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Research Article

Resistance profiling, evolution of biofilm formation and genetic diversity of *Vibrio alginolyticus* isolated from aquaculture systems

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Abstract: This study analyzes 26 presumptive *Vibrio alginolyticus* strains extracted from two fish farms (Khenis and Hergla, East Tunisia) that raised sea bream (*Sparus aurata*) and sea bass (*Dicentrarchus labrax*). In addition, from a shellfish farm located in Menzel Jmil, North Tunisia that raises mussels (*Mytilus edulis*) and oysters (*Crassostrea gigas*).
We have evaluated (i) the discriminatory power of PCR-RFLP for identification of closely related strains, (ii) the antibiotic resistance of the *V. alginolyticus* strains and (iii) their ability to form a biofilm in aquaculture farm.

Results showed that there is great heterogeneity in the diversity observed via the PCR-RFLP method related to the number of 20 genotypes generated by the two enzymes *Sdu*I and *Faq*I tested. The heterogeneity was observed in both fish (aquaculture farm) and bivalve (shellfish farm) origins, as well as in the same sample.

For antibiotic resistance, all isolates present a high resistance to ampicillin, erythromycin, cefotaxime, kanamycin and doxycycline. The resistance is displayed by 15 distinct profiles. The Multiple Antibiotic resistance (MAR) index was ranged from 0.55 to 0.80 for the isolates from the aquaculture farm of Hergla followed by the index aquaculture farm of Khenis (0.55 to 0.75). While for the shellfish farm of Menzel Jmil the index ranges from 0.50 to 0.65. This finding indicated high-risk sources of antibiotic contamination in the three locations. Isolates from aquaculture systems with strong biofilm formation have been found to be resistant to antibiotics, which may allow them to survive longer in these environments.

Present findings could be relevant in aquaculture systems and underscore the importance of the linkage between adhesion, antibiotic susceptibility, and genetic diversity of these pathogenic bacteria to avoid fish and shellfish diseases. The results will provide helpful guidance on how to use antibiotics to control *Vibrio alginolyticus* diseases in aquaculture to be healthy.

Keywords: *Vibrio alginolyticus,* Aquaculture systems, PCR-RFLP, Antibiotic resistance, MAR, Biofilm formation, Tunisia

1. Introduction

Aquaculture worldwide has a high mortality rate due to *Vibrio alginolyticus (*Marhual et al., 2010*).* This bacterium is an economically catastrophic for marine fish, invertebrates and large marine mammals (Xie *et al.*, 2020). In addition, *V. alginolyticus* has been reported to be involved in new human infections that may cause otitis externa, food poisoning related to consumption of raw or undercooked sea products, causing gastroenteritis and extra-intestinal diseases (Fu *et al.*, 2016; Jacobs Slifka *et al.*, 2017).

In Tunisia, *V. alginolyticus* was isolated for the first time in 1987 from diseased farmed gilthead sea bream (*Sparus aurata)* and sea bass (*Dicentrarchus labrax)* (Bakhrouf *et al.,* 1995). This pathogenic bacterium affects mainly larvae of sea bream and sea bass and was associated with high fish mortality in aquaculture systems causing important economic losses (Ben Kahla *et al.*, 2009). Since the incidence of epizootics outbreaks has been on the increase. More recently, several cases of *V. alginolyticus* associated mortality have been reported in reared Chequered carpet shell (*Ruditapes decussatus)* larva and juvenile causing major economic losses to the infected hatchery (Mechri *et al.*, 2017). Foremost this situation, several molecular typing techniques have been used for *Vibrio* species subtyping and for the study of their microbial epidemiology and ecology (Ben Abdallah *et al*., 2010; Lajnef *et al*., 2012; Thompson *et al.,* 2004). RFLP, also known as restriction fragment length polymorphism (RFLP), is a method that uses variations in homologous DNA sequences. It concerns the variations among samples of homologs of restriction enzyme sites. Moreover, the PCR-RFLP is a variation of RFLP in which restriction. analysis is performed on PCR amplicons obtained using primers for specific sequences of interest and could serve as a rapid tool to estimate the rapid tool to *estimate* the approximate phylogenetic relationship of isolates, without need for 16S rRNA sequencing (Urakawa *et al.,* 1997). PCR-RFLP is the most recommended technique for characterization of microorganisms due to its speed, reliability, and sensitivity (Felix *et al.*, 2011). RFLP and PCR-based techniques are a great complement to traditional techniques when it comes to detecting antibiotic multi resistant (AMR) genes. They pinpoint genes that transfer resistance to antimicrobials, while also accurately identifying the species of isolates studied (Galhano *et al*., 2021) Knowing that Vibriosis is recognized as one of the most prominent diseases frequently affecting a wide variety of cultured species all over the world and it recognized by specific receptors belong to the pathogen recognition receptor system (Abouelmaattiet *et al.*, 2013) ; the application of antibiotics in aquaculture especially used in fish farms either as feed additives or immersion baths to achieve either prophylaxis or therapy has led to the development of antibiotic-resistant bacteria and poses a serious challenge in the treatment of infectious diseases (Muhammed *et al.*, 2019). In this context, higher frequency of multidrug-resistant *V. alginolyticus* has been reported by Lajnef *et al.*, (2012), these gram-negative bacteria produce an extended-spectrum β-lactamases (ESBLs) which is a significant resistance-mechanism and present a serious threat to the currently available antibiotic armory (Shaikh *et al.,* 2015).

