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Phenotypic characterisation and strain differentiation of *Vibrio alginolyticus* isolates from Muttukadu brackishwater lagoon

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Vibrio alginolyticus has been described as a pathogenic species that can cause vibriosis in shrimp and many marine fishes. A total of nine *V. alginolyticus* isolates were segregated from 25 water samples collected from various locations at Muttukadu brackishwater lagoon in Tamil Nadu, India. The isolates were separated and characterised phenotypically and genotypically and tested for antibiotic resistance against a range of 14 antimicrobial agents. All the isolates were resistant to the penicillin group of antibiotics. The isolates exhibited higher rates of resistance against antibiotics such as chloramphenicol, nalidixic acid, cefuroxime, cefoperazone, and cefazolin and moderately resistant against tetracycline, tobramycin, gentamicin, cefepime, and showed lower degree resistance against doxycycline, ciprofloxacin and gatifloxacin. The MAR index of the isolates (0.285 to 0.5) indicated that they have originated from high-risk sources of contamination. Genome fingerprinting with RAPD and ERIC-PCR showed that RAPD was highly discriminatory than ERIC-PCR for the tested isolates. Further, both fingerprinting analyses revealed a high level of genetic diversity among the isolates. These data unequivocally indicated that the population of *V. alginolyticus* in a brackishwater environment was not dominated by any environmentally adapted strains and there was mixing and dispersal of genotypes within water samples.

[**Keywords**: Antibiogram, ERIC-PCR, MAR index, RAPD, *Vibrio alginolyticus*]

Introduction

A wide group of bacteria have been responsible for various diseases incultured aquatic organisms and this has been one of the major limitations in aquaculture. Amongst these, vibriosis has been recognized as a significant constraint to the development of shrimp culture practice throughout the world[1]. Vibrios are usually considered autochthonous flora and can survive in different environments like seawater, brackishwater, etc. and thus, tolerate varying saline conditions. For many aquatic animals, vibrios are generally considered to be opportunistic pathogens and hence, trigger mortality due to stressful events, such as sudden fluctuations in temperature and salinity. In shrimp aquaculture, vibrios are often seen to be present among the normal bacterial flora of the cultural population and the habitat[2] from which *V. harveyi*, *V. alginolyticus* and *V. parahaemolyticus* are most frequently isolated. *V. alginolyticus*, a ubiquitous organism in seawater, is a natural host of estuarine and coastal waters and has been almost impossible to observe their absence from these environments. Studies revealed that *V. alginolyticus* is considered to be the most frequent species living freely in water and sediments[3] and can survive in seawater even under conditions of nutrient stress while maintaining their virulence[4]. It is one of the most important opportunistic pathogens and is reported to cause septicaemia, ulcer, exophthalmia and corneal opaqueness in fish, and is associated with the white spot in shrimp. *V. alginolyticus* is an important pathogen that has to be closely monitored and controlled in the mariculture industry because of its strong pathogenicity, quick onset after infection and high mortality rate in aquatic animals[5].

Several epidemiologic and disease control studies have applied various molecular methods like Ribotyping, Random Amplified Polymorphic DNA (RAPD), Restriction Fragment Length Polymorphism (RFLP), Amplified Fragment Length Polymorphism (AFLP) and Enterobacterial Repetitive Intergenic Consensus PCR (ERIC-PCR) for genotyping of *V. alginolyticus*. However, all these methods have their advantages and disadvantages. RAPD is a rapid subtyping method to characterize genetic differences and has been used to fingerprint a variety

of bacterial species including *V. alginolyticus* and *V. parahaemolyticus*[6]. Another PCR-fingerprinting technique used for genotypic characterisation is ERIC-PCR, but it is not arbitrary as the primer is designed for a known target sequence. The position of ERIC elements in enterobacterial genomes varies among different species and has been used as a genetic marker to characterize isolates within bacterial species[7].

This study also explored some of the above-said methods and used RAPD and ERIC-PCR for genotypic characterisation. The study also aimed to characterize the strains of *V. alginolyticus* obtained from brackishwater environment by phenotypic and antimicrobial susceptibility patterns and to provide a basis for epidemiological study as well as drug sensitivity pattern of this species.

Materials and Methods

Sample collection

Twenty-five water samples were collected for a week from various locations of Muttukadu Brackishwater lagoon (12°48'30.1"N; 80°14'43.1"E), an important fishing hamlet for many aquaculture activities. The water sample was collected using sterile containers and brought to the laboratory in ice immediately for bacterial enrichment and isolation.

Bacterial isolation

The collected water samples were enriched by inoculating them into alkaline peptone water and incubated overnight at 30°C. The culture was streaked on Thiosulfate Citrate Bile Sucrose (TCBS) plates to get individual colonies of *Vibrio* sp. The individual colonies were then streaked on Zobell Marine Agar (ZMA) plates and further subcultured to get pure colonies. Selected bacterial isolates were subjected to a series of generally followed tests for their identification.

