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Intraspecific characterization of *Vibrio alginolyticus* isolates recovered from aquaculture systems and marine biotopes in Tunisia by PCR-RFLP, ECP and OMPs profiling.

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Abstract: A total of 54 presumptive *Vibrio alginolyticus* strains isolated from a variety of Tunisian marine biotopes, such as seawater, sediment samples from some bathing areas, and aquaculture farms, were characterized and identified by several biochemical tests. DNA polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP) analysis, extracellular products (ECP) and outer membrane protein (OMP) profiling were used to evaluate their usefulness as a tool to investigate the *Vibrio alginolyticus* diversity within this complex group. Results showed that there is great heterogeneity in the diversity observed via the PCR-RFLP method related to the number of genotypes generated by the two enzymes *SduI* and *FaqI* tested. This heterogeneity was observed not only according to the origin (seawater, sediment, fish and bivalve aquaculture farms) but also within the same type of sample. The two other methods, ECP and OMP resulted in 32 and 26 profiles, respectively. The discriminatory index determined in this study highlighted the good ability of the PCR-RFLP method to discriminate similar *V. alginolyticus* strains.
***Keywords*:** *Vibrio alginolyticus*; extracellular products (ECP); Outer membrane protein (OMP); PCR-RFLP; Tunisia

Sažetak: INTRASPECIFIČNA KARAKTERIZACIJA IZOLATA *VIBRIO ALGINOLYTICUS* PRIKUPLJENIH IZ SUSTAVA AKVAKULTURE I MORSKIH BIOTOPA U TUNISU KORIŠTENJEM PCR-RFLP, ECP I OMPS PROFILIRANJA. Ukupno 54 vjerojatna soja *Vibrio alginolyticus* izolirana su iz raznih morskih biotopa u Tunisu, iz morske vode i sedimenta s nekoliko kupališta i uzgajališta te su karakterizirani i identificirani pomoću nekoliko biokemijskih testova. Istraživana je prikladnost metoda kao što su analiza polimorfizma duljine restrikcijskih fragmenata PCR proizvoda (PCR-RFLP), profiliranje izvanstaničnih proizvoda (ECP) i proteina vanjske membrane (OMP) kao alata za istraživanje raznolikosti *Vibrio alginolyticus* unutar ove složene skupine. Rezultati su pokazali da postoji velika heterogenost u raznolikosti određenoj metodom PCR-RFLP koja se odnosila na broj genotipova generiranih s dva testirana enzima *SduI* i *FaqI*. Ova heterogenost je uočena ne samo prema podrijetlu (morska voda, sediment, uzgajališta riba i školjkaša), već i unutar istog tipa uzorka. Druge dvije metode, ECP i OMP rezultirale su s 32, odnosno 26 profila. Indeks diskriminacije utvrđen u ovoj studiji istaknuo je dobru sposobnost PCR-RFLP metode u razlikovanju sličnih sojeva *V. alginolyticus*.
Ključne riječi: *Vibrio alginolyticus*; izvanstanični produkti (ECP); protein vanjske membrane (OMP); PCR-RFLP; Tunis

INTRODUCTION

Vibrio alginolyticus is considered the main representative of halophilic *Vibrio* in seawater and is an important marine fish and bivalve pathogen (Gómez-Léon *et al.,* 2005). This bacterium is a common inhabitant of the marine environment in both temperate and tropical waters (Zanetti *et al.,* 2000) and it has a broad geographical distribution in marine and estuarine waters, especially in bathing areas (Barbieri *et al.,* 1999). This bacterium is responsible for high mortality rates in aquaculture worldwide (Marhual *et al.,* 2010). It is economically catastrophic for marine fish, invertebrates and large marine mammals as well.

Vibrio alginolyticus remains among the causes of human infections related to consumption of raw or undercooked sea products, causing gastroenteritis and extra-intestinal diseases. These diseases occur frequently during the summer correlated with an increase in seawater temperature (Croci *et al.,* 2001; Thompson *et al.,* 2004).

In Tunisia, *V. alginolyticus* was associated with high fish mortality in aquaculture systems causing important economic losses (Bakhrouf *et al.,* 1995; Ben Kahla-Nakbi *et al.*, 2009). This pathogenic bacterium affects mainly larvae of *Sparus aurata* and *Dicentrarchus labrax* (Ben Kahla-Nakbi *et al.*, 2007). Foremost this situ-

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ation, several molecular typing techniques have been used for *Vibrio* species subtyping and for the study of their microbial epidemiology and ecology (Thompson *et al.,* 2004, Ben Kahla-Nakbi *et al.*, 2006; Snoussi *et al.,* 2008, Ben Abdallah *et al.,* 2010, Lajnef *et al*., 2012). These techniques assessed genetic relatedness of strains, the source of infection and particularly the detection of virulent strains, as well as the study of their geographical and host distribution (Thompson *et al*., 2004; Chen *et al*., 2011).

16S rRNA targeted polymerase chain reactions (PCR) (Kim and Jeong, 2001), the 16S rDNA genotyping using the polymerase chain reaction/restriction fragment length polymorphisms (PCR/RFLP) (Urakawa *et al*., 1997) were used for taxonomic studies of *Vibrio* strains. This 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 approximate phylogenetic relationship of isolates, without the need for 16S rRNA sequencing (Urakawa *et al*., 1997). Moreover, this method (PCR-RFLP) can identify a genetic correlation among pathogenic bacteria and is used in molecular epidemiology studies of different genera and species (Pereyre *et al*., 2013). The analysis of 16S–23S rDNA intergenic spacers (Kong *et al.*, 1999), the random amplified polymorphic DNA (RAPD) /PCR analysis (Sudheesh *et al.,* 2002) and the DNA–DNA hybridization assays (Costa *et al.,* 1998), have been developed and applied in many bacteriological studies.

