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Antimicrobial resistance and Plasmid profile of *Vibrio alginolyticus* isolated from Malaysian seawater

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Abstract : The present study was aimed to detect the presence of multiple antibiotic resistance, antibiotic resistance genes and plasmid profile of *Vibrio alginolyticus* isolated from seawater and sediment of different beaches in Malaysia. Forty five isolates, including 24 seawater and 21 sediment isolates of *V. alginolyticus* were tested against 14 antibiotics for the antibiogram profile and the presence of the plasmids. Polymerase chain reaction (PCR) was conducted to elucidate the presence of 7 antibiotic resistance genes including Streptomycin resistance (*str*B), β-lactamase resistance (*bla*P1), Chloramphenicol Resistance (*flo*R), Tetracycline Resistance (*tet*A), Erythromycin resistance (*erm*B), Quinolone resistance protein (*qnr*A) and Aminoglycosides resistance (*aac*(3)-*IIa*). Antibiotic resistance studies revealed that in seawater isolates, the highest percentage of antibiotic resistant was obtained against erythromycin E and penicillin P (100%), whereas the lowest antibiotic resistant percentage was obtained from both chloramphenicol C and nalidixic acid NA (16.66 %). The sediment isolates of *V. alginolyticus* showed 100% resistance against both penicillin P and ampicillin AM and the lowest percentage was of gentamycin CN (0 %). There were 17 different antibiotic patterns were observed from the *V. alginolyticus* in this study. The plasmid size was ranged from 2.3 Kb to 21.6 Kb, while there was no detection of plasmid in19 isolates. The highest resistance gene percentage of seawater isolates was found to be *erm*B (91.66%) which was followed by *blap*l with 70.83% of resistance gene. The lowest percentage of resistance gene was *flo*R with 16.66% of resistance gene. The highest percentage of resistance gene in seawater isolates was found to be *tet*A with 61.9% of resistance gene and the lowest percentage was obtained from *floR* which had 14.28% resistance gene. The finding of this study was showen high percentage of resistance genes in seawater than sediment isolates. These results suggest that the *V. alginolyticus* isolated from seawater and sediments observed in this study were pathogenic, and involved a source of antibiotic resistance genes that could be transmitted to other population of bacteria through mobile genetic elements.

Key Words : *V. alginolyticus*; Antibiotic resistance; Resistance gens; Plasmid profiling.

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Introduction:

Vibrionaceae is a marine group of bacteria. Members of the family are Gram-negative, facultative, non-spore forming mostly characterized with "comma" shaped rod. The members of the *Vibrio* genus are gram-negative, halophilic bacteria known to be indigenous to coastal marine systems [1]. This group of bacteria is commonly found in fresh and salt water. This requirement for growth is simple involving synthetic media having glucose as sole carbon and energy sources and requiring seawater or salty water for optimal growth in the isolation media. While these common bacteria persist as an indigenous component of the coastal marine system, a small proportion of environmental isolates have been reported to carry the genetic determinants for human pathogenicity[2, 3, 4]. Antimicrobial resistance is one of the most challenging public health problems that are directly associated with management and control of diseases [5]. In the course of treating various bacterial diseases, antimicrobials like doxycycline, tetracycline, streptomycin, and erythromycin are utilized [6], resistance to which have been documented in several bacteria including *Vibrio* species [5, 7, 8]. Increasingly, higher frequency of drug-resistant *Vibrio* species has been documented by scholars [5, 9].On annual bases, increasing number of pathogenic *Vibrio* species are developing resistance toward most of the clinically utilized antibiotics [10]. Most of the genetic determinants that confer antibiotic resistance on the species are found in the plasmid. Plasmids are one of the vital modulators that enhance the transfer of antibiotic resistant genes and they are transmissible to the next generation via vertical gene transfer or exchanged with other bacteria via horizontal gene transfer [11, 12].The smaller chromosome was also discovered to have an integron island or super-integron, described as a capture system, whose genes are typically found on plasmids [13]. Therefore, epidemiological monitoring of drug-resistant strains of Vibrios have to be conducted to make the prevalence of multi-drug resistance that is related to the presence plasmids, and to get a new methods to prevent the spread of these drug-resistant strains. In this background, the present study is designed to assess the presence of antibiotic resistant strains and plasmids and their relationship with the antibiotic resistance in *Vibrio alginolyticus* isolated from seawater of different coastal area in Malaysia.