Bacterial staining and motility test

Gram stained[8] bacterial smears were observed under an oil immersion microscope (Nikon AX, Japan).

Phenotypic identification

Presumptive identification of nine isolates of *V. alginolyticus* was done by performing tests such as oxidase and motility test, Arginine Dihydrolase (ADH), Lysine and Ornithine Decarboxylase (LDC, ODC) assays, and a salt tolerance's test by growing in a series tube of alkaline peptone water containing NaCl (0, 3, 6, 8 and 10%). The identification was then continued only with motile, oxidase and gram-positive strains, negative for ADH, positive for LDC and ODC by performing the following biochemical tests like sugar fermentation tests (sucrose, arabinose, mannitol and glucosamine Voges-Proskauer (VP) test, indole test (Kovac's method), urease test, oxidase test, citrate utilization test, o-nitrophenyl β-d-galactosidase (ONPG) test, gelatinase test, o/129 sensitivity (2,4 diamino 6,7 di-isopropyl pteridine) as per the protocols of Smibert & Krieg[8]. The isolates were identified using the keys described by Alsina & Blanch[9] and Baumann & Schubert[10].

Antibiotic sensitivity profile of *V. alginolyticus*

Antibiotic sensitivity test was performed using Disc diffusion technique[11]. A total of 14 antibiotics from various groups were studied. The antibiotic groups tested belonged to penicillin, fluroquinolones, cephalosporins, tetracycline and phenicol. The antimicrobial agents tested were cefuroxime (30µg), cefepime (30µg), cefazolin (30µg), cefoperazone (75µg), ampicillin (10µg), gentamicin (10µg), tetracycline (30µg), gatifloxacin (5µg), doxycycline hydrochloride (30µg), tobramycin (10µg), nalidixic acid (30µg), carbenicillin (100µg), chloramphenicol (30µg) and ciprofloxacin (5µg) (Himedia, Mumbai, India). In vitro antimicrobial activity was screened by using Mueller Hinton Agar (MHA) (Himedia, Mumbai). The zones of inhibition were interpreted as resistantor sensitive using the interpretation chart of the zone sizes of the Kirby-Bauer sensitivity test method[12].

Multiple antibiotic resistance index

Multiple Antibiotic Resistance (MAR) index of nine *V.alginolyticus* isolates against the tested antibiotics was calculated based on the following formula[13].

$$\text{MAR index} = a/b$$

Where, a = total cases of antibiotic resistance of a particular isolate, and b = total number of antibiotics used in the study.

A MAR index value of equal to or less than 0.2 is considered a low-risk antibiotic exposed, where antibiotics were rarely used or never used. A MAR index value of greater than 0.2 is considered a high-risk antibiotic exposed source.

Bacterial DNA extraction

The bacterial culture was inoculated in nutrient broth supplemented with 1 % NaCl at 30°C for 24 h

in an orbital shaker. From this, 1.5 ml of the bacterial culture was used and centrifuged at 10000 rpm for 5 min to get bacterial pellets. The bacterial genomic DNA was extracted from the pellets by the n-CetylTrimethyl Ammonium Bromide (CTAB) method. The DNA pellet was resuspended in 100µl of TE buffer and stored at -20°C[14].

DNA quantification

The extracted DNA samples were quantified using a UV spectrophotometer (Eppendorf, Germany). Sterile double distilled water was used as a blank and DNA concentration was determined in ng/µl. The protein-to-DNA ratio (A260/A280) was also detected in a UV-visible spectrophotometer to determine the purity.

Strain differentiation

Genome fingerprinting by ERIC-PCR

ERIC-PCR was performed using primers described by Yuan *et al*.[15]. The PCR amplification was performed in 25µl reaction volume containing 2.5µl of 10x PCR reaction buffer, 0.5µl dNTPs (10mM each dNTP), 1µl of each 10µM forward and reverse primers, 1µl of Taq DNA polymerase (1U/µl), 1µl of template DNA and the total volume was compensated through sterile deionized water. PCR was done using a gradient thermocycler (Eppendorf, Germany). The thermocycling profile was as follows; initial denaturation at 95°C for 5 min followed by 35 cycles of denaturation at 92°C for 45 sec, annealing at 52°C for 1 min, extension at 70°C for 10 min and final extension at 70°C for 20 min. The amplicons were maintained at 4°C.

Genome fingerprinting by RAPD

RAPD primers as described by Yuan *et al*.[15] was utilized to carry the reaction. The PCR amplification was performed in 25µl reaction volumes as mentioned above and only one primer was used. Thermocycling was done using gradient PCR (Eppendorf, Germany) with following reaction conditions; initial denaturation at 95°C for 5 min followed by 40 cycles of denaturation at 94°C for 1 min, annealing at 36°C for 1 min, extension at 72°C for 1 min and final extension at 72°C for 10 min. The amplicons were maintained at 4°C.