Several typing methods have been used to elucidate the epidemiology and the pathobiology of several *Vibrio* species; however, only a few studies have been conducted on the characterization of *V. alginolyticus* strains isolated from cultured fish or marine water (Zorrilla *et al.,* 2003). On the other side, many studies were conducted in order to characterize the *Vibrio* species, such as *V. anguillarum* (Kao *et al.,* 2009), *V. alginolyticus* and *V. parahemolyticus* (Ben Abdallah *et al.*, 2010; Marhual *et al.*, 2012) by using enzymatic and proteomic approaches. It is well known that the outer membranes of the Gram-negative bacterial cell play an important role in infection with and pathogenicity of the bacteria (Tsolis, 2002), since they are thought to relate to some important antigenic determinants (Zorrilla *et al.,* 2003; Akayli *et al*., 2008). Also, these outer membrane proteins (OMPs) are assumed to play a key role in the adaptation to the changes of the environment of the bacteria, because of their position in the outermost region of the cell (Xu *et al*., 2005). It has been confirmed that when bacteria are transported to a new environment, the synthesis of their OMPs changes (Kustos *et al.,* 2007). Therefore, there is an increasing interest in interpreting the biological function of OMPs and their immunogenicity characteristics in immune responses (Maftuch *et al.,* 2013). These authors explored the effect of OMPs isolated from the *V. alginolyticus* cell wall, on the immune response in tiger shrimp (*Penaeus monodon*), as well as on the resistance against *V. harveyi*. The outer membrane proteins (OMPs) and the extracellular products (ECP) of *V. harveyi* are good candidates for finding immunogen antigens (Medina *et al*., 2015). Therefore, Ajadi *et al*. (2018) demonstrated that the administration of OMPs led to an increase in the total haemocyte count of the prawns, knowing that haemocytes play significant roles in the recognition of antigens, phagocytosis, encapsulation, cytotoxicity, melanization and cell to cell communication.

OMP are highly immunogenic components with exposed epitopes on the cell surface and the homology among OMP may explain the cross-reactions between Gram negative bacteria (Lun *et al*., 2014). Therefore, OMP profiling is valuable method for typing and differentiating between bacterial fish pathogens (Marhual *et al*., 2012), which is in line with our work, in which we study the OMP profiles for differentiation of the species *V. alginolyticus*.

The study of Peng *et al.* (2021) aimed to identify broad, cross protective antigens from the extracellular secretory proteome of the marine bacterium *Vibrio alginolyticus.* The authors demonstrated that among the 16 DNA vaccines, 3 (AT730_21605, AT730_22220, and AT730_22910) are potential polyvalent vaccine candidates against bacterial infections and their results indicate that the *V. alginolyticus* extracellular secretome is an ideal source for the identification of cross-protective immunogens against bacteria in aquaculture.

The purpose of the present study is to investigate the molecular and phenotypic diversity of *V. alginolyticus* strains recovered from two fish species (*Sparus aurata* and *Dicentrachus labrax*), two bivalve species (*Mytilus edulis* and *Magellana gigas*), seawater and sediments. Strains were characterized by PCR-RFLP, OMP and ECP profiling in order to scrutinize the origins and the relationships among investigated isolates.

MATERIAL AND METHODS

Fifty-four bacterial strains were isolated from four different marine biotopes including two fish aquaculture stations rearing *S. aurata* and *D. labrax* (Khenis and Hergla regions), one bivalve aquaculture station (*M. edulis* and *M. gigas*) and the river Oued Soltane which is in connection with the Mediterranean seawater during the cold seasons (Fig. 1).

Three reference strains of *V. alginolyticus* (CCM 2578^{T}, ATCC 33787, ATCC 17749^{T}) and one reference *V. harveyi* strain (CAIM86) were included in this study. Strains isolated from the marine biotopes 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

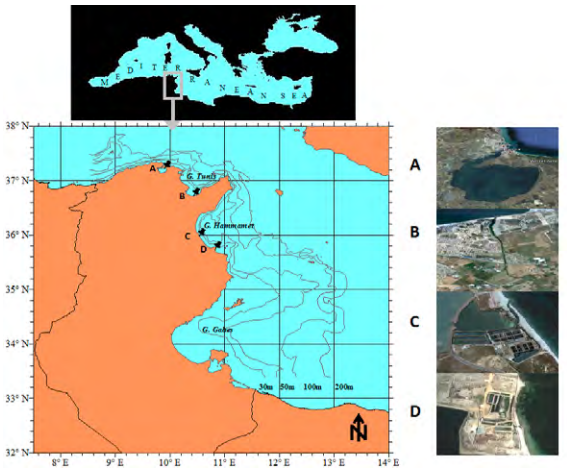

Fig. 1. Map of Tunisia showing the different sites of the *Vibrio alginolyticus* strains isolated from each sample (seawater, fish and bivalve samples). Lac of Bizerte (bivalve aquaculture farm Menzel Jmil) (**A**), Oued Soltane (**B**), aquaculture farm of Hergla (**C**) and aquaculture farm of Khenis (**D**).

test based on the gallery API 20E Kit "galeries 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). Moreover, the determination of different enzymatic activities of each strain was carried out by using API ZYM strips according to the manufacturer's instructions (BioMérieux, Charbonières-Les Bains, France).

DNA Extraction and Specific PCR amplification

Strains were routinely grown on trypticase soy agar (Oxoid Ltd, Madrid, Spain) plates with 1% NaCl at 37°C for 24-48 h, after that colonies were scraped off and suspended in 1 ml of sterile water and centrifuged at 13000 g for 1 min. The supernatant is removed, and the remaining pellet is resuspended in 200 µl of InstaGene Matrix (Bio-Rad, Madrid, Spain) and incubated at 56°C for 30 min. It is shaken using a Vortex for 10 sec and boiled in a water bath for 8 min. The lysate is shaken again and centrifuged at 13000 g for 3 min. The InstaGene DNA preparation is stored at -20°C until being used in PCR amplification.

A *V. alginolyticus*-specific PCR targeting the collagenase gene is used in this study, as described by Di Pinto *et al*. (2006). Forward primer corresponding to nucleotides 1526–1547: VA-F 5'- CGAGTACAGTCACTTGAAAGCC -3'; and reverse primer corresponding to nucleotides 2242–2263: VA-R 5'-CACAACAGAACTCGCGTTACC-3' were used in this investigation. The DNA amplification generated PCR products with molecular weight bands equal to 737 bp. forward and reverse primers VA-F and VA-R were obtained through Invitrogen (Ulm, Germany).

A typical PCR mixture (25 µL) covers 1 mM of the primers, dNTP mix (100 mM each of dATP, dCTP, dGTP and dTTP), 1 U of GO Taq DNA polymerase (Promega), 5 µL green Go Taq buffer (5×), and DNA template (5 µl). PCR is performed in a Gene Amp PCR System 9700 (Applied Biosystems Int., USA). PCR conditions included an initial activation step (95°C for 15 min), followed by 35 cycles of denaturation at 94°C for 30 sec, annealing at 57°C for 30 sec, extension at 72°C for 60 sec, and a final extension at 72°C for 5 min. *V. alginolyticus* (ATCC 33787 and ATCC 17749) reference strains were used as positive controls.

