Detection of virulence genes, antibiotic resistance genes, plasmid profile and molecular typing among *Vibrio vulnificus* isolated from Malaysian seawater and sediment

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ABSTRACT:

This study was aimed to investigate the presence of virulence genes, antibiotic resistance genes and molecular diversity of *V. vulnificus* isolated from several coastal areas in Malaysia. Fifty – five isolates were first tested for the presence of seven virulence genes including Hemolysin (*vvhA*), Extracellular Capsule Polysaccharide (CPS) (*hp1*), Virulence correlated gene (*vcg*), Virulence regulator gene (*toxR_{vv}*), Cytotoxin gene (*rtxA*), Metalloprotease (*vvpE*) and Pili gene (*pilA*). Hundred percent of tested seawater isolates were carrying *pilA* gene, followed by 87.5% for both *vvpE* and *vvhA* genes. While, 100% of sediment isolates were having *vcg* and *vvhA* genes. The presence of 7 antibiotic resistance genes including Streptomycin (*strB*), β -lactamase (*blaP1*), Chloramphenicol (*floR*), Tetracycline (*tetA*), Erythromycin (*ermB*), Quinolone resistance protein (*qnrA*) and Aminoglycosides (*aac(3)-IIa*) were investigated. In seawater, 65% of isolates were having *blaP1* gene, and the lowest percentage was found to be 7.5% of the *ermB* gene while 66.66% of sediment isolates were carrying *blaP1* gene and the lowest percentage was 13.33% of *strB* gene. The plasmid sizes that detected in this study were ranging from 2.7kb to 25.4kb even though 6 of the isolates were plasmidless. The RAPD-PCR analysis of typable *V. vulnificus* isolates showed 6 clusters and 3 single isolates. The ERIC-PCR analysis of typable *V. vulnificus* has divided the isolates into 11 clusters and 10 single isolates. The results of this research have given information of the presence of *V. vulnificus* that carry the factors of virulence and resistance to many classes of antibiotics are directed in the recreational areas of what causes a potential risk of infections and this bacteria might driven from the sewage and /or medical waste disposal and suggesting the regular monitoring of this bacteria in coastal areas.

Key word: *Vibrio vulnificus*, virulence genes, antibiotic resistance genes, plasmid profile, RAPD and ERIC PCR.

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INTRODUCTION

Vibrio vulnificus is a gram negative an opportunistic human pathogen that naturally inhabits estuarine and coastal waters worldwide. This bacterium has been ubiquitous in these environments and isolated from seawater, sediments, fish, and shellfish such as bivalve molluscs aquatic beards and mammals (including humans) being found as free living bacteria in marine waters [1]. This organism is halophilic, and it is routinely found in waters of estuarine environments as part of the normal microflora, as well as in oysters. Other shellfish inhabiting brackish waters in estuaries is a diverse species of moderately halophilic marine bacteria that is found in tropical and warm waters with morphology of curved motile rods and a single motile polar flagellum. More than 100 strains of this bacterial species have been reported and it is rational that several thousands of them still exist [2]. The species also displays diverse lifestyles. Previous studies have reported that *V. vulnificus* strains possess pathogenic potential for humans and aquatic animals, such as shrimp [3] and eels [4, 5, 6, 7].

V. vulnificus is virulent for humans and fish. In terms of virulence, *V. vulnificus* isolates are classified into three biotypes, two of which, biotypes 1 and 3 are classified as human isolates and biotype 2 is classified a fish and eel isolates. There are various factors implicated as possible virulence determinants for *V. vulnificus*, CPS is considered a major virulence factor of *V. vulnificus* which allows survival of the bacteria in the human host [8]. The epimerase gene is essential for capsule expression in *V. vulnificus* [9], extracellular enzymes include hemolysin, cytolysin, and elastolytic protease. The ability of *V. vulnificus* to cause a disease is linked to the production of a large cytotoxin called the "multifunctional-autoprocessing RTX" (MARTXVv) toxin [10, 11, 12]. The capability of bacterial adherence to the host cells is another important virulence factor for many pathogens, including *V. vulnificus*. Moreover, *NanA* gene is obviously crucial for the growth and adhesion of *V. vulnificus* during infection of mice [13].

