higher MAR index indicates that the samples originated from contaminated sources where there is a frequent and indiscriminate use of antibiotics. The occurrence of multi-antibiotic resistant bacteria in seafood and aquatic environments is a major concern and can pose a great risk to human health (Elmahdi *et al.*, 2016). This warrants a necessity for stringent supervision, restricted use of antibiotics and frequent surveillance of *V. parahaemolyticus* strains for resistance to antibiotics (Drais *et al.*, 2016; Park *et al.*, 2018). Majority of the strains in our study showed resistance to ampicillin and vancomycin. A high prevalence rate of ampicillin and vancomycin resistance in *V. parahaemolyticus* strains

isolated from oyster aquaculture sources in Korea have been reported recently (Jun *et al.*, 2012; Kang *et al.*, 2016). This indicates that ampicillin and vancomycin may no longer be effective in treating infections caused by this organism. Our studies showed that the null hypothesis holds good and the presence or absence of CRISPR did not affect the antimicrobial resistance capacity of strains. Similar studies conducted earlier in *E. coli* concluded that the CRISPR system lacked the capacity to resist the spread of antibiotic resistance among strains (Touchon *et al.*, 2012). This is in contradictory to the observations in *Enterococcus faecalis* strains, where the absence of CRISPR contributes to the development of multidrug resistant strains (Price *et al.*, 2018). An inverse correlation between the presence of a CRISPR-*cas* locus and horizontally transferred

antibiotic resistance in *E. faecalis* has been reported earlier (Palmer and Gilmore, 2010). Earlier studies suggested a positive correlation between virulence and antibiotic resistance owing to selection pressure and underlying genetic mechanisms (Zhang *et al.*, 2015). However, our studies demonstrated no significant association between antibiotic resistance and the presence of virulence gene *tdh* in *V. parahaemolyticus*. This is in accordance to an earlier study which reported absence of a significant correlation between antimicrobial resistance and virulence or genetic diversity in *V. parahaemolyticus* strains (Li *et al.*, 2017).

However, in our study a significant association was detected between the CRISPR and the virulence factor *tdh*. This further corroborates the earlier findings which reported a significant association between these two elements of *V. parahaemolyticus* (Sun *et al.*, 2015). Earlier studies in *V. cholera* had shown the co-acquisition of a

possessed 1-2 spacers with 3 showing homology to self spacers of *V. parahaemolyticus*, 2 to plasmid sequences of *V. alginolyticus* and one having no match in database (Table 4) which suggests genetic exchange between species as well as strains in the aquatic environment. Although in this study, a lack of correlation was observed between antibiotic resistant genotypes to acquisition of CRISPR, this needs further investigation by taking into consideration respective antibiotic resistance genes for exactly elucidating their correlation. However, owing to its close association with the virulence genes, it might have a role in modulation of pathogenicity. As with previous studies, the presence of CRISPR loci in strains was found to be significantly associated to the presence of virulence gene *tdh* (Sun *et al.*, 2015), which probably implicates the two to be linked and probably involved in modulating the pathogenicity, which requires further study.

Table 4. Sequence match to *V. parahaemolyticus* CRISPR spacer sequences

Accession No.	No. of Spacers	Spacer sequence	Sequence match
MG765517	2	TAATTCTCACGATCTAATTACAGTTGGTCAC	<i>V. alginolyticus</i> strain K08M3 plasmid pL300
		AGTCGGTCAACTGAGAATACGTTGTTGCCAA	<i>V. alginolyticus</i> strain K08M3 plasmid pL300
MG765518	2	TAGATAACCACGGTCTAATTACAGTTGGTCAC	No match
		AGTCGGTCAACTGAGAATACGTTGTTGCCAA	<i>V. alginolyticus</i> strain K08M3 plasmid pL300
MG765519	1	CTGATAATAAGATACCACAGGCTCAAGCAGATGCTAACAG	<i>V. alginolyticus</i> strain K08M3 plasmid pL300
MG765520	1	TCATTCTCACGATCTAATTACAGTTGGTCAC	<i>V. alginolyticus</i> strain K08M3 plasmid pL300
MG765521	1	CCACTAACCGATACCCACCGACTAGACTACATTTG	No match

virulence factor type III secretion system (T3SS) and Type IF CRISPR-Cas systems as a complete entity (Carpenter *et al.*, 2017). Type I-F CRISPR-cas system in *Pseudomonas aeruginosa* has been implicated in the regulation of virulence (Wiedenheft and Bondy-Denomy, 2017). The CRISPR-Cas system is believed to be involved in biofilm formation and swarming behaviours of *P. aeruginosa* (Zegans *et al.*, 2009).

An analysis of the sequenced *V. parahaemolyticus* CRISPR loci in this study, showed it to belong to the type IF CRISPR-Cas system. This is in accordance to previous reports wherein majority of *V. parahaemolyticus* are known to belong to the type IF system (Sun *et al.*, 2015). The sequence pertaining to the direct repeat as well as spacers is presented in Table 4. The strains

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