Outer membrane protein profiling**:** The OMP patterns were determined using the method previously described by Crosa and Hodges (1981). Outer membranes (OMs) were obtained by treatment of total cell envelops with Sarcosyl [1.5% (wt/vol)] in 10 mM Tris–HCl (pH 8.0) for 20 min at room temperature (25°C). After centrifugation (100000 g, 1 h at 4°C), OM pellet is washed twice with 10 mM Tris-HCl (pH 8.0) and stored at -20°C and later is examined by sodium dodecyl sulfate (SDS) - polyacrylamide gel electrophoresis (PAGE) (Laemmli, 1970), with 12.5% acrylamide in the separating gel and 4.5% acrylamide in the stacking gel. The proteins were stained with Coomassie brilliant blue R-250 (Sigma, Chemical Co., St Louis, MO, USA) and the molecular weights were determined by comparison with a mixture of commercial markers (SDS-6H) (Sigma).

The ECP of *V. alginolyticus* strains were produced by the cellophane overlay method recommended by Liu *et al*. (2001). Briefly, sterilized cellophane sheets were placed on tryptic soy agar plates with 2% NaCl and inoculated with 200 µl portions of overnight cultures grown in tryptic soy broth supplemented with 2% NaCl. After incubation for 72 h at 28°C, the bacteria grown on the cellophane together with associated ECP were scraped into 1.5 ml of ice-cold, sterile, isotonic, calcium-free marine saline (MS) (0.4 M NaCl, 9 mM KCl, 30 mM $NaSO_4$, 3 mM $NaHCO_3$, pH 7.4). Following centrifugation at 12000 g for 30 min at 4°C, the supernatant (ECP) was sterilized by filtration (0.45 µm-pore-size filters followed by 0.22 µm-pore-size filters). Aliquots of ECP were stored at −80°C until they were used. For heat treatment experiments, ECP was heated at 100°C for 30 min and centrifuged at 12000 g for 10 min before it was used to remove any precipitate. The protein concentration of the ECP was determined by a commercial bicinchoninic acid (BCA) assay (Pierce Warriner, Chester, United Kingdom) by using bovine serum albumin as a standard according to the manufacturer's instructions. The protein concentration of most batches of ECP was adjusted to a. 2.0 mg ml^{-1} prior to use. Global enzymatic activities of ECP were evaluated using the API ZYM system (BioMérieux). A 65 µl volume of each ECP sample was inoculated in each well and the gallery was incubated at 22°C for 18 h. The results were recorded following the manufacturer's instructions.

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 a boiled colony suspension. 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.

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, ECP and OMP assays. The number and the size of fragments were evaluated by visual inspection and using Gel Pro Analyzer 3.2 software. For each method, computed similarities among strains were estimated by means of the Jaccard's coefficient (SJ). Cluster analysis and dendrograms were obtained on the basis of the unweighted average pair group method (UPGMA), using Multivariate Statistical Package, version 3.1.

The discriminatory power of the three methods 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:

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

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 n_j is the number of the strains belonging to the j^{th} type. A D value of > 0.9 is required for a highly discriminatory typing method, with segregation results interpreted with confidence (van Belkum *et al.,* 2007; 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.

RESULTS

Biochemical and phenotypical identification

Fifty-four strains of *V. alginolyticus* were isolated from four different marine Tunisian biotopes. 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. Most strains (42/54) are positive for Voges-Proskauer and lysine decarboxylase tests. Further, only six strains are ornithine decarboxylase positive, and all strains are negative for arginine dihydrolase.

By assessing the enzymatic activities of all *Vibrio* isolates *via* the use of API ZYM strips, a wide variability was noticed with this approach (Table 1). All strains showed 11 common enzymatic activities: alkaline phosphatase, esterases (C4), esterase lipase (C8), leucine arylamidase, trypsin, phosphatase acid and naphthol-AS-BI-phosphohydrolase and were negative for cysteine arylamidase, α-galactosidase, β-galactosidase and β-glucosidase activities. In addition, these strains showed variability in lipase (C14), Valine arylamidase, α-chymotrypsin, β-glucuronidase, N-acetyl-b-glucosaminidase, α-mannosidase and α-fucosidase activities. The exoenzymes found in the ECPs of *V. alginolyticus* ATCC 17749 were as follows: Phosphatase alkaline, Esterase, Esterase Lipase, Leucine-arylamidase, Trypsin, α-chymotrypsin, Phosphatase acid, Naphthol-AS-BI-phosphohydrolase activities, and for *V. alginolyticus* ATCC 33787: Phosphatase alkaline, Esterase, Esterase Lipase, Lipase, Leucine arylamidase, Valine arylamidase, Trypsin, α-chymotrypsin, Phosphatase acid, Naphthol-AS-BI-phosphohydrolase activities.

In order to verify and confirm the *V. alginolyticus* identity, specific PCR amplification was performed. A unique fragment of approximately 737-bp was amplified with the *V. alginolyticus*-specific primers for all the tested isolates (Fig. 2). Outer membrane protein (OMP)

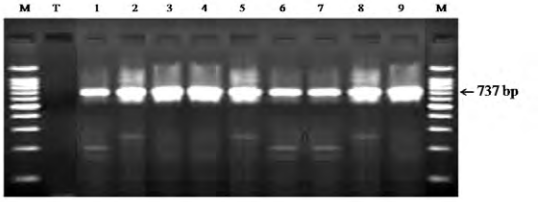

Fig. 2. Representative agarose gel electrophoresis (1.5% agarose) of the amplification products obtained for collagenase genes. (M): 100 bp (Invitrogen, USA); (1): Control; (2,3): *V. alginolyticus* ATCC 17749 and 33787; (4): 36 isolates from seawater Khenis: (5): S50 isolates from the kidney of *Dicentrarchus labrax* (farm Hergla); (6): 57 isolates from juvenile sea bream Khenis; (7): H11 isolates from Oued Soltane; (8): A16 isolates from *Mytilus edulis* (bivalve aquaculture farm Menzel Jmil); (9): A37 isolates from *Magellana gigas* (bivalve aquaculture farm Menzel Jmil).

Table 1. Api Zym profiling of the different *Vibrio alginolyticus* isolates tested.