In addition, *V. alginolyticus* can be distinguished from other species by its ability to possess polar and lateral flagella which has been proven to be associated with adhesion to surfaces and biofilm formation (Chen *et al.*, 2017).

According to Tang *et al*. (2009), bacterial adhesion and colonization are influenced by several factors. Examples of things to consider include bacterial species, bacterial surface, and chemical and physical interactions between the potential substrate and the polymeric adhesive. This adhesion can be a prerequisite for successful infection. After adhesion has taken place, bacteria could stimulate the expression of additional virulence genes and enliven host cell signaling pathways (Ovando Fraiha *et al*., 2019). Moreover, bacteria in biofilms can be 1,000-fold more resistant to environmental stress (Broun *et al.*, 2000) and to antibiotics and biocides (Rogers *et al.*, 2010) than planktonic cells are, so the environmental survival, infectivity and transmission are enhanced due to strong biofilm formation ability of this pathogen (Elexson *et al.*, 2014). According to Deng *et al*. (2020), aquaculture animal disease detection and pathogen resistance development can be promoted by recommending the 'Drug Reduction in Aquaculture' and 'Ecological Health Breeding' initiatives. These authors recommended monitoring, determination of suitable local antimicrobial profiles for aquaculture, provision of guidance for scientific medication, and promotion of vaccine use for disease prevention.

This study focused on multi resistant *Vibrio alginolyticus* species isolated from Tunisian aquaculture systems with the aim of exploring their prevalence and susceptibility, their ability to form a biofilm and their genetic diversity.

2. Materials and Methods

2.1. Sampling sites and strains identification

A total of twenty-six bacterial strains were isolated from two different fish farms (Khenis and Hergla) and one shellfish farm (Menzel Jmil) (Figure 1).

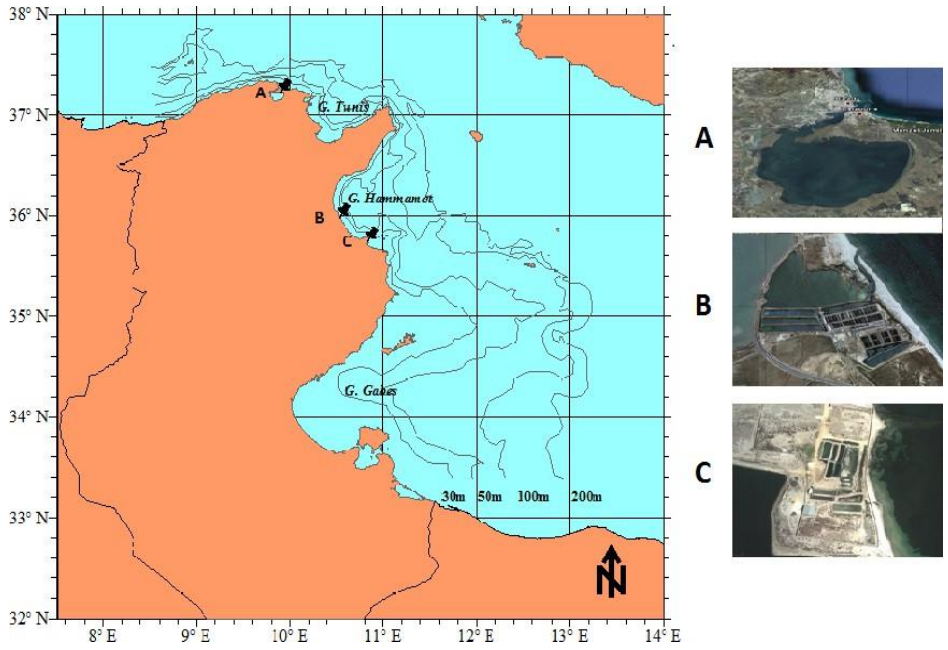

Figure 1. Map of Tunisia showing the different sites of *V. alginolyticus* strains isolated from each sample

Strains isolated from the aquaculture systems were identified as belonging to *V. alginolyticus* using several biochemical tests (Thompson *et al*., 2004): KOH method (Gram non-staining) (Fluharty and Packard, 1967), cell morphology, motility, oxidase test, growth on Thiosulfate Citrate Bile Sucrose (TCBS), susceptibility to the vibriostatic agent 0/129 (10 and 150 µg/disc) (Alsina and Blanch, 1994), production of arginine dihydrolase, lysine and ornithine decarboxylase, glucose

fermentation, indole, hydrolyses of gelatin, of starch, of esculin and of Tween 80, reduction of nitrates to nitrites, production of gas from glucose, Methyl-Red test based on the gallery API 20E Kit “galleries API® 20E™ - biomérieux® SARCS LYON 67362039969280 Marcy-l'Etoile / France”, growth at different temperatures (4°C, 37°C, 44°C) and salinities (0, 6, 8 and 10) (Krieg and Holt, 1984).

The DNA extraction and molecular identification of *V. alginolyticus* strains was done according to the protocol described by Di-Pinto *et al.* (2005) targeting the collagenase gene.

2.2. PCR-RFLP Analysis

The fragment was amplified using the universal primers SD-Bact-0008-a-S20 (5’AGA GTT TGA TCC TGG CTC AG 3’) and SD-Bact-1492-a-A-19 (5’GGT TAC CTT GTT ACG ACT T 3’) (Kim and Austin, 2006). Polymerase chain reactions were carried out in a 50 µl reaction mixture that included 5 pmol of each primer, 200 µM dNTPs, 1×PCR buffer, 2 mM $MgCl_2$, 1 U BIOTAQ™ DNA polymerase (Bioline, London, UK) and 1 µl of extracted DNAs The PCR profile was as follows: 2 min at 95°C and 35 cycles of 30 s at 95°C, 30 sec at 52°C and 1.3 min at 72°C and a final step 5 min at 72°C. Polymerase chain reaction products were electrophoresed on a 1% agarose gel and visualized via ultraviolet trans-illumination.