Agarose gel electrophoresis

An aliquot of PCR product was resolved on 1.5% agarose in Tris–acetate–EDTA (TAE) buffer containing 0.5µg/ml ethidium bromide alongside a 100 bp and 1 kb DNA ladder and the amplified DNA was visualized using a gel documentation system (Bio-Rad Laboratories, USA).

ERIC-PCR and RAPD fingerprint analysis

The banding patterns of ERIC-PCR and RAPD fingerprints were analysed with the fingerprinting II, Informatrix software (Bio-Rad Laboratories, USA). Cluster analysis and calculation of similarity scores between the different banding patterns were performed using Dice's similarity with 1% position tolerance and the Unweighted Pair Group Method with Arithmetic mean (UPGMA).

Calculation of discriminatory index

The index of discrimination (D) was calculated for each ERIC and RAPD genotyping method using Simpson's index of diversity[16] which was developed for the description of species diversity within an ecological habitat. This index can be derived from the following equation:

$$D = 1 - \left[\frac{1}{N(N-1)}\sum_{j=1}^{s} nj\ (nj-1)\right]$$

Where, N - is the total number of strains in the sample population, S - is the total number of types described, and nj - is the number of strains belonging to the j^{th} type.

Results

Bacterial isolation

A total of 25 water samples were tested using the standard protocol for the selective isolation and identification of *V. alginolyticus*. Out of 25, nine samples were found to have *V. alginolyticus* with a prevalence of 36%. All the isolates had uniform physical characteristics and all appeared yellow in colour, round, milky and non-luminescent on the TCBS agar plates. All nine colonies were selected for further biochemical characterization.

Bacterial staining and motility tests

All nine bacterial isolates were found to be gram-negative and motile.

Phenotypic identification

All the isolates were identified as *V. alginolyticus* and showed identical biochemical profiles, details of which are shown in Table 1.

Antibiotic sensitivity profile of *V. alginolyticus*

The antibiotic resistance pattern of nine *V. alginolyticus* isolated from a brackishwater environment showed varying degrees of resistance to

different classes of antibiotics and the results are depicted in Figures 1 & 2. All the isolates were resistant to ampicillin and carbenicillin. A higher percentage of bacteria were resistant to chloramphenicol (89%), nalidixic acid, cefuroxime, cefoperazone (78%), cefazolin (67%) and a moderate percentage of bacteria were resistant to tetracycline, tobramycin (33%), gentamicin, cefepime (22%), whereas a low percentage of bacteria were resistant to doxycycline, ciprofloxacin, gatifloxacin (11%).

Antibiotic resistance pattern against a group of antibiotics

In the present study, nine *V. alginolyticus* isolates displayed complete resistance (Figs. 1 & 2) against the penicillin group of antibiotics such as ampicillin and carbenicillin (100%). Three isolates showed a moderate degree of resistance to the tetracycline group of antibiotics and a lower degree of resistance to doxycycline hydrochloride (one isolate). It gave a higher degree of resistance to the phenicol group of antibiotics such as chloramphenicol (eight isolates). A moderate degree of resistance was indicated towards the aminoglycoside group of antibiotics such as tobramycin (three isolates) and gentamicin (two isolates). It presented a higher degree of resistance to first-generation fluroquinolones antibiotic nalidixic acid (seven isolates), a lower degree of resistance to second and third-generation antibiotics such as ciprofloxacin (one isolate) and gatifloxacin (one

Table 1 — Biochemical profile of nine *V. alginolyticus* isolates

	Biochemical reactions																				
							Fermentation of											Salt tolerance			
Sl. No.	Growth on TCBS	Arginine decarboxylase	Lysine decarboxylase	Ornithine dihydrolase	Indole	VP	Sucrose	Mannitol	Glucosamine	Arabinose	ONPG	Gelatinase	Citrate	Oxidase	Urease	O/129	0%	3%	6%	8%	10%
1	+	-	+	+	+	+	+	+	+	-	-	+	+	+	-	+	-	+	+	+	+
2	+	-	+	+	+	+	+	+	+	-	-	+	+	+	-	+	-	+	+	+	+
3	+	-	+	+	+	+	+	+	+	-	-	+	+	+	-	+	-	+	+	+	+
4	+	-	+	+	+	+	+	+	+	-	-	+	+	+	-	+	-	+	+	+	+
5	+	-	+	+	+	+	+	+	+	-	-	+	+	+	-	+	-	+	+	+	+
6	+	-	+	+	+	+	+	+	+	-	-	+	+	+	-	+	-	+	+	+	+
7	+	-	+	+	+	+	+	+	+	-	-	+	+	+	-	+	-	+	+	+	+
8	+	-	+	+	+	+	+	+	+	-	-	+	+	+	-	+	-	+	+	+	+
9	+	-	+	+	+	+	+	+	+	-	-	+	+	+	-	+	-	+	+	+	+