Strains	Origin	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20
112	*S. aurata* (D)	-	+	+	+	+	+	-	-	+	+	+	+	-	-	-	-	-	-	-	+
36	Sea water (D)	-	+	+	+	-	+	+	-	+	-	+	+	-	-	-	-	-	+	-	-
38	Sea water (D)	-	+	+	+	+	+	-+	-	+	-	+	+	-	-	-	-	-	-+	-	-
118	*S. aurata* (D)	-	+	+	+	-	+	-	-	+	-	+	+	-	-	+	-	-	-	+	-
57	Juvenile Sa (D)	-	+	+	+	-	+	-	-	+	+	+	+	-	-	-	-	-	-	-	+
56	Juvenile Sa (D)	-	+	+	+	-	+	+	-	+	+	+	+	-	-	+	-	-	+	+	+
213	*S. aurata* (D)	-	+	+	+	+	+	-	-	+	+	+	+	-	-	-	-	-	-	-	+
58	Sea water (D)	-	+	+	+	+	+	-	-	+	+	+	+	-	-	-	-	-	-	-	+
K_{11}	Sea water (D)	-	+	+	+	-	+	-	-	+	-	+	+	-	-	+	-	-	-	+	-
K_9	Sea water (D)	-	+	+	+	+	+	+	-	+	+	+	+	-	-	+	-	-	+	+	+
P'7	Sea water (D)	-	+	+	+	+	+	-	-	+	+	+	+	-	-	-	-	-	-	-	+
225	Sea water (D)	-	+	+	+	-	+	-	-	+	-	+	+	-	-	+	-	-	-	+	-
50	Sea water (D)	-	+	+	+	+	+	+	-	+	-	+	+	-	-	-	-	-	+	-	-
226	Sea water (D)	-	+	+	+	+	+	+	-	+	-	+	+	-	-	+	-	-	+	+	-
K_8	Sea water (D)	-	+	+	+	-	+	+	-	+	+	+	+	-	-	-	-	-	+	-	+
K_6	Sea water (D)	-	+	+	+	+	+	+	-	+	+	+	+	-	-	-	-	-	+	-	+
K_3	Sea water (D)	-	+	+	+	-	+	-	-	+	-	+	+	-	-	-	-	-	-	-	-
EM2	Sea water (D)	-	+	+	+	-	+	-	-	+	+	+	+	-	-	+	-	-	-	+	+
EM3	Sea water (D)	-	+	+	+	-	+	-	-	+	-	+	+	-	-	+	-	-	-	+	-
K_{11}	Sea water (D)	-	+	+	+	-	+	-	-	+	-	+	+	-	-	-	-	-	-	-	-
A_{13}	Sea water (A)	-	+	+	+	+	+	-	-	+	-	+	+	-	-	-	-	-	-	-	-
A_{12}	*M. edulis*(A)	-	+	+	+	-	+	-	-	+	-	+	+	-	-	-	-	-	-	-	-
A_{16}	*M. edulis* (A)	-	+	+	+	-	+	-	-	+	-	+	+	-	-	-	-	-	-	-	-
A_{23}	*M. edulis* (A)	-	+	+	+	+	+	-	-	+	+	+	+	-	-	+	-	-	-	+	+
A_{14}	*M. edulis* (A)	-	+	+	+	+	+	-	-	+	+	+	+	-	-	+	-	-	-	+	+
A_{40}	*M. edulis* (A)		+	+	+	-	+	-	+	+	+	+	+	-	-	-	-	-	-	-	+
A_{41}	*M. edulis* (A)	-	+	+	+	+	+	+	-	+	+	+	+	-	-	-	-	-	+	-	+
A_{26}	*M. edulis* (A)		+	+	+	-	+	+	-	+	-	+	+	-	-	-	-	-	+	-	-
A_{28}	*M. edulis* (A)	-	+	+	+	-	+	-	-	+	+	+	+	-	-	-	-	-	-	-	+
A_{30}	*M. gigas* (A)	-	+	+	+	-	+	-	-	+	-	+	+	-	-	-	-	-	-	-	-
A_{27}	*M. gigas* (A)	-	+	+	+	-	+	-	-	+	-	+	+	-	-	+	-	-	-	+	-
A_{37}	*M. gigas* (A)	-	+	+	+	-	+	-	-	+	+	+	+	-	-	+	-	-	-	+	+
S_{38}	*D. labrax* (C)	-	+	+	+	-	+	-	-	+	-	+	+	-	-	-	-	-	-	-	-
S_{50}	*D. labrax* (C)	-	+	+	+	+	+	+	-	+	-	+	+	-	-	+	-	-	+	+	-
S_{55}	*D. labrax* (C)	-	+	+	+	-	+	+	-	+	+	+	+	-	-	-	-	-	+	-	+
S_{57}	*D. labrax* (C)	-	+	+	+	+	+	+	-	+	-	+	+	-	-	+	-	-	+	+	-
S_{32}	*S. aurata* (C)	-	+	+	+	+	+	+	-	+	+	+	+	-	-	+	-	-	+	+	+
DV3	*S. aurata* (C)	-	+	+	+	+	+	-	-	+	-	+	+	-	-	-	-	-	-	-	-
H_1	Sediment (B)	-	+	+	+	+	+	-	-	+	+	+	+	-	-	-	-	-	-	-	+
H_3	Sediment (B)	-	+	+	+	+	+	-	-	+	+	+	+	-	-	-	-	-	-	-	+
H_4	Sediment (B)	-	+	+	+	-	+	-	-	+	+	+	+	-	-	-	-	-	-	-	+
H_6	Sediment (B)	-	+	+	+	+	+	+	-	+	+	+	+	-	-	+	-	-	+	+	+
H_8	Sediment (B)	-	+	+	+	-	+	-	-	+	+	+	+	-	-	-	-	-	-	-	+
H_{10}	Sediment (B)	-	+	+	+	+	+	-	-	+	+	+	+	-	-	+	-	-	-	+	+
H_{11}	Sediment (B)	-	+	+	+	+	+	-	-	+	+	+	+	-	-	+	-	-	-	+	+
H_{12}	Sediment (B)	-	+	+	+	-	+	+	-	+	+	+	+	-	-	+	-	-	+	+	+
H_{20}	Sediment (B)	-	+	+	+	-	+	-	-	+	+	+	+	-	-	-	-	-	-	-	+
H_{21}	Sediment (B)	-	+	+	+	-	+	+	-	+	-	+	+	-	-	-	-	-	+	-	-
I_{12}	Sediment (B)	-	+	+	+	+	+	-	-	+	+	+	+	-	-	-	-	-	-	-	+
I_{14}	Sediment (B)	-	+	+	+	-	+	-	-	+	+	+	+	-	-	-	-	-	-	-	+
ATCC 33787	Type strain	-	+	+	+	-	+	-	-	+	-	+	+	-	-	+	-	-	-	+	-
ATCC 17749	Type strain	-	+	+	+	+	+	-	-	+	-	+	+	-	-	-	-	-	-	-	-
CAIM 86	Type strain	-	+	+	+	+	+	-	-	+	+	+	+	-	-	-	-	-	-	-	+
CCM2578	Type strain	-	+	+	+	-	+	-	-	+	-	+	+	-	-	-	-	-	-	-	-