The PCR products (10µl) were digested separately with *Faq*I (BsmFI) and *Sdu*I (Bsp1286I) following the recommendations of the manufacturers (Thermo Scientific Fermentas Fast Digest Restriction Enzymes) and the reaction was stopped by addition of 15 µl of stabilized solution. Digested PCR products were electrophoresed in 2% agarose gels AGAROSE TYPE II-A MEDDIUM EEO (Sigma-Aldrich) in TAE buffer at 50V for 6 h. After electrophoresis, the gel was visualized *via* ultraviolet trans-illumination.

2.3. Determination of antibiotic susceptibility

The antibiotic susceptibility was determined by using the Kirby-Bauer method and Mueller–Hinton agar plates supplemented with 1 % NaCl as described by Ottaviani *et al.* (2001). Antibiotics tested are as follow: Ampicilline (AMP) 10 lg, Cefotaxime (CTX) 30 lg, Chloramphenicol (C) 30 lg, Fosfomycin (FOS) 200 lg, Gentamycin (CN) 10 lg, Imipenem (IMI) 10 lg, Kanamycin (K) 30 lg, Nalidixic Acid (NA) 30 lg, Norfloxacine (NOR) 10 lg, Streptomycin (S) 10 lg, Sulfamethoxazole (SMX) 50 lg, Trimethoprime (TM) 5 lg, Doxycycline (DXT) 30 lg, Nitrofurantoine (F) 300 lg, Cephalothin (KF) 30 lg, Erythromycin (E) 15 lg, Ticarcilline (TC) 75 lg, Ciprofloxacin (CIP) 5 lg, Co-Trimoxazole Trimethoprime + Sulfamethoxazole (SXT) 25 lg, Amikacin (AK) 30 lg (Liofilchem s.r.l., Roseto, Italy). After incubation at 37°C for 18–24 h, the diameter of the inhibition zone was measured with 1 mm flat rule and the diameters were interpreted according to Performance Standards for Antimicrobial Disk and Dilution Susceptibility Tests for Bacteria Isolates from Animals (CLSI, 2008). For the two antibiotics (Fosfomycin and Doxycycline), results of the diameters of inhibition were interpreted according to the diameters indicated by the Liofilchem company.

2.4. Multiple antibiotic resistance (MAR) index

The MAR index of the isolates against the tested antibiotics was calculated based on the following formula: MAR Index of an isolate is defined as a ratio (a/b), where (a) represents the number of antibiotics to which the isolate was resistant and (b) represents the number of antibiotics to which the isolate was subjected (Jayaraman *et al.*, 2008). MAR index values equal to or less than 0.2 were defined as those antibiotics that were rarely or never used for the animal in terms

of treatment, however, MAR index value greater than 0.2 was considered an indicator of high risk of exposure to those antibiotics received by the animals (Sarter *et al.*, 2007).

2.5. Phenotypic characterization of slime-producing bacteria

Qualitative detection of biofilm formation was studied by culturing the strains on Congo red agar (CRA) plates as described previously (Freeman *et al.*, 1989) *Vibrio* strains were inoculated into the surface of CRA plates, prepared by mixing 0.8 g Congo red with 36 g saccharose (Sigma) in 1 L of brain heart infusion agar, and were incubated for 24 h at 30ºC under aerobic conditions and followed overnight at room temperature (Chaieb *et al.*, 2007). Slime producing bacteria appeared as black colonies, whereas non-slime producers remained non pigmented (Subashkumer *et al.*, 2006).

2.6. Quantitative adherence assay

Biofilm production by *Vibrio* strains was determined using a semi-quantitative adherence assay on 96-well tissue culture plates, as described previously (Chaieb *et al.*, 2007). Strains were grown in Trypticase Soy Broth supplemented with 1% (w/v) NaCl (TSB 1%, Pronadisa, Spain), following overnight incubation at 30ºC, the optical density at 600 nm (OD 600) of the bacteria was measured. An overnight culture, grown in TSB 1% at 30ºC, was diluted to 1:100 in TSB supplement with 2% (w/v) glucose. A total of 200 µl of cell suspensions was transferred in a U bottomed 96-well microtiter plate (Nunc, Roskilde, Denmark).

Each strain was tested in triplicate. Wells with sterile TSB alone were served as controls. The plates were incubated aerobically at 30ºC for 24 h. The cultures were removed, and the microtiter wells were washed twice with phosphate-buffered saline (7 mM Na_2HPO_4, 3 mM NaH_2PO_4 and 130 mM NaCl at pH 7.4) to remove non-adherent cells and dried in an inverted position. Adherent bacteria were fixed with 95% ethanol and stained with 100 µl of 1% crystal violet (Merck, France) for 5 min. The excess stain was rinsed and poured off and the wells were washed three times with 300 µl of sterile distilled water. The water was then cleared, and the microplates were air-dried. The optical density of each well was measured at 570 nm (OD 570) using an automated Multiskan reader (GIO. DE VITA E C, Rome, Italy).