VP- Voges-Proskauer test, ONPG- o-nitrophenyl β-d-galactosidase test, O/129 - 2,4-diamino-6,7-diisopropyl pteridine

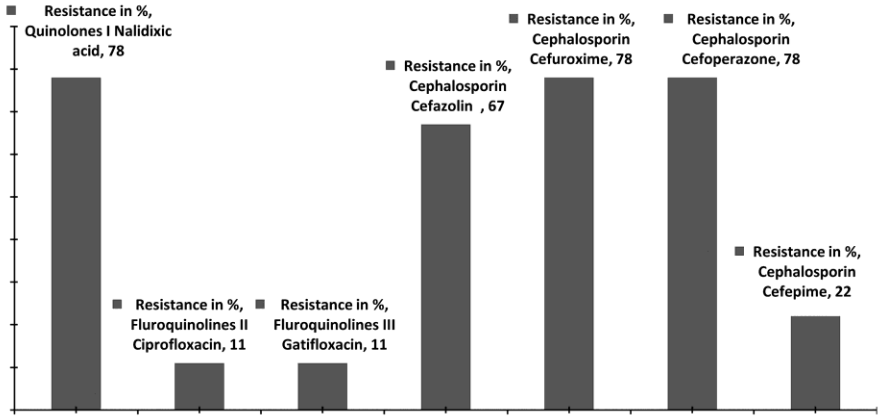


Fig. 1 — Comparison of antibiotic resistance of *V. alginolyticus* against Quinolones, Fluroquinolines II, Fluroquinolines III and Cephalosporin group of antibiotics.

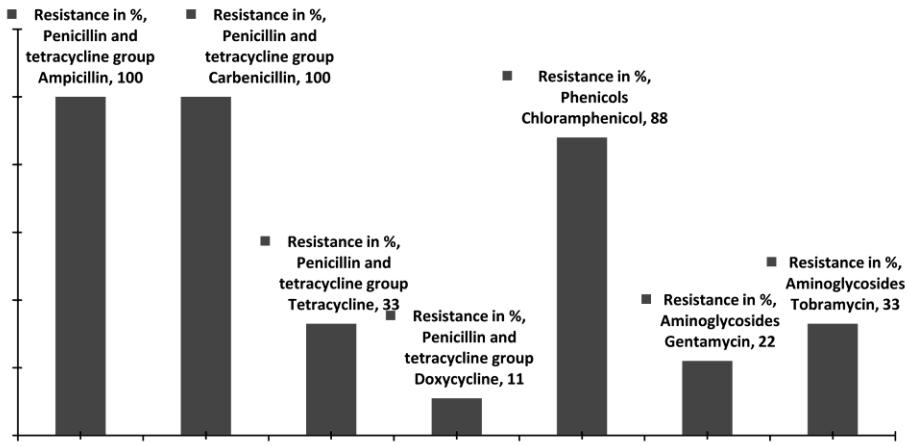


Fig. 2 — Comparison of antibiotic resistance of *V. alginolyticus* against Penicillin, tetracycline, phenicols and aminoglycoside group of antibiotics.

isolate). A higher degree of resistance to first, second and third-generation cephalosporin groups of antibiotics such as cefazolin (six isolates), cefuroxime (seven isolates) and cefoperazone (seven isolates) wasseen but a moderate degree of resistance to fourth generation antibiotic cefepime (two isolates).

Similarly, four cephalosporin groups of antibiotics were studied belonging to second, third and fourth generations, two isolates exhibited sensitivity to cefuroxime, a second-generation cephalosporin and seven isolates were resistant to this antibiotic. Further, seven of the isolates were sensitive to fourth-generation cephalosporin namely cefepime and two isolates were resistant. Among the third-generation cephalosporin, two of isolates were sensitive to cefoperazone. Six of the isolates were resistant to cefazolin. Two tetracycline groups of antibiotics namely tetracycline and doxycycline hydrochloride were studied. Doxycycline gave 88% sensitivity whereas tetracycline exhibited 66% sensitivity. Towards the aminoglycosides group tested, seven *V. alginolyticus* isolates exhibited sensitivity to gentamicin whereas six isolates gave sensitivity to tobramycin. Phenicols group of antibiotics like chloramphenicol was found to be highly resistant, eight isolates were highly resistant and only one was sensitive.

Multiple antibiotic resistance index

Antibiogram patterns of 14 antibiotics against nine isolates were analysed. The study isolates showed resistance to 3 to 7 antibiotics tested. The MAR index of *V. alginolyticus* ranges from 0.285 to 0.5 with an average value of 0.35 (Table 2). The study displayed the occurrence of higher MAR index among the isolates of *V. alginolyticus*.