1: Control, 2: Phosphatase alcaline, 3: Esterase (C4), 4: Esterase lipase (C8), 5: Lipase, 6: Leucine arylamidase, 7: Valine arylamidase, 8: cystine arylamidase, 9: trypsine, 10: α chymotripsin, 11: phosphatase acid, 12: Naphtol AS-BI phosphohydrolase, 13: α galactosidase, 14: β galactosidase, 15: β glucuronidase, 16: α glucosidase , 17 : β glucosidase, 18: N acetyl β glucosaminidase,19: α mannosidase, 20: α fucosidas, Juvenile Sa: Juvenile *S. aurata*

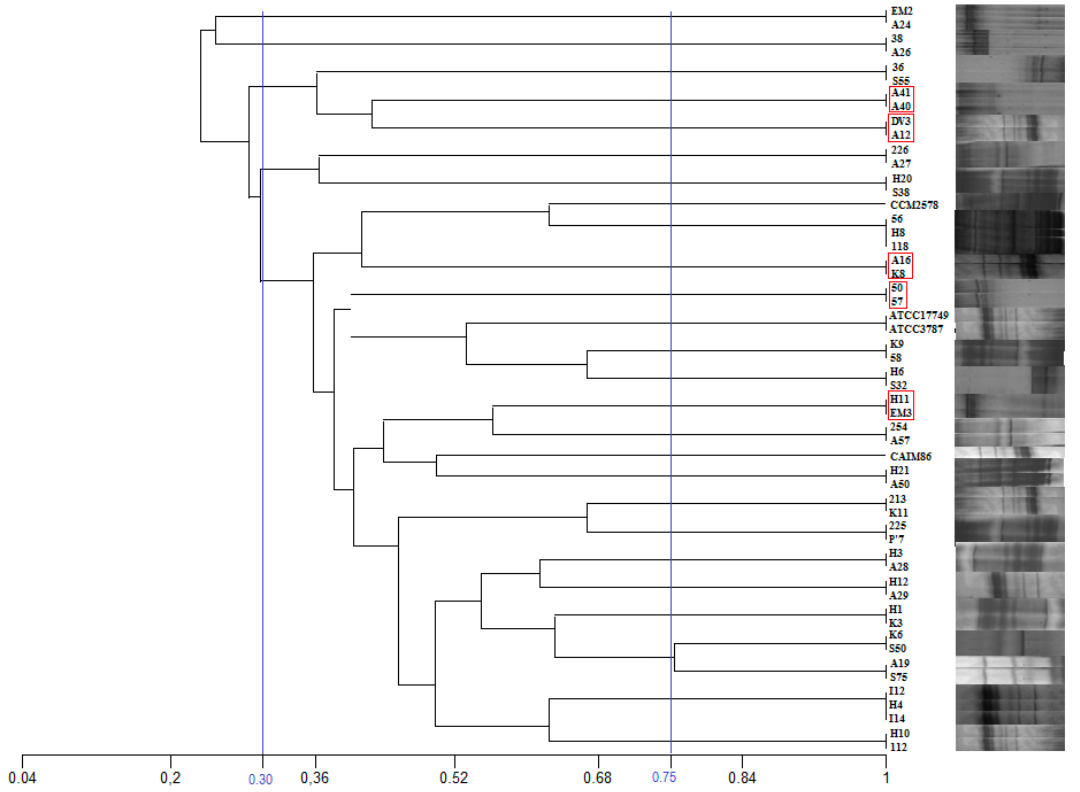

Fig. 3. Cluster analyses of OMP profiles showing the diversity of *Vibrio alginolyticus* strains. The dendrogram was obtained from similarity coefficient (Dice) calculations, and clustering was done using the unweighted pair-grouping method based on the arithmetic averages (UPGMA) algorithm using MVSP software.

analysis; the SDS-PAGE (10%) analysis of OMPs of *V. alginolyticus* strains allowed to obtain 11 to 15 distinct bands, ranging from approximately 10 to 200 KDa. All tested strains showed stable and reproducible patterns. Interestingly, the OMP of *V. alginolyticus* revealed several differences in their profiles, whereas the strains shared common OMP bands such as 75, 60, 45, 30, 27, and 15 KDa.

Cluster analysis based on the pairwise coefficient similarity with UPGMA of OMP resulted in 5 distinct clusters with 30% similarity coefficients and permitted the delineation of 26 distinct OMP profiles among 54 tested strains based on the degree of similarity greater than 0.75 (Fig. 3). Referring to this figure, the dendrogram obtained showed 5 clusters I, II, III, IV and V containing related isolates whose number is 2,2,6,4 and 40 respectively. According to these results, the two strains A40 and A41 grouped into the cluster III were isolated from the same origin (*M. edulis* in bivalve aquaculture farm of Menzel Jmil) and were closely related to the strains (DV3 and A12) which were isolated from *S. aurata* in the fish farm of Hergla and from *M. edulis* in bivalve aquaculture farm of Menzel Jmil respectively. The cluster V showed many closely related strains and had been recovered from the same origin such as the strains 50 and 57 which were obtained from juvenile sea bream from the farm of Khenis. Moreover, this cluster showed strains from different origins (sea water of Khenis and from bivalve aquaculture farm of Menzel Jmil) which were closely related such as the case of K8 and A16; also, the case of the strains (EM3 and H11) which were isolated from seawater of Khenis and from Oued Soltane's sediments respectively. The discriminatory power of OMP technique was calculated to be 0.84.

