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Genotypic Diversity, Antimicrobial Resistance and Screening of *Vibrio cholerae* Molecular Virulence Markers in *Vibrio alginolyticus* Strains Recovered from a Tunisian *Ruditapes decussatus* Hatchery

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Abstract

In this study, a total of 54 *Vibrio alginolyticus* strains were analyzed. The isolates were recovered from different compartments of the *Ruditapes decussatus* hatchery in the National Institute of Marine Sciences and Technologies, Monastir, Tunisia. All isolates were biochemically identified (API 20E and API ZYM strips), characterized by amplification of the Hsp-40 gene polymerase chain reaction (PCR) and analyzed by enterobacterial repetitive intergenic consensus (ERIC)-based genotyping to evaluate genetic relationship between the isolated strains. We also looked for the presence of ten *V. cholera* virulence genes (*toxRS*, *toxR*, *toxT, toxS, tcpP*, *tcpA*, *ace, vpi*, *zot* and *ctxA*) in the genomes of *Vibrio* isolates. The antibiotics susceptibility, exoenzymes production and *in vitro* cytotoxic activitiy against HeLa cell line were also carried out for all tested bacteria. Most of *V. alginolyticus* isolates showed significant antimicrobial resistance rates to at least ten antibacterial agents. For most isolates, the minimum inhibitory concentration (MIC) data showed that tetracyclin and streptomycin were the most effective antibiotics. Construction of the phylogenetic dendogram showed that studied isolates were in general genetically heterogeneous; however some *Vibrio* strains were present in different structures of the *R. decussatus* hatchery. The *V. cholerae* virulence genes investigation showed a wild distribution of *toxS* (49/54), *toxR* (45/54) and *toxT* (22/54) genes among *V. alginolyticus* strains isolated from the *R. decussatus* rearing system. Cytotoxic effects of several *Vibrio* extracellular products (28/54) were also observed on HeLa cells.

Key words: *Vibrio alginolyticus*, *Ruditapes decussatus*, hatchery, ERIC-PCR, virulence genes

Introduction

The grooved carpet shell, Ruditapes decussatus (Linnaeus, 1758) belonging to the Venerideae family is common in estuaries and lagoons of most of the Mediterranean (Parache, 1982; Lubert, 1984). In Tunisia, it is present on almost all the coast, especially in the Gulf of Gabes (Medhioub, 1983) and represents the most socioeconomically important shellfish in terms of both employment and value (Hamida *et al.*, 2004). In the last decade, the *R. decussatus* clam has been produced in the experimental hatchery of the National Institute of Marine Sciences and Technologies where epizootic mortality have been occurred during the larval and post-larval stage.

Generally, molluscan larvae are susceptible to bacterial pathogens, especially during their temporary fixation on the bottom of the tank where they are exposed to high concentration of potential pathogenic bacteria associated with the tank surface, moribund larvae or organic detritus (Sutton and Garrick, 1993). *Vibrio alginolyticus* is considered to be a part of marine biota (Vandenberghe *et al.*, 1998; Xie *et al.*, 2005; Mechri *et al.*, 2011). However some works have shown them as one of the most pathogenic bacteria for bivalve larval cultures (Luna-González *et al.*, 2002; Anguiano *et al.*, 1998).

The enterobacterial repetitive intergenic consensus (ERIC) sequences are present in many copies in the genomes of enterobacteria and *Vibrionaceae* bacteria (Versalovic *et al.*, 1991; Hulton *et al.*, 1991; Fabiano *et al.*, 2004). The ERIC-PCR fingerprinting technique is usually used as genetic marker to characterize isolates within *Vibrio* species (Marshall *et al.*, 1999; Ashraf *et al.*, 2001; Ben Kahla-Nakbi *et al.*, 2006).