Materials and Methods:

Sampling sites:

Water and sediment samples were collected from 4 coastal sites of 4 different public beaches of Malaysia (Bachok, Kelantan; Mersing, Johor; Port Klang, Selangor; Port Dickson, Negeri Sembilan). Water samples were collected using sterile 500 mL Schott bottles and transferred to the laboratory within 2-5 h. Sediment samples were collected using sterile 100 ml vials and disposable plastic containers.

Bacterial isolation and identification:

Serial dilution method and spreading plate technique were used for growing *V. alginolyticus*. Two media: Thiosulfate Citrate Bile Salts Sucrose Agar -TCBS (Oxoid, UK) and Chromogenic Vibrio Agar CV (Titan Media, India) were used for isolation and identification of bacteria. The colonies that were suspected to be *V. alginolyticus* that have shown yellow color on TCBS agar and colorless on CV agar were subjected to biochemical tests that designed by following Bergey's manual and FDA manual [14].

Antibiotic resistance test:

Identified *V. alginolyticus* were tested for antimicrobial resistance using the disc diffusion method [15]. A pure broth cultures of *V. alginolyticus* were then diluted with sterile broth to obtain turbidity comparable with a 0.5 McFarland standard (BD Diagnostic system, US). The surfaces of prepared Mueller-Hinton agar plates (Oxoid, UK) were inoculated by streaking the entire surface by sterile cotton swab that was dipped into the bacterial suspension, then left to dry for 10 min before placing the antimicrobial sensitivity discs. The inoculated plates with the disks were incubated invertedly at 35 °C for 24 hours. The *V. alginolyticus* were tested against the following 14 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). The American Type Culture Collection ATCC (*V. alginolyticus* ATCC 17749) were used as control organisms. The zone of

inhibition was measured in millimeter (mm) and the results were interpreted based on the recommendations of National Committee for Clinical Laboratory Standards for antimicrobial susceptibility tests [16].

DNA extraction:

The DNA extraction was conducted by DNA Purification Kit (Promega, USA) as described in the manufacturer's manual. The extracted genomic DNA was then stored at 4 ^{o}C for further studies.

Plasmid isolation:

For plasmid extractions, *V. alginolyticus* were grown overnight in Luria Bertani LB broth (Oxoid, UK) with an addition of 3% (w/v) NaC1 at 30 °C, and the plasmids were extracted with the PureYield^{TM} Plasmid Miniprep System (Promega, Madison, WI, USA) to get the high-quality plasmid DNA following manufacturer's instruction. The eluted plasmid DNA was stored at -20°C until use for further analysis. After the plasmid DNA was isolated, the gel electrophoresis was carried out according to the method described previously to analyze the DNA pattern on 1% (w/v) agarose gel. The gel electrophoresis was run at 85 V for 1 hour, and 1 kb Extend DNA ladder (New England BioLabs, USA) was used as a DNA marker. The gelwas then visualiz under UV light through gel documentation of GeneSys G: BOX EF2 (Syngene, USA). In addition, the gel image was recorded, and the plasmid DNA bands produced were analyzed [17].

PCR amplification of resistance gens:

Primers used in this study to detect different antibiotic resistance genes are listed in Table 1. The primers and reagents for PCR were purchased from FIRST BASE LABORATORIES SDN BHD (Malaysia). The conditions used for the PCR are described in Table 2. All reactions were conducted in a Mastercycler® thermal cycler (Eppendorf, Germany). Positive control (*V. alginolyticus* ATCC 17749) and negative control (E.coli ATCC 25992) were included for each set of amplifications. A 100 bp DNA ladder (Invitrogen, Belgium) was used as a molecular size marker. 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 80-90 V in 1x Tris-Acetate - Ethylenediaminetetraacetic acid (TAE) buffer (FirstBase Sdn Bhd, Malaysia). The 100bp DNA ladders (Invitrogen, Belgium) was used as a molecular size marker.