The SDS-PAGE (10%) analysis of ECPs revealed that the *V. alginolyticus* strain pattern consisted of 10 to 18 bands ranging from 10 to 200 KDa. All the tested strains showed stable and reproducible patterns. Cluster analysis based on the pairwise coefficient similarity with UPGMA of ECP differentiated 4 distinct clusters at a 30% of the similarity coefficient and revealed the occurrence of 32 different profiles among the 54 tested strains based on the degree of similarity greater than 0.75 (Fig. 4). Referring to this figure, the dendrogram obtained showed 4 clusters I, II, III and IV containing related isolates whose number is 1, 2, 2 and 49 respectively. Taking count of the dendrogram, the strains A 14 and A12 which were isolated from *M. edulis* (bivalve aquaculture farm of Menzel Jmil), were closely related and grouped into cluster II. In the same context, the strains 36 and 58 isolated from sea water of Khenis were grouped in the same cluster IV. This cluster showed many closely related strains recovered also from different origin (Oued Soltane's sediment, *S. aurata* from the fish aquaculture farm of Khenis and seawater of Khenis) for the case of (H11, DV3 and K11); also, the case of

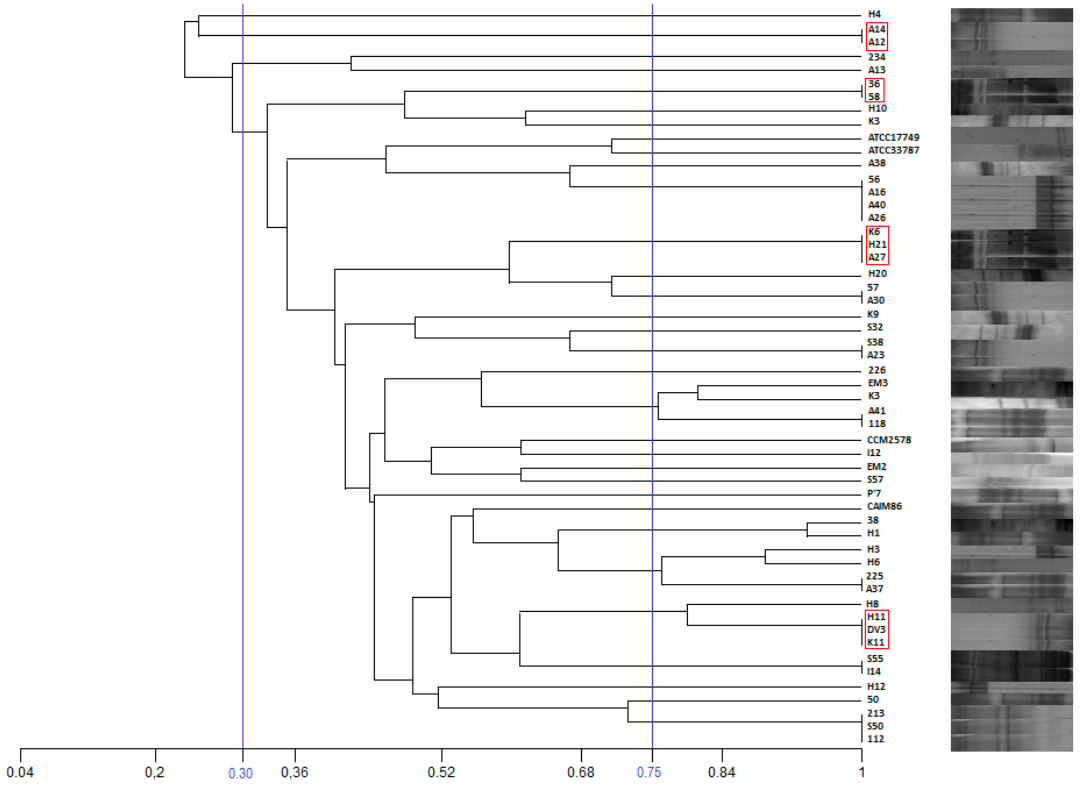

Fig. 4. Cluster analyses of ECP profiles showing the diversity of *Vibrio alginolyticus* strains. The dendrogram was obtained from similarity coefficient (Dice) calculations, and clustering was done using the unweighted pair-grouping method based on the arithmetic averages (UPGMA) algorithm using MVSP software.

the strains (K6, H21 and A27) which were isolated from seawater of Khenis, from Oued Soltane's sediments and from *M. gigas* sampled from the bivalve aquaculture in Menzel Jmil respectively. The discriminatory power of ECP technique was calculated to be 0.90**.**

PCR-RFLP analysis

The PCR-RFLP analysis is performed by using two types of enzymes: *Faq*I (BsmFI) and *Sdu*I (Bsp1286I). The restriction patterns of these two enzymes generated polymorphic banding patterns with 3 to 10 bands ranging from 110 to 1450 bp for *Sdu*I (Bsp1286I) and from 120 to 1350 bp for *Faq*I (BsmFI), respectively. A common band was observed at 500 bp for the different strains profile. These two enzymes *Sdu*I and *Faq*I generated polymorphic banding patterns and produced 23 and 28 different restriction profiles, respectively (Fig. 5). Cluster analysis based on the pairwise coefficient similarity with UPGMA of PCR-RFLP differentiated 5 distinct groups at a 30% similarity coefficient and showed the presence of 44 different haplotypes produced among 54 tested strains based on the degree of similarity greater than 0.75 (Fig. 5). Referring to this figure, the dendrogram obtained showed 5 clusters I, II, III, IV, and V containing related isolates whose number is 2, 1, 2, 41 and 8 respectively. The clusters I, III, IV and V showed closely related strains recovered from different origin, showing the high heterogeneity of *V. alginolyticus* strains not only *per* origin (seawater, sediments, aquaculture farms and shellfish stations), but also within the same type of sample. The discriminatory power of PCR-RFLP technique was calculated to be 0.99**.**

DISCUSSION

Vibrio alginolyticus continues to be a serious pathogen in aquaculture systems including hatcheries. Many controls were practiced in coastal aquaculture systems to avoid and to fight this pathogenic bacterium, and due to its unknown diversity, the current study was performed in order 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. A unique fragment of approximately 737-bp was amplified with the *V. alginolyticus*-specific primers for all the tested isolates (showed in the Fig. 2). This result is in conformity with those of Di Pinto *et al.* (2006) when they described a collagenase-targeted multiplex-polymerase chain reaction (m-PCR) for the identification of *V. alginolyticus*, of *V. cholerae* and of *V. parahaemolyticus* isolated from shellfish samples enriched in alkaline peptone water.