V. alginolyticus has a high rate of recombination with the diverse, closely related marine bacterial strains (González-Escalona *et al.*, 2006). In fact several studies have reported that *V. alginolyticus* represents an

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important recipient of some *V. cholerae* and *V. parahaemolyticus* virulence genes transfers (Sechi *et al.*, 2000; Xie *et al.*, 2005; Snoussi *et al.*, 2008b; Ben Abdallah *et al.,* 2009). The mobility of the virulence-factor genes and a successful transfer may cause the transformation of a nonpathogenic strain to a pathogenic one (Boyd *et al.*, 2000; Hentschel *et al.*, 2000; Faruque and Nair, 2002).

The purpose of this study was to determine the relationship between 54 *V. alginolyticus* strains isolated from a Tunisian *R. decussatus* hatchery using ERIC-PCR fingerprinting technique and to investigate the presence of 10 *V. cholerea* virulence-factor genes (*toxRS*, *toxR*, *toxT, toxS, tcpP*, *tcpA*, *ace, vpi*, *zot* and *ctxA*) among these strains. In addition, we tested the antibacterial susceptibility, the enzymatic and the cytotoxic activities.

Experimental

Material and Metods

Bacterial strains. A total of 54 *V. alginolyticus* isolates collected from a Tunisian *R. decussatus* hatchery were analyzed in this study, including 14 strains from post-larva and rearing tanks, 12 from larva and rearing tanks, 10 from broodstock and breeder tanks, 10 were isolated from sea water inside the hatchery, 5 from larva food and 3 from broodstock food. All the *V. alginolyticus* isolates were screened by colony color on thiosulfate citrate bile salt sucrose (TCBS) medium (supplemented with 2% NaCl) and were further identified by the biochemical tests as described by Mechri *et al.* (2011).

Antibacterial susceptibility. Bacterial susceptibility to antimicrobial agents was performed by disc diffusion method on Mueller Hinton agar plates (bioMérieux, France) supplemented with 1% NaCl as described by Ottaviani *et al.* (2001). A total of 20 antibiotics were used on the basis of previous reports (Ottaviani *et al.*, 2001; Ben Kahla *et al.*, 2006; Snoussi *et al.*, 2008a). These antibiotics are: ampicillin (10 µg), chloramphenicol (30 µg), co-trimoxazole (25 µg), ciprofloxacin (5 µg), gentamicin (10 µg), flumequine (30 µg), nalidixic acid (30 µg), streptomycin (10 µg), tetracyclin (30 µg) erythromycin (15 µg), polymyxin B (300 units), cephalothin (30 µg), , imipenem (10 µg), doxycyclin (30 units), kanamycin (30 µg), carbenicillin (100 µg), norfloxacin (5 µg), nitrofurantoin (300 µg), oxolonic acid (10 µg), colistin-sulphate (25 µg). After incubation at 37°C for 18–24 h, the diameters of the inhibition zone were interpreted according to the "Comité de la Société Française de l'Antibiogramme" (Cavallo *et al.*, 2006) and following by the recommendations of the National Committee for Clinical Laboratory Standards (NCCLS, 2002), the strains were categorized as susceptible or resistant to the drug. *Escherichia coli* ATCC 25922 was used as a control throughout all the experiments.

Minimum Inhibitory Concentration (MIC). The minimum inhibitory concentration of four antibiotics (ampicillin, erythromycin, tetracyclin and streptomycin) was determined using the broth microdilution method in Muller Hinton broth (bioMérieux, France) supplemented with 2% NaCl (pH 7,2) as described by the Clinical and Laboratory Standards Institute recommendations (M7-A7; CLSI, 2006). *Vibrio* strains were streaked on Trypticase Soy Agar plates (TSA) supplemented with 2% NaCl and incubated at 30°C for 24–48 h. Single colonies were suspended in 0.85% saline and the turbidity was compared with the 0.5 McFarland standard, to produce a bacterial suspension of 10^8 CFU/ml. Last inoculums concentration of 10^5 CFU/ml was obtained in sterile U shaped bottom 96-well microtiter plates containing the test concentrations of antibiotics (0.125–256 mg/l) and incubated at 35°C for 18–20 h. *Escherichia coli* ATCC 25922 was used as a control microorganism.