Table 1 Primers used for detection of antibiotic resistance genes in *V. alginolyticus*

Gene name	Primer name	Primer sequence (5' to 3')
Streptomycin resistance (*strB*)	*str*B-F	CCGCGATAGCTAGATCGCGTT
	*str*B-R	CGACTACCAGGCGACCGAAAT
β-lactamase resistance (*blaP1*)	*bla*P1-F	GGCATCCAAGCAGCAAG
	*bla*P1-R	CTGGTTCATTTCAGATAGCG
Chloramphenicol Resistance (*floR*)	*flo*R-F	TTATCTCCCTGTCGTTCCAGCG
	*flo*R-R	CCTATGAGCACACGGGGAGC
Tetracycline Resistance (*tetA*)	*tet*A-F	GTAATTCTGAGCACTGTCGC
	*tet*A-R	CTGCCTGGACAACATTGCTT
Erythromycin resistance (*ermB*)	*erm*B-F	AGACACCTCGTCTAACCTTCGCTC
	*erm*B-R	TCCATGTACTACCATGCCACAGG
Quinolone resistance protein (*qnrA*)	*qnr*A-F	AATTTTAAGCGCTCAAACCTCCG
	*qnr*A-R	TCCTGTTGCCACGAGCATATTTT
Aminoglycosides resistance (*aac(3)-IIa*)	*aac*(3)-IIa-F	CGGCCTGCTGAATCAGTTTC
	aac(3)-IIa-R	AAAGCCCACGACACCTTCTC

Table 2 PCR conditions for detection of antibiotic resistance genes in *V. alginolyticus*

Gene	Product Size (bp)	Primer Conc.	dnTP (µM)	*Taq* poly (units)	$MgCl_2$ (mM)	Buffer	Anneal Temp (°C)	Reference
strB	515	0.5	200	0.04	2.0	1x	64	**18**
blaP1	874	0.5	200	0.04	2.0	1x	55	**8**
floR	526	0.5	200	0.04	2.0	1x	64	**19**
tetA	950	0.5	200	0.04	2.0	1x	60	**20**
ermB	640	0.5	200	0.04	2.0	1x	63	**21**
qnrA	521	0.5	200	0.04	2.0	1x	62	**22**
aac(3)-IIa	436	0.5	200	0.04	2.0	1x	61	**23**

Results and Discussions:

Antibiotic resistance test:

The data analysis of results based on the distribution of antibiotic resistant percentages among seawater and sediment isolates of *V. alginolyticus* demonstrated a higher degree of resistance in seawater compared to sediment isolates (Figure1). The highest percentage was obtained from erythromycin and penicillin (100%) in seawater samples. Whereas the lowest antibiotic resistant percentage of *V. alginolyticus* isolated was from seawater was obtained from both chloramphenicol C and nalidixic acid NA (16.66 %). The sediment isolates of *V. alginolyticus* showed 100% resistance against both ampicillin AM and penicillin P and the lowest value was the percentage of gentamycin CN (0 %). The higher percentage of resistance by *V. alginolyticus* isolates against ampicillin as observed in this study and the variations in resistance rates against the other antibiotics agrees with the findings of previous study [24]. The incidence of infection caused by *Vibrio* species is usually companion with high populations of *Vibrio* species that present in seawater environment. In the present study, Chloramphenicol C, Nalidixic acid NA and Gentamycin CN were effective in controlling *V. alginolyticus* growth which is agreed with previous study. [25]