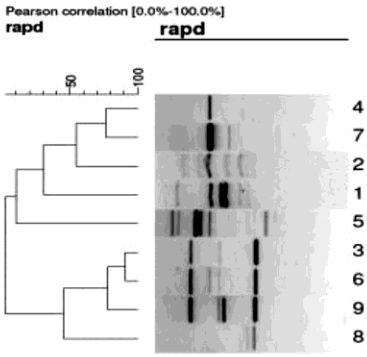
Table 2 — MAR index values of the *V. alginolyticus* isolates

Isolate	No of antibiotics to which the isolate was resistant	MAR index a/b
1	6	0.42
2	6	0.42
3	6	0.42
4	3	0.214
5	7	0.5
6	6	0.42
7	4	0.285
8	6	0.42
9	6	0.42

Strain differentiation

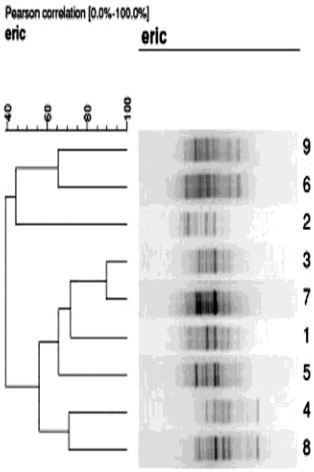
Genome fingerprinting by RAPD

RAPD of genomic DNA from nine *V. alginolyticus* isolates resulted in the amplification of 1 – 9 fragments of DNA ranging from 300 bp to 3000 bp in size. The banding patterns generated by RAPD are presented as a dendrogram (Fig. 3). The RAPD fingerprinting exhibited high polymorphism (5 to 90% index) among the *V. alginolyticus* isolates analysed. The comparison of the banding patterns revealed nine genotypes and by using a cut-off value of 50% similarity, five clusters could be delineated. These five clusters were designated as C_1, C_2, C_3, C_4 and C_5. There are two major clusters comprised of three isolates each. Three isolates occupied a separate position in the dendrogram. C_1 formed of three *V. alginolyticus* isolates namely 2, 4 and 7, these isolates were clustered at the linkage level of 54 to 76%. C_4 was composed of three isolates namely 3, 6 and 9 and clustered at the linkage level of 78 to 90%. Three isolates namely 1, 5 and 8 occupied separate positions in the dendrogram as C_2, C_3 and C_5, respectively.



	AMP	CAR	CFZ	CXM	FEP	CFP	GEN	TOB	TET	DOX	CHL	NAL	CIP	GAT
4	R	R	S	R	S	I	S	R	S	S	S	S	S	S
7	R	R	S	S	S	R	S	I	I	S	I	R	I	I
2	R	R	R	R	I	R	S	S	S	S	I	R	S	S
1	R	R	R	R	S	R	R	S	S	S	I	S	S	S
5	R	R	R	R	S	S	R	S	S	S	R	I	S	S
3	R	R	S	R	S	R	S	S	S	S	R	R	S	S
6	R	R	R	R	R	S	S	S	S	S	R	I	S	S
9	R	R	R	S	S	R	S	S	I	R	I	R	S	S
8	R	R	R	R	S	R	S	I	I	R	I	I	S	S

Fig. 3 — Comparison of dendrogram showing genetic relatedness of nine *V. alginolyticus* isolates determined by analysis of RAPD fingerprinting patterns using Discs similarity coefficient and UPGMA cluster methods as well as the isolates behaviour towards antibiotics (R= resistance, S= Sensitive, I= Intermediate).



	AMP	CAR	CFZ	CXM	FEP	CFP	GEN	TOB	TET	DOX	CHL	NAL	CIP	GAT
9	R	R	R	S	S	R	S	S	I	R	I	R	S	S
6	R	R	R	R	R	S	S	S	S	S	R	I	S	S
2	R	R	R	R	I	R	S	S	S	S	I	R	S	S
3	R	R	S	R	S	R	S	S	S	S	R	R	S	S
7	R	R	S	S	S	R	S	I	I	S	I	R	I	I
1	R	R	R	R	S	R	R	S	S	S	I	S	S	S
5	R	R	R	R	S	S	R	S	S	S	R	I	S	S
4	R	R	S	R	S	I	S	R	S	S	S	S	S	S
8	R	R	R	R	S	R	S	I	I	R	I	I	S	S

Fig. 4 — Comparison of dendrogram showing genetic relatedness of nine *V. alginolyticus* isolates determined by analysis of ERIC-PCR fingerprinting patterns using Discs similarity coefficient and UPGMA cluster methods as well as the isolates' behaviour towards antibiotics (R= resistance, S= Sensitive, I= Intermediate).