The optical density (OD_{595nm}) was measured spectrophotometrically. Bacteria were interpreted as (−) non- biofilm forming $OD_{595} \leq 1$, (+) weak biofilm forming $1 < OD_{595} \leq 2$, (++) medium biofilm forming $2 < OD_{595} \leq 3$, or (+++) strong biofilm forming $OD_{595} > 3$ (Snoussi *et al*., 2009) Each essay was performed three times.

2.7. Data analysis and discriminatory power of the methods

To determine significant differences in the patterns, the reproducibility of results was evaluated by repetition of at least three independent RFLP, assays. The number and the size of fragments were evaluated by visual inspection and using Gel Pro Analyzer 3.2 software. Computed similarities among strains were estimated by means of the Jaccard's coefficient (SJ). Cluster analysis and dendrograms were obtained based on the unweighted average pair group method (UPGMA), using Multivariate Statistical Package, version 3.1.

The discriminatory power of this method was calculated by the application of Simpson numerical index of diversity (Hunter and Gaston, 1988). This index was used to compare the typing methods and to select the most discriminatory system for the molecular differentiation of isolates.

The discriminatory index (***D***) of each method was calculated using the standard

formula for this metric. This Discriminatory Power (***D***), as shown by Hunter and Gaston (1988) can be expressed by the formula of Simpson's index of diversity, which reads:

$$D = 1 - \frac{1}{N(N-1)} \sum_{j=1}^{S} n_j(n_j - 1)$$

In this equation, *N* is the total number of *V. alginolyticus* strains in the sample population used for the chemometric model, *S* is the total number of *V. alginolyticus* types involved in this model, and *nj* is the number of the strains belonging to the j^{t} type. A *D* value of > 0.9 is required for a highly discriminatory typing method, with segregation results interpreted with confidence (Willemse-Erix *et al.,* 2009). The discriminatory index of each method was calculated using the standard formula for this metric, where a value of 1 is highly discriminatory and a value of 0 is not discriminatory.

3. Results and Discussion

3.1. Biochemical and phenotypical identification

Twenty-six strains of *V. alginolyticus* were isolated from two aquaculture systems and their phenotypic and biochemical identification is based on some specific characters. Yellow colonies obtained from the modified TCBS agar are identified as Gram-negative motile fermentative rods, with positive, catalase and oxidase activities and susceptible to *Vibrio* static compounds O/129 (150 µg/disk). These colonies could grow in peptone water prepared with 3%, 8% and 10% of NaCl, respectively.

3.2. *Molecular identification and PCR-RFLP*

Many controls were practiced in coastal aquaculture systems to avoid and to fight this pathogenic bacterium, due to its unknown diversity. The current study was performed to compare different methods and allow the detection of some similarities and differences among *V. alginolyticus* isolates. First, we confirmed the *V. alginolyticus* identity by specific PCR amplification as recommended in the research of Di Pinto *et al.*, (2005).

The PCR-RFLP was able to distinguish closely related *V. alginolyticus* strains, allowing deducing phylogenetic relationships and investigating their diversity in various ecosystems. In this study, the two enzymes *Sdu*I and *Faq*I generated polymorphic banding patterns and produced different restriction profiles (Figure 2 a - b). Cluster analysis revealed the existence of 20 haplotypes among 26 strains investigated with different incidence showing the high heterogeneity of *V. alginolyticus* strains. This heterogeneity not only per the origin (fish aquaculture farms and bivalve aquaculture stations), but also within the same type of sample (Figure 3).