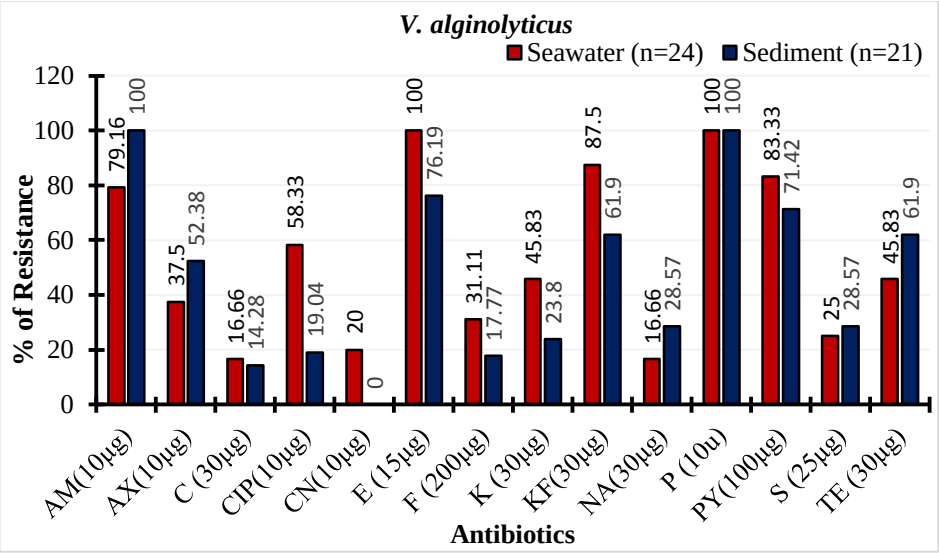

Figure 1 Percentage distribution of antibiotic resistance of *V. alginolyticus* isolates from seawater and sediments against selected antibiotics

Antibiotic resistance patterns and plasmid profiling of *V. alginolyticus*

The antibiotic resistance patterns and plasmid profiling of *V. alginolyticus* isolated in this study is presented in Table 3. All the *V. alginolyticus* isolates demonstrated multi-antibiotic resistance to at least five of the selected antibiotics, with some isolates (VAEW1, VAIW2, VAJW4, VAKW1) being resistant to 13 different antibiotics. Seventeen different antibiotic patterns were observed from the *V. alginolyticus* isolated in this study. These findings agree with the findings reported in an earlier reported study[26], where *V. alginolyticus* isolates were reported to have 20 different antibiotic resistance patterns. The plasmid size of the *V. alginolyticus* isolated in this study ranged from 2.3 Kb to 21.6 Kb, while 19 isolates having no plasmids. This result corroborate with the findings of Jacintha[25], where twenty seven *V. alginolyticus* isolates have no plasmids. These sizes of plasmids observed in this study are comparatively very low compared to the plasmid range of 68 kb to 126 Kb reported in another related study[27].

Table 3 Antibiotic resistance patterns and plasmid profiling of *V. alginolyticus*

Antibiogram	Isolate codes	Pattern	Plasmid Size (kb)
AM, AX, C, CIP, E, F, K, KF, NA, P, PY, S, TE	VAEW1, VAIW2, VAJW4, VAKW1	A	2.3, 6.5,13.5, 21.6
AM, AX, CIP, CN, E, F, KF, P, PY, S	VAIW1, VAJW2	B	3.5, 21.6
AM, CIP, E, F, K, KF, P, PY, TE	VAKW2, VALW1	C	2.3, 3.5, 13.5
AM, CIP, CN, E, F, K, P, PY	VADW2, VAHW1, VAJW3	D	6.5, 11.8
AM, CIP, E, F, KF, P, PY	VAAW1, VAFW5, VAHW4	E	N.D
AX, CN, KF, P, TE	VACW1, VAGW2, VAJW1	F	11.8
AM, E, KF, P, PY	VADW1, VAFW2, VAGW7, VAIW3, VALW2	G	N.D
CN, K, KF, P, TE	VADW3	H	N.D
K, KF, P, PY, TE	VAEW2	I	N.D
AM, AX, CIP, E, F, K, KF, P, PY, S, TE	VAKS1	J	5.7, 11.8, 19.3
AM, AX, C, CIP, E, F, K, KF, NA, P, S	VAIS3, VAJS2, VALS1	K	3.5, 6.5,21.6
AM, AX, E, F, K, KF, P, PY, S, TE	VADS1	L	5.7
AM, AX, F, KF, P, PY, S, TE	VAIS4	M	2.3, 6.5
AM, AX, F, KF, P, PY	VAES1, VAIS2	N	N.D
AM, AX, KF, NA, P	VADS2, VAHS3, VAIS6	O	3.5, 11.8
AM, KF, P, PY, TE	VABS1, VACS2	P	N.D
AM, P, PY, TE	VAAS1, VACS1, VACS3, VACS4, VACS5, VAIS5, VAJS3, VAJS4	Q	N.D