ECPs preparation and enzymatic profile. The extracellular products (ECPs) were obtained by the cellophane plate technique (Liu, 1957; Amaro *et al.*, 1992) by spreading 1 ml of an overnight culture of the isolated strains over sterilized cellophane sheets placed on TSA plates supplemented with 1% NaCl. After incubation for 24 h at 25°C, bacterial cells were washed off the cellophane sheets with phosphate-buffered saline (PBS – 1% NaCl), (pH 7.0). The respective suspensions were centrifuged at 12000 g for 30 min at 4°C. The supernatants obtained were sterilized by means of a 0.45 µm pore size nitrocellulose filters (Millipore, Germany). ECP samples were stored at – 20°C until used. The API ZYM system (bioMérieux, France) was used for characterization of the global enzymatic activities of the *V. alginolyticus* strains ECPs. Assays were performed according to the manufacturer's recommendations at 25°C.

Genotypic confirmation. The biochemically typical isolates of *V. alginolyticus* were subjected to PCR for further confirmation of their identity. DNA was extracted from the bacterial strains using Wizard genomic DNA purification kit (Promega, France) according to the manufacturer's instructions. In this study we targeted a *dnaJ* sequence, a housekeeping gene that encodes heat shock protein 40, VM-F (5'-CAGGTTTGYTGCACGGCGAAGA-3') and V.al2-MmR (5'-GATCGAAGTRCCRACACTMGGA-3'). These primers amplify a 144 bp long fragment (Nhung *et al.*, 2007). Amplification reactions contained 5x PCR buffer (Promega, France), 200 µmol/l of each desoxyribonucleotide triphosphate, 1.5 mmol/l of $MgCl_2$, 1U Taq polymerase (Promega, France), 1 µmol/l of each primer, and 2 µl of the template in a final reaction volume of 25 µl. PCR amplifications were carried out in a thermal cycler (Eppendorf, Mastercycler personal) as follows: 3-min initial denaturation step, followed by 35 cycles

of 94°C for 30 s, 60°C for 30 s, and 72°C for 1 min, with a final extension step of 7 min at 72°C.

ERIC PCR. PCR was performed with the following primer sequences ERIC 1 (5'-ATGTAAGCTCCTGGGGATTCAC-3') and the reverse primer ERIC 2 (5'AAGTAAGTGACTGGGGTGAGCG) (Versalovic *et al.,* 1991). Reactions were performed in a 50 µl volume in an Eppendorf, Mastercycler personal thermocycler. Amplification reactions contained 5x PCR buffer (Promega, France), 200 µmol/l of each desoxyribonucleotide triphosphate, 1.5 mmol/l of $MgCl_2$, 1 U Taq polymerase (Promega, France), 1 µmol/l of each primer, and 2 µl of the template. Amplifications were made with 1 cycle at 95°C for 5 min, 30 cycles at 90°C for 30 s, 52°C for 30 s, 70°C for 1 min, and 1 cycle at 70°C for 10 min. PCR products were visualized under UV-light after electrophoresis on a 1.2% agarose gel by ethidium bromide staining and photographed using Gel Doc XR apparatus (Biorad, Milan). The DNA fingerprints obtained were evaluated for similarity using both Gel Pro Analyser 4.0 software and by visual inspection of band patterns. Dendrogram was constructed by the unweighed pair group method of arithmetic averages (UPGMA) and Jaccard's correlation coefficient using the MVSP 3.2 software (Snoussi *et al.*, 2008a).