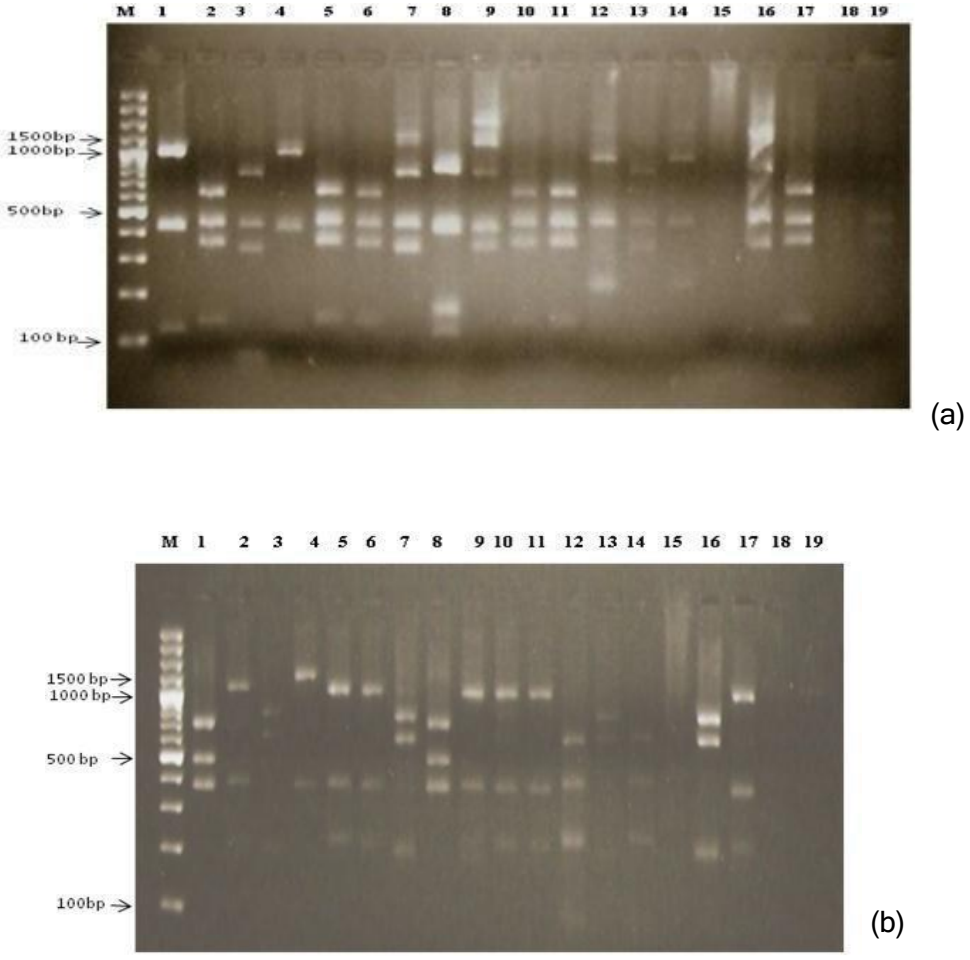

Figure 2. (a) Different profiles obtained by PCR-RFLP using the SduI enzyme (Bsp1286I) on the different isolates of *V. alginolyticus*. (b). Different profiles obtained by PCR-RFLP using. the FaqI enzyme (BsmFI) on the different isolates of *V. alginolyticus*

M: 50-2000 bp DNA molecular size marker; Lanes: 1: CM4 (Conchylicole station-Menzel Jmil), 2: CM13 (Conchylicole station- Menzel Jmil), 3: AKh1(Aquaculture farm-Khenis), 4: AH4 (Aquaculture farm-Hergla), 5:CM2 (Conchylicole station-Menzel Jmil), 6: Akh5 (Aquaculture farm-Khenis), 7: CM7 (Conchylicole station-Menzel Jmil), 8: AH3 (Aquaculture farm-Hergla), 9: AH11 (Aquaculture farm-Hergla), 10:CM9 (Conchylicole station-Menzel Jmil), 11: Akh6 (Aquaculture farm-Khenis), 12: Akh3 (Aquaculture farm-Khenis), 13: CM1(Conchylicole station-Menzel Jmil), 14: AH2 (Aquaculture farm-Hergla), 15: AH6 (Aquaculture farm-Hergla), 16: CM8 (Conchylicole station-Menzel Jmil), 17: CM12 (Conchylicole station-Menzel Jmil), 18: Akh7 (Aquaculture farm-Khenis), 19: CM10(Conchylicole station-Menzel Jmil).

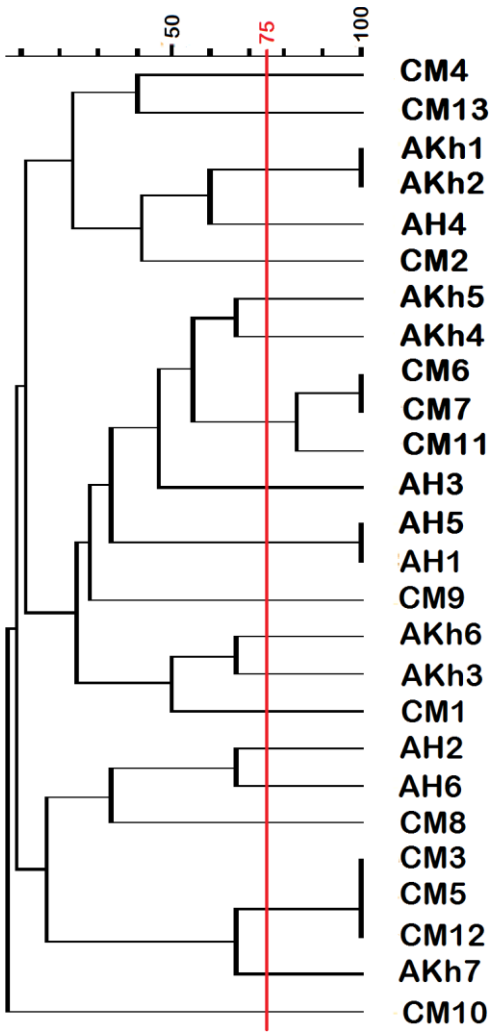

Figure 3. Clusters analyses of RFLP fingerprints showing the genotypic diversity of *V. alginolyticus* strains

Similar results were found by Hossain *et al.* (2014), who demonstrated that PCR-RFLP was more reliable than PCR-based methods. This approach was successfully used to identify *Vibrio cholera* non O1/non0139 and to differentiate among pathogenic *Vibrio* species and can potentially be complementary methods to identify and characterize *V. parahemolyticus* strains by PCR-RFLP of *V. parahemolyticus* MAM-7 gene (Elola-Lopez *et al.*, 2015). This method can be employed for accurate detection of *Vibrio* species including those closely related. Using the PCR-RFLP method, it is possible to recognize closely related *V. alginolyticus* strains, which enables us to deduce phylogenetic relationships and examine their diversity in different ecosystems (Silvester *et al.*, 2017). Whereas Bughe *et al.* (2020) showed that a significant distinction between *V. alginolyticus* and *V. parahaemlyticus* isolates could not be achieved using RFLP 16Sr RNA gene analysis, indicating the need for more sensitive methods. Or, that RFLP and the phylogeny cladogram should be considered in combination.

3.3. Antibiotic resistance and MAR index

Twenty antibiotics were tested on the 26 isolates of *V. alginolyticus*. A total of 70% of *V. alginolyticus* strains were resistant to 11 antibiotics, this resistance is presented by 15 different profiles (Table 1).