Out of 54 isolates tested, the PCR-RFLP, ECP, and OMP techniques discerned respectively 44, 32 and 26 different profiles. The discriminatory power of the three typing methods can be compared by calculating the discrimination value. Hunter and Gaston (1988) recommended a value greater than 0.9 for better differ-

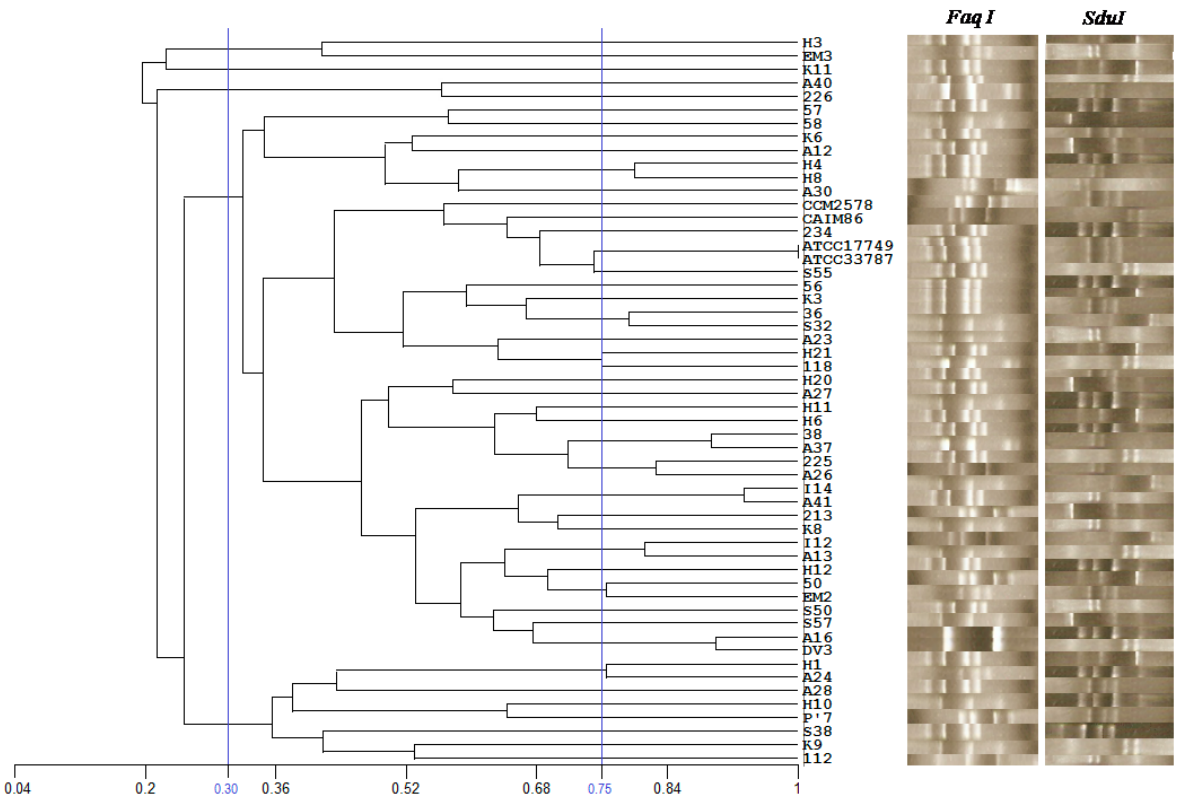

Fig. 5. Cluster analyses of RFLP fingerprints showing the genotypic diversity of *Vibrio alginolyticus* strains. The dendrogram was obtained from similarity coefficient (Dice) calculations, and clustering was done using the unweighted pair-grouping method based on arithmetic averages (UPGMA) algorithm using MVSP software.

entiation. Based on these criteria, PCR-RFLP using two primers *SduI* (Bsp1286I) and *FaqI* (BsmFI) showed the highest discriminatory power and polymorphism (0.99) compared to the ECP and OMP methods which showed discriminatory values of 0.90 and 0.84 respectively and will differentiate the 54 *V. alginolyticus* isolates into 32 and 26 different patterns.

DNA-based approaches have increasingly been applied to microbial identification and classification. In fact, the PCR-RFLP could 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 23 and 28 different restriction profiles, respectively. Cluster analysis permitted the definition of 43 haplotypes with different incidences, showing the high heterogeneity of *V. alginolyticus* strains not only *per* origin (seawater, sediments, fish aquaculture farms and bivalve aquaculture stations), but also within the same type of sample with higher discriminatory index (0.99). This is in accordance with the work of Urakawa *et al*. (1997), who indicated that it is more reliable to use a minimum number of restriction enzymes to examine the phylogenetic affiliation of several environmental bacteria and showed that PCR-RFLP is a rapid tool to calculate the approximate phylogenetic relationship between bacterial isolates without the need of the 16S rRNA sequencing. Furthermore, Maeda *et al.* (2003) showed that the PCR-RFLP clustering method using *ScaI* and *BlnI* tends to be less time-consuming for differentiating *V. parahemolyticus, V. campbelli, V. carchariae, V. harveyi* and *V. natriegens* in 14 groups among 46 *Vibrio* species. According to Hossain *et al*. (2014), PCR-RFLP was more reliable than PCR-based methods. In fact, a PCR-RFLP based strategy was used to screen for an array of pathogenic *Vibrio* species using groEL gene product. This approach was successfully used to identify *Vibrio cholerae* non O1/non0139 and to differentiate among pathogenic *Vibrio* species (Hossain *et al.,* 2014) and can potentially be complementary method to identify and characterize *V. parahemolyticus* strains by PCR-RFLP of *V. parahemolyticus* MAM-7 gene (Lopez *et al.,* 2015). Recently, Silvester *et al.* (2017) showed the efficiency of groEL PCR-RFLP for detection and accurate differentiation of known pathogens among *Vibrio* species. Species such as *V. cholerae, V. parahemolyticus, V. vulnificus, V. mimicus, V. fluvialis, V. alginolyticus,* and *V. anguillarum* were evaluated. PCR amplified groEL gene fragment of each *Vibrio* species was digested separately using 5 restriction enzymes Hha1, Rsa1, Alu1, Dde1 and Mbo1. The method proved to be efficient for detection and differentiation of *Vibrio* species and can be employed for accurate detection of *Vibrio* species including those closely related. Phylogenetic analysis also revealed the groEL gene to be a better

phylogenetic marker for *Vibrio* compared to 16S rRNA. This method can be employed for accurate detection of *Vibrio* species including those closely related. The PCR-RFLP could distinguish closely related *V. alginolyticus* strains, allowing deducing phylogenetic relationships and investigating their diversity in various ecosystems (Silvester *et al.,* 2017).