Plasmids are important in certain bacteria since they code for proteins, especially enzymes, which can confer resistance to antibiotics. Strains of *V. vulnificus* possess one or more virulence plasmids with molecular mass that ranges between 68 and 70 kb. Virulence in fish seems to rely on a recently described plasmid that can be transmitted between strains by a conjugative plasmid [14, 15]. *V. vulnificus* strains were also found to carry more than plasmids with diverse sizes that carrying virulent elements [16].

Traditionally, *Vibrio* is highly susceptible to virtually all antimicrobials. However, recently, antimicrobial resistance has emerged and evolved in many bacterial genera due to the excessive use of antimicrobials in human, agriculture, and aquaculture systems [17]. In contrast, previous research has not documented the awareness of antimicrobial-resistant bacteria in the aquatic environment adequately. The only extensive investigation of antimicrobial susceptibility in *V. parahaemolyticus* in the United States had occurred in 1978, even before *V. vulnificus* was first recognized as a food-borne pathogen [18]. A few recent studies examining the antimicrobial susceptibilities of non-cholerae *Vibrio* spp. and other aquatic bacteria have been carried out in other countries, but with a very limited number of either *V. parahaemolyticus* or *V. vulnificus* isolates isolated from Saudi Arabia included in their investigation [16, 19, 20].

Molecular techniques for *Vibrio* identification and subtyping have been developed, including DNA microarray technologies and PCR-based methods which target species-specific determinants. PCR-based molecular-typing studies have been performed with amplified fragment length polymorphism (AFLP), enterobacterial repetitive intergenic consensus (ERIC)-PCR and randomly amplified polymorphic DNA (RAPD) analysis [21]. Therefore, the current study aimed to determine the presence of virulence genes, antibiotic resistance genes, plasmid and study the molecular characterization of *V. vulnificus* from seawater and sediment samples from Malaysia.

Materials and Methods

Sampling sites and samples collection:

Sampling sites were carefully selected and included ten recreational sites of four different public beaches of West Malaysia (Bachok, Kelantan; Port Klang, Mersing, Johor; Selangor; Port Dickson, Negeri Sembilan) see figure 1. Typically, water samples were collected mornings (9-11 am) at 50 cm water depths using sterile 500 mL Schott bottles following the hand-dip method [22]. Sediment samples were collected as described by [23], sterile disposable plastic containers and 100 ml vials were used to collect sediments from the sampling stations and carefully forcing them into the sand at about 10 cm depth. All Samples were maintained at a temperature of 1 to 10 °C in a pre-chilled cooler containing sufficient cold artificial ice and transferred to the laboratory within 2-5 h.

Fig 1. Map of Malaysia and sampling station sites; 1: Bachok, Kelantan; 2: Port Klang; 3: Mersing, Johor; 4: Port Dickson, Negeri Sembilan

Bacterial isolation and identification:

For isolation and identification, serial dilution method and spreading plate technique were used for growing *V. Vulnificus*. Two media were used for isolation and identification: Thiosulfate Citrate Bile Salts Sucrose Agar -TCBS (Oxoid, UK) and Chromogenic Vibrio Agar CV (Titan Media, India). The typical colonies that were assumed to be *V. Vulnificus* that have shown green color on TCBS agar and mauve on CV agar were then subjected to biochemical tests that designed by following Bergey's manual and FDA manual [24]. All the isolates were maintained on Trypticase Soy Agar TSA (Oxoid, UK) plates at 4 °C and for long-term storage in Trypticase soy broth TSB (Oxoid, UK) containing 40 % glycerol at -70 °C [25].

DNA extraction:

DNA extraction was conducted by DNA Purification Kit (Promega, USA) as described in the manufacturer's manual. The identified *V. vulnificus* isolated were then grown in 5 ml of Lauria Bertani (LB) broth (Oxoid, UK) at 35 °C for 20 h. The extracted genomic DNA was then stored at 4 °C for further studies.

Plasmid isolation:

For plasmid extractions, *V. vulnificus* were grown overnight in LB broth with an addition of 4 % (w/v) NaCl at 35 °C, and the plasmids were extracted with the FavorPrep™ Plasmid Extraction Mini Kit (FAVORGEN BIOTECH CORP., Taiwan) to get the high-quality plasmid DNA following manufacturer's instruction. The yield of this process were then loaded in 1% (w/v) agarose gel. The gel electrophoresis was run at 80 V for 1 hour, and DNA marker, 1 kb Extend DNA ladder (New England BioLabs, USA) was used. The gel was then visualiz under UV light through gel documentation of GeneSys G: BOX EF2 (Syngene, USA). The gel image was recorded and analyzed [26]. The plasmid DNA products were stored at -20°C for further analysis