N.D: Not Detected

Percentage of Antibiotic Resistance Genes of *V. alginolyticus*:

The percentages of seven resistance genes of *V. alginolyticus* isolates from seawater, sediment and all isolates were evaluated (Figure 2 and 3). No gene of isolates from seawater had 100% resistance as the highest resistance gene was found to be *ermB* which had 91.66% of resistance gene (Table 4). This was followed by *blap*l with 70.83% of resistance gene. The gene with the lowest percentage of resistance was *flo*R with 16.66% of resistance gene. No gene from sediment isolate had a high percentage of resistance as the highest percentage of resistance gene was found to be *tet*A with 61.9% of resistance gene. The rest of the genes under this category had a low percentage of resistance genes with the least being from *flo*R which had 14.28% resistance gene. Similarly, no gene from all isolates has a high percentage of resistance gene with the highest percentage of resistance being from *erm*B with 60% resistance gene. This was followed by *blap*l and *tet*A with 55.33% resistance gene each. Just as the seawater and sediment isolate, the gene with the least percentage of resistance in this group was found to be from floR with 15.55% resistance gene. Generally, isolates from seawater had relatively higher percentage of resistance genes compared to isolates from sediment and all isolates. Other related studies[28], have reported the presence of *tet*A and *flo*R in *Vibrio* isolates but not in percentages as reported in this study. This study would suggest that *V. alginolyticus* can be a store that can be shared for these genes in the aquatic environment.

Table 4 Antibiotic resistance gene patterns of *V. alginolyticus*

Resistance Gene Profile	Isolate codes	Pattern
aac(3)-IIa, ermB, floR, qnrA, strB, tetA	VAEW1, VAIW2, VAJW4, VAKW1	A
aac(3)-IIa, blaP1, ermB, qnrA, strB	VAIW1, VAJW2	B
ermB, qnrA, tetA	VAKW2, VALW1	C
blaP1, ermB, qnrA	VADW2, VAHW1, VAJW3	D
blaP1, ermB, qnrA	VAAW1, VAFW5, VAHW4	E
blaP1, tetA	VACW1, VAGW2, VAJW1	F
blaP1, ermB	VADW1, VAFW2, VAGW7, VAIW3, VALW2	G
aac(3)-IIa, blaP1, tetA	VADW3	H
tetA	VAEW2	I
aac(3)-IIa, blaP1, ermB, qnrA, strB, tetA	VAKS1	J
aac(3)-IIa, ermB, floR, qnrA, strB	VAIS3, VAJS2, VALS1	K
blaP1, ermB, strB, tetA	VADS1	L
blaP1, strB, tetA	VAIS4	M
blaP1	VAES1, VAIS2	N
blaP1, qnrA	VADS2, VAHS3, VAIS6	O
tetA	VABS1, VACS2	P
tetA	VAAS1, VACS1, VACS3, VACS4, VACS5, VAIS5, VAJS3, VAJS4	Q