Virulence genes. The oligonucleotide primers positions and sources for each of the selected virulence-associated factors were listed in table I. All the PCR reactions were performed in a 50 µl volume with 10 µl of 5x PCR reaction buffer (Promega, France), 200 µmol/l of each of the four dNTPs, 1.5 mmol/l $MgCl_2$ (Promega, France), 1 µmol/l of each primer, 1 µl extracted DNA, 1.25 U Taq polymerase (Promega, France) and sterile ultrapure water. The mixtures were incubated for 5 min at 94°C, followed by 35 cycles of amplification. Apart from the primer annealing temperature, each cycle consisted of denaturation at 94°C for 40 sec, annealing for 40 sec, and primer extension at 72°C for 1 min then the mixtures were kept at 72°C for 10 min. The annealing temperature was 54°C for *toxRS*, *toxR* and *toxT*, 58°C was used for *tcpP*, *tcpA*, *toxS* and *ace* whereas the temperature was 60°C for *vpi*, *zot* and *ctxA*. The amplification products were separated by agarose gel electrophoresis (1.6%), visualized following ethidium bromide staining, and photographed under UV-light using Gel Doc XR apparatus (Bio-Rad, Milan).

***In vitro* cytotoxicity assay.** HeLa (human cervical epitheloid carcinoma) cells were grown in MEM (Minimum Essential Medium, Sigma) supplemented with 10% of foetal calf serum (Sigma), 1% of antibiotic solution (Streptomycin-Penicillin 5000 U, Sigma), and 1% of non-essential aminoacids (Sigma). Cells were seeded on 24-well tissue culture plates (2×10^4 cell/ml), and incubated at 37°C in 5% CO_2 for 24 h. *Vibrio* strains were grown on TCBS agar (Scharlau Microbiogy, Spain), at 37 °C for 18–24 h. *V. alginolyticus* isolates were drawn and inoculated in TSB (Bio-Rad, France) supplemented with 1% of NaCl, and incubated at 37°C for 18–24 h. At the end of incubation, the flask contents were transferred to sterile 50 ml test tubes and

Table I
PCR primers used in this study

Target genes	PCR primer sequences (5'-3')	Product size (bp)	Reference
***tox*RS**	toxR0, ATGAGTCATATTGGTACTTAAATT toxS2, AACAGTACCGTAGAACCGTGA	1397	Sechi *et al.* (2000)
***tox*T**	toxT1, TTGCTTGGTTAGTTATGAGAT toxT2, TTGCAAACCCAGACTGATAT	581	Sechi *et al.* (2000)
***tox*R**	toxR1, CCT TCG ATC CCC TAA GCA ATA C toxR2, AGG GTT AGC AAC GAT GCG TAA G	779	Rivera *et al.* (2001)
***tox*S**	toxS1, CCACTGGCGGACAAAATAACC toxS2, AACAGTACCGTAGAACCGTGA	640	Sechi *et al.* (2000)
zot	zot1, ACGTCTCAGACATCAGTATCGAGTT zot2, ATTTGGTCGCAGAGGATAGGCCT	198	Colombo *et al.* (1994)
ace	ace1, GCTTATGATGGACACCCTTTA ace2, TTTGCCCTGCGAGCGTTAAAC	284	Colombo *et al.* (1994)
***tcp*P**	tcpP1, CGAATGCAGTAATCAAGTCT tcpP2, CAGTCAGCTTCATCAACAAT	320	Sechi *et al.* (2000)
***tcp*A**	tcpA1, CACGATAAGAAAACCGGTCAAGAG tcpA2, ACCAAATGCAACGCCGAATGGAGC	617	Keasler and Hall (1993)
vpi	VPI1, GCAATTTAGGGGCGCGACGT VPI2, CCGCTCTTTCTTGATCTGGTAG	680	Sechi *et al.* (2000)
***ctx*A**	ctx2, CGGGCAGATTCTAGACCTCCTG ctx3, CGATGATCTTGGAGCATTCCCAC	563	Field *et al.* (1992)

centrifuged at 3000 rpm for 15 min. The supernatant was filtered through a 0.45 and 0.22 µm pore size filter membrane (Millipore, Germany). The bacterial filtrates (diluted 1:10, 1:50, 1:100), were added to HeLa cell monolayers, previously washed in PBS, and incubated at 37°C in 5% CO2 for 24 h (Baffone *et al.*, 2005). At the end of incubation, cells were observed for the presence of cytotoxic effect (rounding to ≥ 50% of cells). All assays were repeated three times and the readings were averaged. The filtrates showing cytotoxic effect at a 1:10 dilution were considered to be weak (W) producers of toxin, those at a 1:50 dilution were moderate (M) producers, and those at a 1:100 dilution were strong (S) producers (Barbieri *et al.*, 1999).