Table 1. Results of resistance patterns, MAR index, biofilm formation on polystyrene 96-well plates and slime production on Congo Red Agar

Aquaculture systems	Origin	Strains	Resistance patterns	MAR Index	Polystyrene DO_{595} ± DS	Estimation of biofilm formed	Phenotype of strains (CRA)
Khenis-Monastir Aquaculture farm	Fish (Liver *of S. aurata*)	Akh 1	R2: AMP-CTX-C-IMI-K-S-SMX-DXT-KF-E-TC-CIP: (12)	0.60	4.350 ± 0.208	(+++)	Black
	Fish (Gill of *S. aurata*)	Akh 2	R9: AMP-CTX-C-FOS-CN-IMI-K-NA-SMX-TM-F-KF-E-TC-AK: (15)	0.75	6.512 ± 0.264	(+++)	Black
	Fish *(S. aurata*)	Akh 3	R5: AMP-CTX-K-NA-K-S-TM-DXT-F-KF-E-TC-CIP: (13)	0.65	4.350 ± 0.208	(+++)	Black
	Fish (Juvenile of *S. aurata*)	Akh 4	R3: AMP-CTX-C-FOS-CN-K-TM-DXT-KF-CIP-AK: (11)	0.55	2.261 ± 0.116	(++)	Black
	Fish *(S. aurata*)	Akh 5	R14: AMP-CTX-FOS-CN-IMI-K-NA-SMX-TM-DXT-K-KF-E-TC-CIP: (15)	0.75	6.512 ± 0.264	(+++)	Black
	Fish (Juvenile of *S. aurata*)	Akh 6	R14: AMP-CTX-FOS-CN-IMI-K-NA-SMX-TM-DXT-K-KF-E-TC-CIP (15)	0.75	5.423 ± 0.245	(+++)	Black
	Fish *(S. aurata*)	Akh 7	R12: AMP-CTX-CN-IMI-K-DXT-F-KF-E-TC-CIP:(11)	0.55	1.801 ± 0.152	(+)	White
Menzel Jmil Shellfish Farm	*M. edulis*	CM 1	R4: AMP-CTX-C-FOS-CN-IMI-K-S-SMX-DXT:(10)	0.50	2.524 ± 0.148	(++)	White
	M. edulis	CM 2	R5: AMP-CTX-K-NA-K-S-TM-DXT-F-KF-E-TC-CIP:(13)	0.65	2.177 ± 0.009	(++)	Black
	M. edulis	CM 3	R3: AMP-CTX-C-FOS-CN-K-TM-DXT-KF-CIP-AK :(11)	0.55	2.122 ± 0.193	(++)	Black
	M. edulis	CM 4	R4: AMP-CTX-C-FOS-CN-IMI-K-S-SMX-DXT: (10)	0.50	1.661 ± 0.032	(+)	White
	M. edulis	CM 5	R3: AMP-CTX-C-FOS-CN-K-TM-DXT-KF-CIP-AK: (11)	0.55	1.454 ± 0.116	(+)	White
	M. edulis	CM 6	R11: AMP-CN-IMI-K-NA-S-TM-DXT-F-E-TC-AK: (12)	0.60	2.092 ± 0.032	(++)	Black
	M. edulis	CM 7	R10: AMP-CN-IMI-K-S-SMX-TM-DXT-F-E-TC-CIP-SXT:(13)	0.65	2.177 ± 0.009	(++)	Black
	M. edulis	CM 8	R8: AMP-FOS-IMI-K-TM-DXT-F-KF-E-TC-CIP-SXT: (12)	0.60	2.501 ± 0.174	(++)	Black
	M. edulis	CM 9	R10: AMP-CN-IMI-K-S-SMX-TM-DXT-F-E-TC-CIP-SXT:(13)	0.65	2.544 ± 0.071	(++)	Black
	C. gigas	CM 10	R2: AMP-CTX-C-IMI-K-S-SMX-DXT-KF-E-TC-CIP: (12)	0.60	2.261 ± 0.116	(++)	Black
	C. gigas	CM 11	R8: AMP-FOS-IMI-K-TM-DXT-F-KF-E-TC-CIP-SXT: (12)	0.60	2.223 ± 0.089	(++)	Black
	C. gigas	CM 12	R1: AMP-CTX-FOS-K-NOR-S-DXT-F-E-SXT-AK: (11)	0.55	2.231 ± 0.096	(++)	White
	C. gigas	CM 13	R4: AMP-CTX-C-FOS-CN-IMI-K-S-SMX-DXT: (10)	0.50	1.553 ± 0.164	(+)	White
Hergla Aquaculture farm	Fish (*D. labrax)*	AH 1	R6: AMP-CTX-FOS-IMI-K-NA-SMX-TM-DXT-TC-CIP: (11)	0.55	2.230 ± 0.089	(++)	White
	Fish (*D. labrax)*	AH 2	R15: AMP-CTX-FOS-CN-IMI-K-NA-SMX-TM-DXT-K-KF-E-TC-CIP- S: (16)	0.80	6.512 ± 0.264	(+++)	Black
	Fish (Kidney of *D. labrax)*	AH 3	R13: AMP-C-FOS-IMI-K-NA-TM-DXT-F-KF-E-TC-CIP-SXT: (14)	0.70	5.279 ± 0.264	(+++)	Black
	Fish (Kidney of *D. labrax)*	AH 4	R7: CTX-CN-IMI-K-NA-DXT-F-KF-E-TC-CIP: (11)	0.55	2.524 ± 0.148	(++)	White
	Fish (*D. labrax)*	AH 5	R9: AMP-CTX-C-FOS-CN-IMI-K-NA-SMX-TM-F-KF-E-TC-AK: (15)	0.75	5.279 ± 0.264	(+++)	Black
	Fish (*D. labrax)*	AH 6	R15: AMP-CTX-FOS-CN-IMI-K-NA-SMX-TM-DXT-K-KF-E-TC-CIP- S: (16)	0.80	6.654 ± 0.128	(+++)	Black