PCR amplification of virulence genes:

Screening for the presence of virulence genes among 55 *V. vulnificus* isolates (40 recovered from seawater and 15 from sediments) was done. Polymerase Chain Reactions (PCR) were conducted to amplify hemolysin (*vvhA*), Extracellular Capsule Polysaccharide CPS (*hp1*), virulence correlated gene (*vcg*), virulence regulator gene (*toxR_{Vv}*), cytotoxin gene (*rtxA*), metalloprotease (*vvpE*) and pili gene (*pilA*) [27, 28, 29,30 31, 32, 33]. The primes list and PCR reaction conditions were listed in table 1 and table 2, respectively. Positive control (*V. vulnificus* ATCC 27562) and negative control (*E.coli* ATCC 25992) were used in this study. All reactions were conducted in a Mastercycler® thermal cycler (Eppendorf, Germany). Gel electrophoresis assay was done by mixing 10 µL of the amplicon with 2 µL of gel loading dye and electrophoresed in pre-stained 1.0 % agarose (Sigma Aldrich, USA) for 1 h at 85 V in 1x Tris-Acetate - Ethylenediaminetetraacetic acid (TAE) buffer (FirstBase Sdn Bhd, Malaysia). The 100bp and 1 kb DNA ladders (Invitrogen, Belgium) was used as a molecular size marker.

Table 1. Primer used to detect virulence genes in this study

Gene name	Primer name	Primer sequence (5' to 3')
Hemolysin (<i>vvhA</i>)	<i>vvhA</i> -F	CCGCGGTACAGGTTGGCGCA
	<i>vvhA</i> -R	CGCCACCCACTTTCGGGCC
Extracellular Capsule Polysaccharide (CPS) (<i>hp1</i>)	<i>hp1</i> -F	TTTGGGATTTGAAAGGCTTG
	<i>hp1</i> -R	GTGCCTTTGCGAATTTTGAT
Virulence correlated gene (<i>vcg</i>)	<i>vcgC</i> -F	AGCTGCCGATAGCGATCT
	<i>vcgC</i> -R	TGAGCTAACGCGAGTAGTGAG
Virulence regulator gene (<i>toxR_{Vv}</i>)	<i>toxR</i> -F	GASTTTGTTTGGCGYGARCAAGGTT
	<i>toxR</i> -R	AACGGAAGTTAGACTCCGAC
Cytotoxin gene (<i>rtxA</i>)	<i>rtxA</i> -F	CGGGATCCTATGGCGTGAACGGCGAAG
	<i>rtxA</i> -R	CGGGATCCAGCAGCCACAAGCGATTCT
Metalloprotease (<i>vvpE</i>)	<i>vvpE</i> -F	GTGCGGAAGAAGAGCC
	<i>vvpE</i> -R	GGCCGTGAGAGCACTCCGG
Pili gene (<i>pilA</i>)	<i>pilA</i> -F	TGGCTGCTGTTGCTATTC
	<i>pilA</i> -R	GGTCCACCACTAGTACCAAC

Table 2. PCR conditions of detection of virulence genes

Gene name	Size (bp)	Primer Conc.	dnTP (μM)	Taq poly. (units)	MgCl ₂ (mM)	Buflr	Anne Temp (°C)	Reference
<i>vvhA</i>	519	0.5	200	0.04	2.0	1x	57	M34670, NCBI
<i>hp1</i>	342	0.5	200	0.04	2.0	1x	57	DQ360502, NCBI
<i>vcg</i>	99	0.5	200	0.04	2.0	1x	57	AY626575, NCBI
<i>toxR_{Vv}</i>	435	0.5	200	0.04	2.0	1x	57	25
<i>rtxA</i>	1440	0.5	200	0.04	2.0	1x	61	28
<i>vvpE</i>	426	0.5	200	0.04	2.0	1x	55	31
<i>pilA</i>	217	0.5	200	0.04	2.0	1x	50	30

PCR amplification of antibiotic resistance genes:

All *V. vulnificus* isolates (n=55) from both, seawater and sediment samples were screened first phenotypically by using 14 different antibiotics {Ampicillin AM (10μg), Amoxicillin AX (10μg), Chloramphenicol C (30μg), Ciprofloxacin CIP (10μg), Gentamycin CN (10μg), Erythromycin E (15μg), Nitrofurantion F (200μg), Kanamycin K (30μg), Cephalothin KF (30μg), Nalidixic acid NA (30μg), Penicillin P (10u), Carbenicillin PY (100μg), Streptomycin S (25μg) and Tetracycline TE (30μg)} (Oxoid, UK) through disk diffusion method [34] and genotypically by PCR technique to detect the carriage of genes coding for resistance to antibiotics. The following resistant genes streptomycin resistance (*strB*), β-lactamase resistance (*blaP1*), chloramphenicol resistance (*floR*), tetracycline Resistance (*tetA*), erythromycin resistance (*ermB*), quinolone resistance protein (*qnrA*) and aminoglycosides resistance (*aac(3)-IIa*) were employed in this investigation for detection of genes responsible for resistance and previously described by [35, 36, 37, 38]. The primes list and PCR reaction conditions were listed in table 3 and table 4, respectively. All reactions were conducted in a Mastercycler® thermal cycler (Eppendorf, Germany). Positive and negative controls were included for each set of amplifications. A 100 bp and 1 kb DNA ladder (Invitrogen, Belgium) was used as a molecular size marker.

Table 3. Primers used for detection of antibiotic resistance genes in *V. vulnificus*

Gene name	Primer name	Primer sequence (5' to 3')
Streptomycin resistance (<i>strB</i>)	<i>strB</i> -F	CCGCGATAGCTAGATCGCGTT
	<i>strB</i> -R	CGACTACCAGGCGACCGAAAT
β-lactamase resistance (<i>blaP1</i>)	<i>blaP1</i> -F	GGCATCCAAGCAGCAAG
	<i>blaP1</i> -R	CTGGTTCATTTCAGATAGCG
Chloramphenicol Resistance (<i>floR</i>)	<i>floR</i> -F	TTATCTCCCTGTCTCGTTCCAGCG
	<i>floR</i> -R	CCTATGAGCACACGGGGAGC
Tetracycline Resistance (<i>tetA</i>)	<i>tetA</i> -F	GTAATTCTGAGCACTGTCGC
	<i>tetA</i> -R	CTGCCTGGACAACATTGCTT
Erythromycin resistance (<i>ermB</i>)	<i>ermB</i> -F	AGACACCTCGTCTAACCTTCGCTC
	<i>ermB</i> -R	TCCATGTACTACCATGCCACAGG
Quinolone resistance protein (<i>qnrA</i>)	<i>qnrA</i> -F	AATTTTAAGCGCTCAAACCTCCG
	<i>qnrA</i> -R	TCCTGTTGCCACGAGCATATTTT
Aminoglycosides resistance (<i>aac(3)-IIa</i>)	<i>aac(3)-IIa</i> -F	CGGCCTGCTGAATCAGTTTC
	<i>aac(3)-IIa</i> -R	AAAGCCCACGACACCTTCTC

Table 4. PCR conditions for detection of antibiotic resistance genes in *V. vulnificus*

Gene	Product Size (bp)	Primer Conc.	dnTP (μM)	Taq poly. (units)	MgCl ₂ (mM)	Buffer	Annealing Temp (°C)	Reference
<i>strB</i>	515	0.5	200	0.04	2.0	1x	64	49
<i>blaP1</i>	874	0.5	200	0.04	2.0	1x	55	48
<i>floR</i>	526	0.5	200	0.04	2.0	1x	64	50
<i>tetA</i>	950	0.5	200	0.04	2.0	1x	60	51
<i>ermB</i>	640	0.5	200	0.04	2.0	1x	63	52
<i>qnrA</i>	521	0.5	200	0.04	2.0	1x	62	34
<i>aac(3)-IIa</i>	436	0.5	200	0.04	2.0	1x	61	53

Molecular typing of *V. vulnificus* by RAPD-PCR:

To assess the utility of RAPD-PCR in the subtyping of *V. vulnificus* recovered during the study, all isolates were subjected to RAPD-PCR. The Gen1 5'-AGGATACGTG-3' primer [39] was used for the RAPD-PCR reactions. *V. vulnificus* ATCC 27562 was used as a reference, and negative controls were performed by adding 1 μl of sterile ultrapure deionized water [40]. PCR amplifications were performed in 0.2 mL and the total volume of the reaction mixture was 50 μl consisting of 25 μl 10x PCR master mix (EconoTaq® PULS GREEN 2X Master Mix, Lucigen), 0.5 μl of primer and template DNA 1.0 μl, and then, the volume adjusted to the final volume by adding Nuclease Free Water (NFW). PCR conditions adopted involving initial denaturation at 95 °C for 5 min following by 45 cycles of denaturation at 94 °C for 1 min, annealing for 1 min at 35 °C and polymerization at 72 °C for 2 min. Final elongation was at 72 °C for 7 min [40].