Genome fingerprinting by ERIC-PCR

ERIC-PCR of genomic DNA from nine *V. alginolyticus* isolates resulted in the amplification of 9-13 fragments of DNA ranging between 250bp – 4000bp. The relationship among the PCR-generated patterns is represented as a dendrogram (Fig. 4). The comparison of the banding patterns revealed nine genotypes. ERIC-PCR fingerprinting showed high polymorphism (44 to 90% index) among the *V. alginolyticus* isolates analysed. By using a cut-off value of 50% similarity, three clusters could be delineated. These three clusters were designated as C_1 to C_3. There was one major cluster comprised of six isolates and one minor cluster composed of two isolates and one isolate occupied a separate position in the dendrogram. C_1 accounted for two *V. alginolyticus* isolates namely 9 and 6 clustered at the linkage level of 66%. C_2 was composed of a single *V. alginolyticus* isolate namely 2. It clustered with C_1 at the linkage level of 44%. C_3 consisted of six isolates namely 3, 7, 1, 5, 4 and 8 clustered at the linkage level of 44 to 90%. It clustered with C_2 at the linkage level of 40%.

Comparison of ERIC-PCR and RAPD

Using the Simpson index of diversity[17] (DI), in this study DI value obtained by RAPD was 0.833 and that of ERIC-PCR was 0.556, which showed that RAPD is more discriminatory than ERIC-PCR. RAPD fingerprints were highly diverse and had few common fragments that could be employed for pattern comparison. A total of five distinct clusters were detected by RAPD and three distinct clusters were discerned using ERIC-PCR. Very few clusters contained more than three isolates while other clusters comprised one or two isolates. Using RAPD genotyping, two major clusters were obtained consisting of three isolates (33.33% of the total

collection). Using ERIC-PCR genotyping, one major cluster could accommodate six isolates (66% of the total collection).

Discussion

Urbanization has led to the dumping of human and other harmful waste into the brackishwater and marine environment. These harmful wastes harbouring harmful bacteria and other potential pathogens are often discharged into natural water sources including brackishwater through untreated sewage[17]. The occurrence of *V. alginolyticus* in the brackishwater environment in the present study (36%) is comparable to the previously published (34%) studies[18]. However, the bacterial abundance is influenced by several abiotic factors such as sunlight, land runoff and tidal variation[19]. All the identified *V. alginolyticus* isolates showed similar biochemical profiles which were similar to those reported earlier by Ben Kahla-Nakbi*et al.*[20].

The imprudent and widespread usage of antibiotics in all areas of human life has resulted in the development of resistance by bacterial strains against a wide range of antimicrobial substances. *V. alginolyticus* isolates in the present study showed absolute resistance against the penicillin group of antibiotics such as ampicillin and carbenicillin. Similar observations were reported by Zanetti *et al.*[21] wherein the isolates of *V. parahaemolyticus, V. vulnificus* and *V. alginolyticus* isolated from seawater were highly resistant to ampicillin. Two tetracycline groups of antibiotics namely tetracycline anddoxycycline hydrochloride were tested of which three isolates were resistant to tetracycline while one was resistant to doxycycline in this study. This might be because the widespread use of tetracycline constitutes a high selective pressure and as a consequence, bacteria of animal origin have either developed or acquired tetracycline resistance[22]. These antibiotics are the most commonly used worldwide and also in aquaculture. Ampicillin has been used abundantly in animal husbandry as a growth promoter and prophylactic agent. The extensive usage of these antibiotics explains the high selection pressure for these bacteria. In the current study, 88% resistance was found against chloramphenicol. The re-emergence of chloramphenicol-sensitive strains from places where previously resistant strains were reported explains the concept of antibiotic recycling and preserving the use of older antibiotics[23] and tobramycin are the two aminoglycosides group of antibiotics used in the study. The isolates showed 22% resistance to gentamicin and 33% resistance to tobramycin.

The resistance rates of *V. alginolyticus* to first, second and third-generation quinolone and fluroquinolone are 77, 11 and 11%, respectively. Fluroquinolone resistance in gram-negative bacteria is common all over the world. The majority of *V. alginolyticus* (77%) were found to be resistant to first-generation quinolones namely, nalidixic acid. Increased sensitivity to ciprofloxacin (88%) was also observed. The resistance rates of *V. alginolyticus* to first, second, third, and fourth-generation cephalosporin are 66, 77, 77 and 22%, respectively. The increasing use of the third-generation cephalosporin is often associated with an increased frequency of Gram-negative bacteria with class C β-lactamase resistance[24].

Discriminatory power is most conveniently calculated by using Simpson's index of diversity (DI) which takes into account the number of types defined by the method and the relative frequencies of these types[16]. Multiple antimicrobial resistance has been recognized as an emerging worldwide problem in human and veterinary medicine both in developed and developing countries[25]. The effect could be severe in heavily populated countries such as India where there is no strict monitoring programme regarding the use of antibiotics in animals and humans. The higher MAR index of all *V. alginolyticus* isolates indicates that it might have originated from high-risk sources of contamination with potentially hazardous to human health and also these isolates must have been exposed continuously to the antibiotics. The mean MAR index of all the isolates was found to be 0.35. Higher MAR index in *Vibrio* spp. isolated from cultured groupers in Malaysia due to continuous exposure to antibiotics was reported by Amalina *et al*.[26].