Statistical analysis. All data were analyzed with SPSS for Windows, version 16.0. The relationship between isolates and virulence factors was studied by the Crosstabs methods. For all test P-values < 0.05 were considered significant.

Results

Biochemical, enzymatic and molecular characterization. The biochemical profiles obtained from the analysis of 54 *V. alginolyticus* strains tested on API 20E miniaturized system showed heterogeneity of the bacterial population isolated from different compartments of the *R. decussatus* hatchery. In fact, among the studied strains we recognized 15 biotypes (Table II). All tested strains of *V. alginolyticus* were positive to indole test and most of them (48/54) were positive to lysine decarboxylase test while seven strains showed positive result to citrate test. All *Vibrio* strains were able to grow in nutrient broth prepared respectively with 2, 4, 6 and 8% of NaCl and produced acid from glucose and sucrose. Twenty three strains were amygdaline positive and only four strains were positive to sorbitol test. All strains tested amplified a 144 pb size fragment corresponding to the heat shock protein 40 gene specific for this species (Fig. 1).

In the API ZYM system, complete homogeneity was observed among all the isolates for phosphatase alcaline, esterase Lipase (C8), leucine arylamidase, valine arylamidase, trypsin and acid phosphatase. However, over than 75% of the tested strains produced both esterase (C4) and Naphtol-AS-BI-phosphohydrolase. Enzymatic activity was also detected for α-chymotrypsine (37%), N-acétyl-β-glycosaminidase (35%), β-glucosidase (22%), Lipase (C4) (9%), α-mannosidase (7%) and β-glucoronidase (5%).

Antibiotic susceptibility. According to the results, most of *Vibrio* isolates showed strong resistance to at least ten of the following antimicrobials: ampicillin (AM), kanamicin (K), nitrofurantoin (FT), doxycyclin

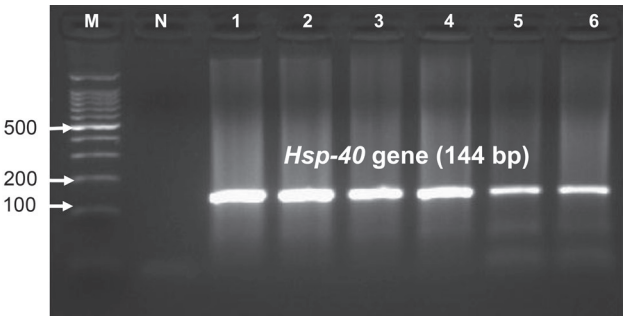

Fig. 1. Agarose gel electrophoresis (2% agarose) of the amplification products obtained for heat shock protein 40 gene. (M): molecular weight marker (100-pb DNA ladder, Promega); (N): negative control; lane (1 to 5): *V. alginolyticus* (strains: L_{12}, PL_{48}, B_5, W_9, LF_4); Lane (6): *V. alginolyticus* ATCC 33787

(DO), carbenicillin (CB), norfloxacin (NOR), erythromycin (E), streptomycin (S), gentamicin (GM), tetracyclin (T) and oxolinic Acid (OA) (Fig. 2). The highest rates of resistance were against AM, K and FT (100%), followed by E (96.3%), CB and DO (94.4%), NOR (90.7%), S (88.8%), GEM (81.5%), T (75.9%), and OA (70.4%). Sixty one percent of the tested strains were resistant to imipenem (IMP), 51.8% to chloramphenicol (C) and 35.2% to co-trimoxazol (STX). The lowest percentages of resistance were observed with nalidixic acid (NA, 16.6%), colistin-sulphate (CT, 9.2%), ciprofloxacin (CIP, 5.5%), cephalotin (CF, 3.7%), polymyxin B (PMB, 1.8%) and flumequin (UB, 1.8%).