Molecular typing of *V. vulnificus* by ERIC-PCR:

The ERIC-PCR assay was performed on all *V. vulnificus* isolates that recovered during the study. The PCR technique was carried out in 0.2 ml microcentrifuge tubes, with a 50 μl reaction mixture consisting of 25 μl of 2X DreamTag Green PCR Master Mix (Thermo Scientific, USA), 1 μl of 100 μM of each ERIC primers as described previously by [39]; ERIC-1 (5'- CAC TTA GGG GTC CTC GAA TGT A -3') and ERIC-2 (5'- AAG TAA GTG ACT GGG GTG AGC G -3') [41], 1 μl of approximately 100 ng DNA template. This was followed by adjusting the volume until 50 μl by adding nuclease-free water (NFW). Both positive and negative DNA controls were included in each reaction. The cycling conditions were as follows; pre-denaturation at 95°C for 7 minutes, denaturation at 90°C for 30 seconds, annealing at 58°C for 1 minute, and extension at 65°C for 8 minutes, with a final extension at 68°C for 16 minutes at the end of 30 cycles.

RAPD and ERIC-PCR analysis:

A 10 μL volume of each PCR product was mixed with 2 μL of loading dye and subjected to a 1% TAE buffer electrophoresis system. In each run, a molecular weight marker 100 bp PCR ladder (Invitrogen) was included. Following photography using a UV transilluminator and the relatedness of the bacterial isolates were estimated by ERIC-PCR and RAPD-PCR according to the photographic image of the gel using ImageJ. The data obtained then were clustered

using average linkage (UPGMA, unweighted group pair method with arithmetic averages) by using PHYLIP (version 3.697) application and the results were performed in dendrogram form.

Results and Discussions

Percentage of virulence genes of *V. vulnificus*:

The percentage of virulence genes of *V. vulnificus* from seawater and sediment isolates are presented in Figure 2 and Table 5. For *V. vulnificus*, seven virulence genes were evaluated, and analysis of the results have shown that only one gene (*pilA*) had 100% virulence while two other genes had a high percentage of virulence and these are genes *vvhA* and *vvpE* which had 87.5% virulence each. The gene with the least virulence from seawater isolates was gene *rtxA*. However, two genes (*vcg* and *vvhA*) from sediment isolates have 100% virulence while *hpl* had 93.33% virulence. The gene with the least percentage of virulence from sediment isolates was still gene *rtxA*. The low percentage of *rtxA* virulence gene as observed in this study is in contrast with those reported by previous study [42], that found 75.5% of virulence gene to be *rtxA* gene. The generality of the results showed that isolates from sediment have a higher percentage of virulence compared to seawater isolates and all isolate groups.

Fig 2. Percentage of virulence genes of *V. vulnificus* from seawater isolates, isolates from sediment and all isolates

Table 5. Percentage of virulence genes of *V. vulnificus*

Gene	Seawater n=40 (%)	Sediment n=51 (%)	All isolates n=55 (%)
<i>hp1</i>	21 (52.5)	14 (93.33)	35 (63.63)
<i>toxRVv</i>	21 (52.5)	12 (80)	33 (60)
<i>vcg</i>	29 (72.5)	15 (100)	44 (80)
<i>vvhA</i>	35 (87.5)	15 (100)	50 (90.9)
<i>rtxA</i>	13 (32.5)	1 (6.66)	14 (25.45)
<i>vvpE</i>	35 (87.5)	10 (66.66)	45 (81.81)
<i>pilA</i>	40 (100)	5 (33.33)	45 (81.81)

Fig 3. Detection of virulence genes in *V. vulnificus* isolates by PCR technique, electrophoresed on 1.5 % (w/v) agarose gel. Lanes 1, 2: vvhA gene in VVAW2 and VVCW1. Lanes 3, 4: hp1 gene in VVKW1 and VVKW3. Lanes 5, 6: vcg gene in VVAW1 and VVAW2. Lanes 7, 8: toxR gene in VVCW3 and VVDW3. Lanes 9, 10: rtxA gene in VVCW3 and VVDW3. Lanes 11, 12: vvpE gene in VVAW2 and VVBW1. Lanes 11, 12: pilA gene in VVDW8 and VVDW9