On the other hand, the API ZYM system has been applied in several studies for the discrimination of closely related microorganisms presenting a high level of homogeneity in their conventional biochemical patterns (Zorilla *et al.,* 2003). The enzymatic activities determined by API ZYM strips revealed a wide variability. All strains showed 11 common enzymatic activities: alkaline phosphatase, esterase (C4), ester lipase (C8), leucine-arylamidase, trypsin, phosphatase acid and naphthol-AS-BI-phosphohydrolase, and were negative for cysteine arylamidase, α-galactosidase, β-galactosidase, β-glucosidase.

All tested strains showed variability in lipase (C14), Valine arylamidase, α-chymotrypsin, β- glucuronidase, N-acetyl-b-glucosaminidase, α-mannosidase and α-fucosidase. The exo-enzymes found in the ECPs of *V. alginolyticus* ATCC 17749 were as follows: Phosphatase alkaline, Esterase, Esterase Lipase, Leucine-arylamidase, Trypsin, α-chymotrypsin, Phosphatase acid, Naphthol-AS-BI-phosphohydrolase. For *V. alginolyticus* ATCC 33787: Phosphatase alkaline, Esterase, Esterase Lipase, Lipase, Leucine-arylamidase, Valine-arylamidase, Trypsin, α-chymotrypsin, Phosphatase acid, Naphthol-AS-BI-phosphohydrolase.

Interestingly, the detection of phosphatase acid and leucine arylamidase confirmed the pathogenicity of *V. alginolyticus* and the invasion of *S. aurata* and *D. labrax*. This result was in accordance with previous studies using the API ZYM system, which demonstrated that the two leucine arylamidase and phosphatase acid enzyme activities have been identified as some disease-related virulence mechanisms in bacterial pathogens (Ridgway *et al.,* 2008).

From these API ZYM results, we showed that ours were consistent in some tests and showed some variability in others by comparing them with the studies of Zorilla *et al.* (2003), Snoussi *et al.* (2008) and Ridgway *et al.* (2008). Additionally, the ECP of *V. alginolyticus* play an important role in the virulence of the cultured Gilthead sea bream (*S. aurata*) (Balebona *et al.,* 1998). In this study, we investigated this important component that showed considerable heterogeneity since 32 different profiles were obtained among the 54 *V. alginolyticus* tested strains. Compared to OMP analysis, this ECP approach discerned more profiles and appeared more discriminative than the OMP approach per its discriminatory index. This discriminatory power of a typing method is defined as its ability to distinguish between unrelated strains (Hunter and Gaston, 1988). It is determined by the number of types defined by the test method and the relative frequencies of these types. These two facets of discrimination are not generally presented as a single numerical value and therefore cannot be used for a straightforward comparison of different methods. Hunter and Gaston (1988) proposed a single numerical index of discrimination, based on the probability that two unrelated isolates would be placed into different typing groups. This probability can be calculated from Simpson's index of diversity. If typing results are to be interpreted with confidence, a DI greater than 0.90 is desirable.

The extracellular products (ECP) produced by pathogenic bacteria may contain different biological activities, such as proteases, cytolysins, hemolysins, siderophores, esterases and phospholipases (Ellis, 1991). In addition, these virulence factors allow the pathogenic bacteria to survive, to proliferate and to invade the host tissues by means of the production of two main effects, toxicity and tissue hydrolysis (Campbell *et al.,* 1990). Also, the study of Borrego *et al.* (1996) showed that the exo-enzymatic content of ECP of some tested strains grouped *per* their geographical site of isolation varied inter- and intra-specifically. This high intraspecific heterogeneous character of these biological activities has been previously reported by Balebona, (1998) in some *Vibrio* strains and by Nieto and Ellis (1991) in other fish pathogens. The extracellular products of *V. alginolyticus* have been reported as having noticeable cytotoxic effects against CHSE-214, EPC, FHM cells (Balebona *et al.,* 1998; Mechri *et al*., 2013**)** and HeLa cell lines (Baffone *et al.,* 2005). These authors suggested that the character of cytotoxic activity towards cell lines could be strongly related to the virulence of the bacteria, such as *V. alginolyticus* strains (Baffone *et al.,* 2005).

Besides ECP, the OMP of *V. alginolyticus* was known to have a significant role in the virulence of the bacterium. The studied strains shared approximately six successive bands of 75, 60, 45, 30, 27 and 15 KDa. The frequent variations in the OMP profiles detected in our work agreed with the result of Akayli *et al*. (2008), whose OMP cluster analysis has shown 28 different profiles. This heterogeneity may be explained by the frequent movement of bacteria between different biotopes and the persistence of *V. alginolyticus* in various aquatic environments may be attributed not only to the high genomic plasticity *via* continuous acquisition of alien genes by diverse mobile genetic elements (MGEs) (Xue et *al.,* 2022). The variety of mobile genetic elements (MGEs), identified by genomic analysis, have been considered to facilitate the evolution and niche adaptation of *Vibrio via* horizontal gene transfer (HGT) (Le Roux and Blokesch, 2018). Furthermore, the study conducted by Zorilla *et al.* (2003) pointed out the presence of a similar OMP band for *V. alginolyticus* isolates irrespective of their geographical origin and the fish species originating from. Nevertheless, the protein profiles allowed grouping the isolates in six OMPs and showed that all strains shared at least four major OMP bands, presenting the molecular masses of 33, 36, 40 and 46 KDa, respectively.

The study of Medina *et al.* (2015) confirmed that OMP and ECP profiles could be considered as potential candidates in the design of vaccines for *Solea senega-*

lensis against *V. harveyi*. The OMPs played a fundamental role in the adaptation to the frequent changes of the environment owing to their position in the outermost region of the cell. In this context, Xu *et al*., (2005) demonstrated that the OMP profile of *V. alginolyticus* is altered at different sodium concentrations. Likewise, the study of Ben Abdallah *et al.* (2010) showed that expression of OmpW, OmpA and peptidoglycan-associated lipoprotein decreased to non-detectable levels when cells were submitted to starvation.

CONCLUSION

Based on all these results, the present investigation established the high degree of intra-species diversity among the *Vibrio alginolyticus* strains isolated from different marine biotopes, such as seawater, fish and bivalve aquaculture farms in Tunisia. The data obtained in this study showed that the PCR-RFLP technique offers an excellent means of discriminating the closely related *V. alginolyticus* species, shown by a wide variation in fingerprinting patterns.

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