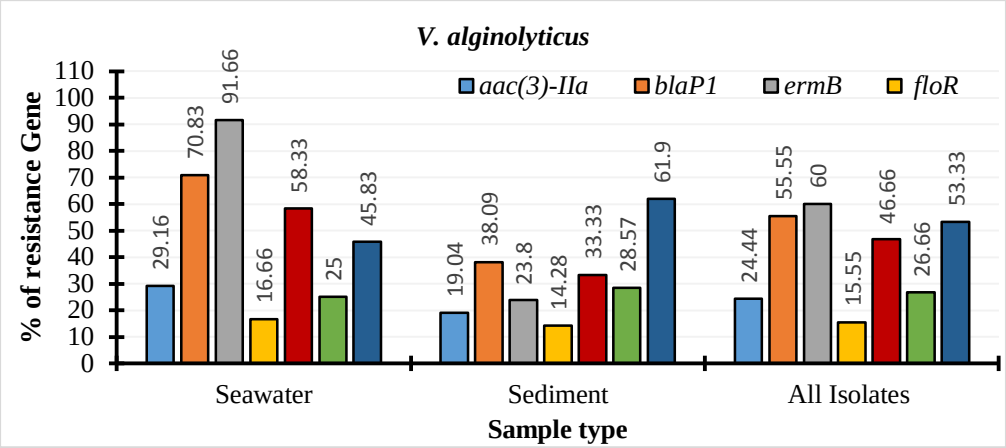

Figure 3 **Percentages of seven resistance genes of *V. alginolyticus* from seawater isolates from sediment and all isolates**

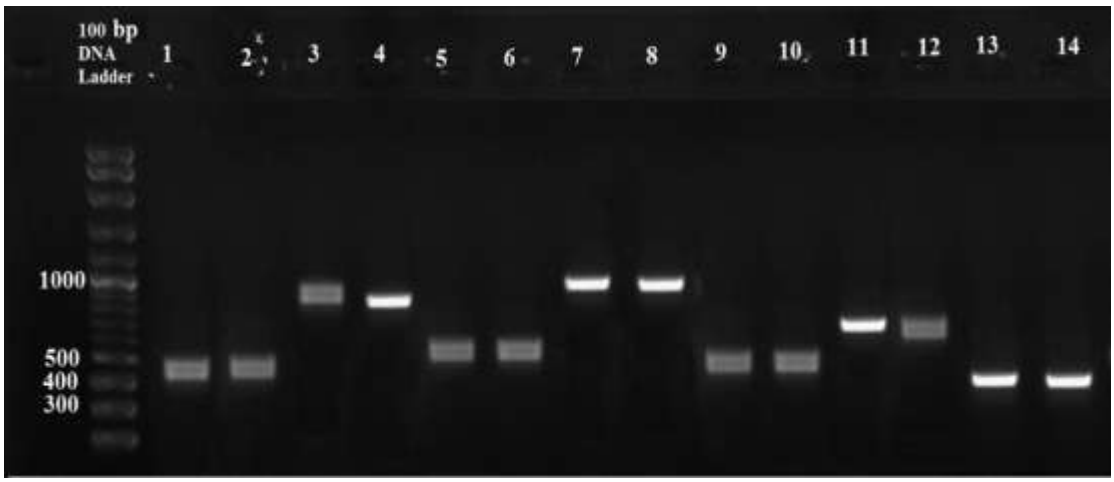

Figure 3 **Detection of antibiotic resistance genes in some *V. alginolyticus* isolates by PCR technique, electrophoresed on 1.5 % (w/v) agarose gel. Lanes 1, 2: *Str*B gene in VADS1 and VAIS4. Lanes 3, 4: *bla*P1 gene in VADW2 and VAHW1. Lanes 5, 6: *flo*R gene in VAJW4 and VAKW1. Lanes 7, 8: *tet*A gene in VACW1 and VAGW2. Lanes 9, 10: *erm*B gene in VAEW1 and VAIW2. Lanes 11, 12: *qnr*A gene in VAEW1 and VAJW4. Lanes 13, 14: *aac(3)-IIa* gene in VAKS1 and VAIS3**

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