In this study, ERIC-PCR and RAPD analysis was carried out to assess the genetic relationship of the *V. alginolyticus* from brackishwater. The non-occurrence of similar isolates suggests the heterogeneity of the source. ERIC-PCR based analysis was found to have 100% reproducibility and a 0.556 discriminatory index. The ERIC-PCR depicted a high diversity of polymorphism between *V. alginolyticus* isolates but lower than RAPD which is in correlation with the findings in *V. alginolyticus* and *V. parahaemolyticus*[27,28]. Xu *et al.*[29] described the importance of bacteria in mariculture and ERIC-PCR fingerprint-based amplification could be

unambiguously used for identifying them. RAPD showed a discriminatory index of 0.833 in the present study. RAPD fingerprinting formed five clusters at the cut-off value of 50% similarity. The major cluster consists of three isolates. The results obtained from both techniques revealed the existence of high diversity among the isolates of *V. alginolyticus.* The approach can also be used to establish DNA fingerprints for *V. harveyi* to evaluate the applicability of these techniques in epidemiological studies. Another study by Shabarinath *et al*.[30] showed that RAPD fingerprinting and ERIC-PCR (both individually and in combination) would be useful tools to study strain differences and helpful for tracing the source of contamination. There was no correlation between antibiotic resistance pattern and ERIC-PCR and RAPD fingerprinting pattern. This may be due to the high genetic diversity among the study isolates.

Conclusion

In conclusion, ERIC-PCR and RAPD are both valuable techniques for characterizing *V. alginolyticus* isolates. Both these methods can be efficiently utilized for epidemiological studies and also for finding heterogeneity. The widespread resistance of *V. alginolyticus* isolates obtained in the current study should raise a concern about the imprudent use of antimicrobials in the country, which is a serious concern for aquaculture and the environment today. Moreover, it may be used as a bioindicator of antibiotic resistance among the bacterial species and for the presence of contaminated effluents in the water bodies.

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Conflict ofInterest

The authors declare that they have no known competing interests.

Author Contributions

All the authors have contributed equally to the article.

References

1 Uma T, Abraham T J, Jayaseelan M J D & Sundararaj V, Effect of probiotic feed supplement on performance on disease resistance of Indian white shrimp *Penaeus indicus* H. Milne Edwards, *J Aqua Trop*, 14 (1999) 159-164.

2 Otta S K, Karunasagar I & Karunasagar I, Bacterial flora associated with shrimp culture ponds growing *Penaeus monodon* in India, *J Aquacult Trop*, 14 (1999) 309-318.

3 Harriague A, Brino M D C, Zampini M, Albertelli G, Pruzzo C, *et al*., *Vibrios* in association with sedimentary crustaceans in three beaches of the northern Adriatic Sea (Italy), *Mar Pollution Bull*, 56 (2008) 574-579.

4 Ben Kahla-Nakbi A, Besbesa A, Chaieba K, Rouabhiab M & Bakhroufa A, Survival of *Vibrio alginolyticus* in seawater and retention of virulence of its starved cells, *Mar Envmntl Res*, 46 (2007) 469-478.

5 Dong Y, Zhao P, Chen L, Wu H, Si X, *et al*., Fast, simple and highly specific molecular detection of *Vibrio alginolyticus* pathogenic strains using a visualized isothermal amplification method, *BMC Vet Res*, 16 (2020) p. 76.

6 Marhual N P, Das B K, Sadique M, Swain A K, Mishra B K, *et al*., Molecular identification and typing of *Vibrio alginolyticus* and *Vibrio parahaemolyticus* strains isolated from black tiger shrimp *Penaeus monodon*, *J Aqua Trop*, 25 (2010) 25-33.

7 Versalovic J, Koeuth T & Lupski J R, Distribution of repetitive DNA sequences in eubacteria and application to fingerprinting of bacterial genomes, *Nucleic Acids Res*, 19 (1991) 6823-6831.

8 Smibert R M & Krieg N R, Phenotypic characterization, In: *Methods for General and Molecular Bacteriology*, edited by Gerhard P, Murray R G, Wood W A & Krieg N R, (American Society of Microbiology, Washington D C),1994, pp. 611-651.

9 Alsina M & Blanch A R, A set of keys for biochemical identification of environmental *Vibrio* species, *J Appl Bacteriol*, 76 (1994) 79-85.

10 Baumann P & Schubert R H W, Family II: Vibrionceae, Veron 1965, In: *Bergey's Manual of Systematic Bacteriology*, edited by Krieg N R & Holt J G, (Williams and Wilkins, Baltimore, USA), 1984, pp. 516-550.

11 Bauer A W, Kirby W M, Sherris J C & Turck M, Antibiotic susceptibility testing by a standardized single disk method, *Am J Clin Pathol*, 45 (1966) 493-496.

12 Cheesbrough M, *District Laboratory Practice in Tropical Countries*, 2nd edn, (Cambridge University Press, Cambridge, UK), ISBN-13: 9781139449298.