Antibiotic used:
AMP Ampicillin (10 lg), CTX Cefotaxim (30 lg), C Chloramphenicol (30 lg), FOS Fosfomycin (200 lg), CN Gentamycin (10 lg), IMI Imipenem (10 lg), K Kanamycin (30 lg), NA Nalidixic Acid (30 lg), NOR Norfloxacin (10 lg), S Streptomycin (10 lg), SMX Sulfamethoxazole (50 lg), TM Trimethoprime (5 lg), DXT Doxycycline (30 lg), F Nitrofurantoine (300 lg), KF Cephalothin (30 lg), E Erythromycin (15 lg), TC Ticarcillin (75 lg), CIP Ciprofloxacin (5 lg), SXT Co-Trimoxazole Trimethoprime+ Sulfamethoxazole (25 lg), AK Amikacin (30 lg)

Interpretation of biofilm formed on polystyrene surface:

- **(-) :** Non biofilm forming, OD595 ≤ 1
- **(+) :** Weak biofilm forming, 1 < OD595≤ 2
- **(++) :** Medium biofilm forming, 2 < OD595 ≤ 3
- **(+++) :** Strong biofilm forming, OD595 > 3

(CRA): Congo Red Agar

Among the antibiotics tested, ampicillin had the highest resistance rate with 92.3%, erythromycin coming in second with 80.8% resistance, cefotaxim third with 77% resistance, kanamycin fourth with 69.2% resistance, doxycyclin fifth with 65.3% resistance, trimethoprim sixth with 57.6% resistance, and gentamycin seventh with 42.3% resistance. Whereas the results recorded by Abdel-Aziz *et al*. (2013) and Sanhoury *et al*. (2021) found that *V. alginolyticus* were resistant to ampicillin, amoxycillin and lincomycin, present a moderate sensitivity against Co- trimoxazole trimethoprime+ sulfamethoxazole and erythromycin and were sensitive to ciprofloxacin, chloramphenicol, gentamicin, enrofloxacin, oxytetracycline, tetracycline. Furthermore, Shahimi et al. (2021) reported that a total of 45.8% of the *V. alginolyticus* isolates were resistant to one or more antibiotics. Moreover, the research of Shahimi *et al.* (2021) showed that a total of 45.8% of the *V. alginolyticus* isolates were resistant to one or more antibiotics. Their study revealed that the greatest resistance was to penicillin, followed by ampicillin, vancomycin and erythromycin.

Kang *et al*. (2016) found identical outcomes, stating that all 15 oyster *V. alginolyticus* isolates in Korea were resistant to ampicillin, vancomycin, and to cephalothin. This antibiotic resistance is often determined by genetic information of plasmid origin in *Vibrio* spp. (Muhammed *et al.*, 2019). Other researchers have reported high levels of antibiotic resistance for *V. alginolyticus* strains by using the minimum inhibitory assay to some antibiotics (Hernández-Robles *et al.*, 2016; Lajnef *et al.*, 2012).

The emergence resistance observed in our work could be linked to the misuse and overuse of antibiotic in aquaculture systems for prophylactic and therapeutic purposes. Additionally, excessive use of antimicrobials could result in a widespread presence of multidrug-resistant bacteria in fish, shellfish, and the surrounding water (Snoussi *et al.*, 2016). Moreover, the studies of Suresh *et al.* (2018) showed that out of 15 *Vibrio* isolates, highest resistance was recorded against ampicillin followed by gentamicin, ceftazidime, amikacin, penicillin, tetracycline and streptomycin and out these 15 isolates, 5 isolates were found positive for ESBLs i.e. 2 (33.33%), 1 (50%), 1 (25%) and 1 (33.33%) were from *V. parahaemolyticus*, *V. vulnificus, V. alginolyticus* and *V. cholerae* respectively by both phenotypic and molecular methods.

In our investigation, it was found that the majority of the isolates were resistant to multiple antibiotics (MAR) with fifteen different profiles. Seven isolates (27%) were resistant to eleven antibiotics, five (19.2%) to twelve antibiotics and four (15.4%) to both thirteen and fifteen antibiotics, respectively. The multiple antibiotic resistance (MAR) index was

ranged from 0.55 to 0.80 for the isolates from the aquaculture farm of Hergla. For the isolates from aquaculture farm of Khenis and from shellfish farm of Menzel Jmil, the MAR index was ranged from 0.55 to 0.75 and from 0.50 to 0.65 respectively (Table 1).

In fact, the isolates from the two aquaculture farm of Hergla and Khenis present the higher value which confirms the overuse of antimicrobials in the aquaculture settings. Thus, “in appropriate used of antibiotic agents provide a favorable condition for resistant bacteria to develop”. These findings were similar to those reported by Lajnef *et al.* (2012) and Manage (2018). Our MAR index value was higher than 0.2, could be originated from high-risk sources of antibiotic contamination where antibiotics are often used, which is in accordance with the studies of Kurdi Al-Dulaimi *et al.* (2019) and Wei *et al.*, (2011).