Antibiotic resistance patterns, plasmid profile of *V. vulnificus*:

The antibiotic resistance patterns and plasmid profile of *V. vulnificus* isolated from this study is presented in Table 6. The results showed that all the isolates were resistant to at least four of the tested antibiotics, implying multi-drug resistance. This agrees with the findings of previous study [43], where multiple antibiotic resistances were reported from *Vibrio* isolates of different sources. Two of the isolates (VVEW1, VVKW2) were found to be resistant to 13 of the tested antibiotics. In a recent study [44], multiple drug resistance was reported from *V. vulnificus* isolates. Twenty different antibiotic resistance patterns were recorded from the *V. vulnificus* isolated in this study. The isolates have plasmid sizes ranging from 2.7kb to 25.4kb even though many of the isolates were plasmidless. Plasmid sizes ranging from 9 kb to 123 kb has been reported in an earlier study on *Vibrio* isolates [45].

Table 6. Antibiotic resistance patterns and plasmid profiling of *V. vulnificus*

Antibiogram	Isolate codes	Patter n	Plasmid Size (kb)
AM, AX, C, CIP, CN, E, F, K, KF, NA, P, PY, S	VVEW1, VVKW2	A	5.4, 13, 25.4, 35
AM, AX, C, CIP, E, F, K, KF, NA	VVDW4, VVEW3, VVFW6	B	4.3, 6.5, 10.4
AM, AX, CIP, F, K, KF, NA	VVJW4, VVKW1, VVLW3	C	7.6, 10.4
AM, CIP, F, K, P, PY, S	VVHW6, VVIW3, VVKW3, VVLW2	D	2.7, 5.4, 24.8
AM, AX, P, PY, S, TE	VVAW1, VVBW1, VVCW2, VVDW3, VVDW6, VVEW2, VVGW1, VVHW3, VVIW1, VVJW3, VVJW6, VVLW1	E	2.7, 6.5, 35
AM, F, KF, P, PY	VVJW5, VVLW4	F	N.D
AM, F, KF, P, S	VVCW3, VVKW4	G	N.D
AM, F, P, PY, S	VVAW2, VVDW5, VVHW2, VVIW5	H	N.D
AM, CIP, F, K	VVCW1, VVDW8, VVFW2,	I	N.D

	VVHW4, VVIW2, VVJW2		
AM, F, P, PY	VVCW5, VVDW9	J	10.4
AM, CN, E, F, K, KF, NA, P, PY, S, TE	VVDS1, VVKS2	K	2.7, 4.3, 5.4, 6.5, 7.6, 10.4, 13, 16.9, 24.8, 25.4, 35
AM, CN, E, F, KF, NA, P, TE	VVDS2	L	4.3, 7.6, 25.4
AM, AX, C, CN, E, F, KF, P	VVJS2, VVLS1	M	2.7, 16.9
AM, AX, CN, E, KF, P, PY	VVES2	N	N.D
AM, AX, F, KF, P, PY	VVHS2	O	4.3, 16.9
AX, CN, E, KF, P, PY	VVES2	P	N.D
AX, CN, E, P, PY	VVBS1, VVLS2	Q	N.D
AX, C, P, PY, S	VVCS2, VVES1, VVIS1	R	N.D
AX, CN, P, PY	VVIS3	S	6.5, 10.4
CN, E, P, PY	VVES3	T	N.D

Percentages of antibiotic resistance genes of *V. vulnificus*:

The rate of resistance genes of *V. vulnificus* from seawater and sediment isolates were evaluated in this study, and the results are presented in figure 4, 5 and Table 7. In case of seawater isolates, the results showed that no gene from seawater isolates has 100% of resistance gene. The highest rate of resistance was obtained from *blaPI* which has 65% of the resistance gene, followed by *strB* with 60% of resistance gene. In a recent study, however, the percentage of resistance of *strB* was found to be 22.2% [45]. The rest from this group have a low rate of resistance genes and the least among them was *ermB* with 7.5% of resistance gene. While, the sediment isolates were also showing no gene from sediment isolates with 100% of resistance gene. The highest rate of resistance was from *blaPI* with 66.66% of resistance followed by *ermB* with 53.33% of resistance gene. The rest of the genes in this group have a low rate of resistance and the least was from *strB* with 13.33% of resistance gene.