13 Riaz S, Faisal M & S Hasnain, Antibiotic susceptibility pattern and multiple antibiotic resistances (MAR) calculation of extended spectrum βlactamase (ESBL) producing *Escherichia coli* and *Klebsiella* species in Pakistan, *Afric J Biotech*, 10 (2011) 6325-6331.

14 Ausubel M F, Brent R, Kingston R E, Moore D D, Seidman J G, *et al*., *Current Protocols in Molecular Biology*, (John Wiley & Sons), 2003.

15 Yuan W, Chai T J & Miao ZM, ERIC-PCR identification of the spread of airborne *Escherichia coli* in pig houses, *Sci Total Environ*, 408 (2010) 1446–1450. http://dx.doi.org/10.1016/j.scitotenv.2009.12.019

16 Hunter P R & Gatson M A, Numerical index of the discriminatory ability of typing systems: an application of Simpson's index of diversity, *J Clin Microbiol*, 26 (1988) 2465-2466.

17 Simpson E H, Measurement of Diversity, *Nature*, 163 (1949) p. 688. https://doi.org/10.1038/163688a0

18 Chandran A, Varghese R, Jyothi S & Hatha A A M, *In situ* survival of indicator bacteria and enteric pathogens in a tropical estuary, *Bioscan*, 1 (2010) 51-56.

19 Mustapha S, Mustapha E M, Brahim B & Nozha C, Characterization of *Vibrio Alginolyticus Trh* positive from Mediterranean environment of Tamouda Bay (Morocco), *World Environ*, 2 (2012) 76-80.

20 Burkhardt W, Calci K R, Watkins W D, Rippey S R & Chirtel S J, Inactivation of indicator microorganisms in estuarine waters, *Water Res*, 34 (2000) 2207-2214.

21 Ben Kahla-Nakbi A, Chaieb K & Bakhrouf A, Investigation of several virulence properties among *Vibrio alginolyticus* strains isolated from diseased cultured fish in Tunisia, *Dis Aquat Organ*, 86 (2009) 21-28.

22 Zanetti S, Spanu T, Deriu A, Romano L, Sechi L A, *et al*., In vitro susceptibility of *Vibrio* spp. isolated from the environment, *Int J Antimicrob Agents*, 17 (2001) 407-409.

23 Roberts M C, Tetracycline resistance determinants: mechanisms of action, regulation of expression, genetic mobility, and distribution, *FEMS Microbiol Rev*, 19 (1996) 1-24.

24 Harish B N & Menezes G A, Preserving efficacy of chloramphenicol against typhoid fever in a tertiary care hospital, India, (*Regional Health Forum, WHO South-East Asia Region*), 15 (2011) 92-96.

25 Kolar M, Karel U & Tomas L, Antibiotic selective pressure and development of bacterial resistance, *Int J Antimicrob Agents*, 17 (2001) 357-363.

26 Sharma R, Verma U, Sharma C L & Kapoor B, Self Medication among urban population of Jammu City, *Indian J Pharmacol*, 37 (2005) 37-45.

27 Amalina N Z, Santha S, Zulperi D, Amal M N A, Yusof M T, *et al*., Prevalence, antimicrobial susceptibility and plasmid profiling of *Vibrio* spp. isolated from cultured groupers in Peninsular Malaysia, *BMC Microbiol*, 19 (2019) p. 251. https://doi.org/10.1186/s12866-019-1624-2

28 Mechri B, Monastiri A, Medhioub A, Medhioub M N & Aouni M, Molecular characterization and phylogenetic analysis of highly pathogenic *Vibrio alginolyticus* strains isolated during mortality outbreaks in cultured *Ruditapes decussatus* juvenile, *Microb Pathog*, 111 (2017) 487-496.

29 Zulkifli Y, Alitheen N B, Son R, Raha A R, Samuel L, *et al*., Random amplified polymorphic DNA-PCR and ERIC-PCR analysis on *Vibrio parahaemolyticus* isolated from cockles in Padang, Indonesia, *Int Food Res J*, 16 (2009) 141-150.

30 Xu X, Liu K, Wang S, Guo W, Xie Z, *et al*., Identification of pathogenicity, investigation of virulent gene distribution and development of a virulent strain-specific detection PCR method for *Vibrio harveyi* isolated from Hainan Province and Guangdong Province, China, *Aquaculture*, 468 (2017) 226-234.

31 Shabarinath S, Sanath Kumar H, Khushiramani R, Karunasagar I & Karunasagar I, Detection and characteri zation of *Salmonella* associated with tropical seafood, *Int J Food Microbiol*, 114 (2) (2007) 227-233.

32 Yang C M, Lin M F, Liao P C, Yeh H W, Chang B V, *et al*., Comparison of antimicrobial resistance patterns between clinical and sewage isolates in a regional hospital in Taiwan, *Lett Appl Microbiol*, 48 (2009) 560-565.