Ottaviani *et al.* (2013) demonstrated in their research that high levels of multiple-antibiotic resistance property could be stated by the furthered chance to exchange genetic resistance determinants spotted on the plasmids among microorganisms, due to the extensive use of antibiotics in fishery and for the treatment of different kinds of infections.

It was demonstrated that the low MAR range (0.15) indicated low risk of contamination, whereas the high MAR range (above 0.25) indicated high risk of contamination (Chitanand *et al.*, 2010). In this study, the higher MAR index values were 0.80, 0.75, and 0.65 (Table 1), indicating the high contamination ability of *V. alginolyticus* isolates; this agrees with the results obtained in other studies (Ahmed *et al.*, 2018; Ashrafudoulla *et al.*, 2019; Kang *et al.*, 2017). Furthermore, Nguyen *et al.* (2014) showed that antibiotic-resistant microbes (ARMs) have become a major concern for public health, and many isolates from seafood have demonstrated a higher degree of resistance against a wide range of antibiotics.

3.4. Determination of slime production

Slime production was assessed by culturing the investigated strains on Congo Red agar (CRA) plates. Among the 26 *Vibrio alginolyticus strains* tested, 18 strains (69.2%) were a slime-producer developing almost black colonies whereas the remaining 8 strains are considered as non-producers since they showed white colonies on CRA plates (Figure 4).

Figure 4. Different morphotypes described on Congo Red Agar showing black and white colonies

These results were nearly similar to those recorded by Ben Abdallah *et al.* (2009) and Abdulhakeem *et al.* (2023). In fact, the slime is used by bacteria as a protective mechanism against external environments and is measured as an important virulence factor in some pathogenic bacteria, including *Vibrio* and *Aeromonas* species, and it could be an indicator of a high-risk contamination (Secchi *et al.*, 2002). The uses of antibiotics often fail to eradicate the pathogen bacteria because of the aggregation strategy that they have in aquaculture. Indeed, after adhesion to host

fish, pathogen bacteria aggregate, produce an extracellular exopolysaccharide (EPS) that form a matrix and serves for biofilm formation and invading (Ben Hamed *et al.*, 2019; Limoli *et al.*, 2015). Furthermore, Slime molecules play a significant role in the initial stages of biofilm development (Rajkumar *et al.*, 2016).

All strains of *V. alginolyticus* showed swarming motility after inducing the expression of the lateral flagella by growth on solid medium. Lateral flagella allow microorganisms to travel on highly dense environments (Hernández-Robles *et al.*, 2016). Therefore, colonization by this microorganism when present on viscous tissues will be favored (Chen *et al.*, 2017).

3.5. Quantitative Estimation of Biofilm Formation by Tested Bacteria on Abiotic Surfaces

On a polystyrene 96-well microtiter plate (U-bottom), 9 strains out 26 (34.61%) were strong biofilm forming with an optical density of about OD595 > 3 (Table 1). These strains were recovered from fish farms were *S. aurata* and *D. labrax* were reared including 5 strains and 4 strains from Khenis and Hergla respectively. In addition, 13 strains (50%) were medium biofilm forming with an optical density of about 2 < OD595≤ 3; most of which were recovered from shellfish station of Menzel Jmil (*M. edulis* and *C. gigas*). Only four strains (15.38%) weakly adhered to polystyrene and formed a weak biofilm (1 < OD595 ≤ 2) on the polystyrene surface (96-well plate). This study showed that almost the tested bacteria adhered to polystyrene, 84.61% were moderately and strongly adherent which is in accordance with the research of Ben Hamed *et al.* (2019). These authors showed in their research that all the tested bacteria adhered to polystyrene, 90% were moderately and strongly adherent. In aquaculture, Ben Hamed *et al.* (2019). suggested that it should be important to monitor the biofilm formation in the tank wall or any other abiotic material other than glass or polystyrene especially without rough surfaces. The reason for this is that surface roughness influences bacteria adhesion (Lorite *et al*., 2011; Dussud *et al*., 2018) and harbored 25 times more bacteria. (Quirynen *et al*., 1993).

Our strains were able to form biofilms on the polystyrene surface (96-well plate) to different degrees. Similar studies showed the capacity of the isolates to form biofilms on different biotic and abiotic surfaces including polystyrene, glass, and plastic (Abdulhakeem *et al.*, 2023 and Odeymi *et al.*, 2017). The moderate and strong adhesion observed in this study, showed a high antibiotic resistance and were slime-producer developing almost black colonies which could show the relationship between biofilm formation and antibiotic resistance Similar results were observed in the work of Lorite *et al.* (2011).

Such studies have shown the close relationship between biofilm and antibiotic resistance (Song et al., 2017). These authors showed that biofilm cell of *V. parahaemolyticus* has great resistant ability to antibiotics and disinfectants than planktonic cells. Abilities of bacteria for biofilm formation increase their resistance to antibiotic, also increase antibiotic usage and concentrations to overcome antibiotic resistance makes the condition to get worse (Sanhoury *et al.*, 2021).

4. Conclusion

The results of this study demonstrated that the *V. alginolyticus* strains investigated had a high multiple antibiotic resistance (MAR) index values and were able to form biofilms on different biotic and abiotic surfaces. This could allow them to survive longer in these environments and increase their resistance to antibiotic which could make the situation worse. In front of this situation other alternatives to antibiotics should be used such as effective probiotics, antibacterial organic materials, and vaccines. Furthermore, the monitoring and

management of antibiotic patterns, the continuous study of genetic diversity of *Vibrio alginolyticus* and avoid polystyrene as abiotic material in aquaculture are important for treatment to increase fish and seafood safety.

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