Fig 4. Percentages of seven resistance genes of *V. vulnificus* from seawater isolates from sediment and all isolates

Fig 5. Detection of antibiotic resistance genes in *V. vulnificus* isolates by PCR technique, electrophoresed on 1.5 % (w/v) agarose gel. Lanes 1, 2: *StrB* gene in VVCW2 and VVDW3. Lanes 3, 4: *blaP1* gene in VVCW5 and VVDW9. Lanes 5, 6: *floR* gene in VVJS2 and VVLS1. Lanes 7, 8: *tetA* gene in VVIW1 and VVJW3. Lanes 9, 10: *ermB* gene in VVDW4 and VVFW6. Lanes 11, 12: *qnrA* gene in VVEW1 and VVKW2. Lanes 13, 14: *aac(3)-IIa* gene in VVDW4 and VVFW6

Table 7. Antibiotic resistance gene patterns of *V. vulnificus*

Resistance Gene Profile	Isolate codes	Pattern
<i>aac(3)-IIa, blaP1, floR, qnrA, strB</i>	VVEW1, VVKW2	A
<i>aac(3)-IIa, blaP1, ermB, floR, qnrA</i>	VVDW4, VVEW3, VVFW6	B
<i>blaP1, qnrA</i>	VVJW4, VVKW1, VVLW3	C
<i>blaP1, strB</i>	VVHW6, VVIW3, VVKW3, VVLW2	D
<i>strB, tetA</i>	VVAW1, VVBW1, VVCW2, VVDW3, VVDW6, VVEW2, VVGW1, VVHW3, VVIW1, VVJW3, VVJW6, VVLW1	E
<i>blaP1</i>	VVJW5, VVLW4	F
<i>blaP1, strB</i>	VVCW3, VVKW4	G
<i>strB</i>	VVAW2, VVDW5, VVHW2, VVIW5	H
<i>blaP1, qnrA</i>	VVCW1, VVDW8, VVFW2, VVHW4, VVIW2, VVJW2	I
<i>blaP1</i>	VVCW5, VVDW9	J
<i>aac(3)-IIa, blaP1, ermB, strB, tetA</i>	VVDS1, VVKS2	K
<i>blaP1, ermB, qnrA, tetA</i>	VVDS2	L
<i>blaP1, ermB, floR</i>	VVJS2, VVLS1	M
<i>blaP1, ermB</i>	VVES2	N
<i>blaP1</i>	VVHS2	O
<i>aac(3)-IIa, blaP1</i>	VVES2	P
<i>ermB</i>	VVBS1, VVLS2	Q
<i>floR, qnrA</i>	VVCS2, VVES1, VVIS1	R
<i>blaP1</i>	VVIS3	S
<i>aac(3)-IIa, blaP1</i>	VVES3	T

Molecular fingerprinting of *V. vulnificus* by RAPD-PCR and ERIC –PCR Typing

The dendrogram of typeable *V. vulnificus* isolates produced from RAPD-PCR and ERIC-PCR analysis using average linkage unweighted group pair method with arithmetic averages (UPGMA) are presented in Figures 6 and 7 respectively. The RAPD-PCR analysis using average linkage unweighted group pair method with arithmetic averages (UPGMA) discriminated typeable *V. vulnificus* isolate into 6 clusters and 3 single isolates, see figure 6. These findings are consistent with the findings of previous researchers [46], whom reported that *Vibrio vulnificus* isolates were discriminated into two clusters by RAPD-PCR analysis.

In another related study [21], *V. vulnificus* isolates were similarly discriminated into 12 clusters by RAPD-PCR analysis. In yet another study [48], *V. vulnificus* isolates were similarly discriminated into 13 clusters by RAPD-PCR analysis. The ERIC-PCR analysis using average linkage unweighted group pair method with arithmetic averages (UPGMA) however discriminated typeable *V. vulnificus* isolates into 11 clusters and 10 single isolates, see figure 7. These results corroborate with the findings of [49], who reported that *V. vulnificus* isolates from different locations in Malaysia were discriminated into 13 clusters by ERIC-PCR analysis.

Fig 6. Dendrogram of typeable *V. vulnificus* isolates produced from RAPD analysis using average linkage unweighted group pair method with arithmetic averages (UPGMA)

Fig 7. Dendrogram of typeable *V. vulnificus* isolates produced from ERIC analysis using average linkage unweighted group pair method with arithmetic averages (UPGMA)

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