The values of the MIC's of ampicillin, erythromycin, tetracyclin and streptomycin against 54 strains of *V. alginolyticus* recovered from *R. decussatus* hatchery are presented in table II. The data showed that tetracyclin (0.5–64 mg/l) and streptomycin (1–64 mg/l) were the most effective antimicrobials against the tested isolates. While most of these strains exhibit a strong resistance to ampicillin (4–256 mg/l) and erythromycin (0.5–256 mg/l). The minimum inhibitory concentration values corresponded to the interpretive MIC breakpoints for susceptibility among *V. cholerae* (CLSI 2006).

ERIC-PCR. The results for ERIC-PCR in this study showed a high diversity of polymorphism among *V. alginolyticus* strains isolated from different compartments of *R. decussatus* hatchery. *Vibrio* strains showed reproducible patterns consisting of 2 to 14 bands over a size range of 0.15 kb–10 kb estimated using the Gel Pro Analyser 4.0 software. ERIC PCR fingerprinting revealed 39 genetic patterns (Fig. 3), *V. alginolyticus* strains isolated from sea water inside the hatchery (40 to 49) had the highest genotypic diversity with nine different patterns followed by post-larva strains (1 to 14), larva strains (15 to 26) and broodstock strains (27 to 36) with eight patterns. Four genotypes were identified from larva food (50 to 54) and three from broodstock food (37 to 39). These results were analyzed using the MVSP 3.2 software to construct the

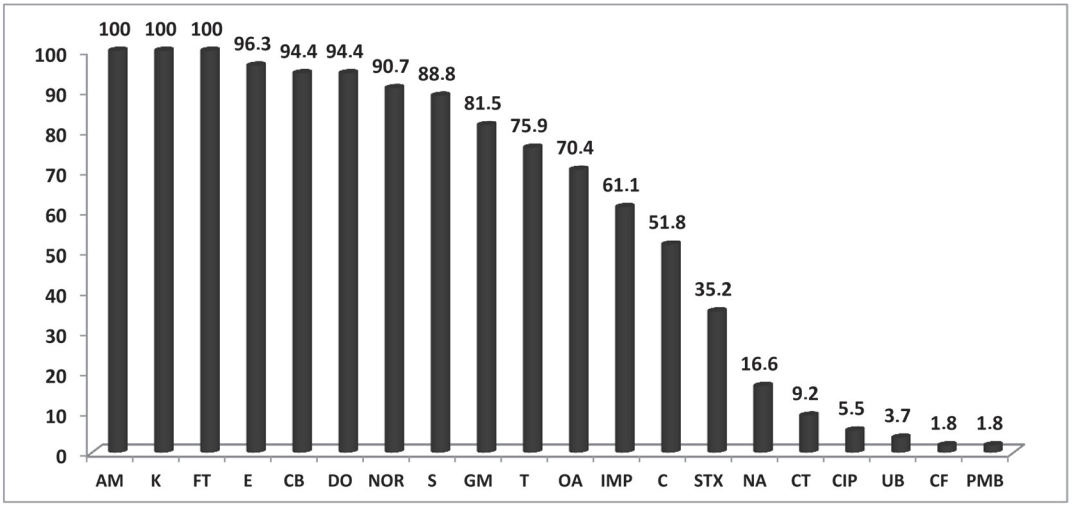

Fig. 2. Antibiotic resistance patterns of selected ***V. alginolyticus*** isolates expressed in %.

AM – ampicillin, K – kanamycin, FT – nitrofurantoin, E – erythromycin, CB – carbenicillin, DO – doxycyclin, NOR – norfloxacin, S – streptomycin, GM – gentamicin, T – tetracyclin, OA – oxolonic acid, IMP – imipenem, C – chloramphenicol, STX – co-trimoxazol, NA – nalidixic acid, CT – colistin-sulphate, CIP – ciprofloxacin, UB – flumequin, CF – cefalotin, PMB – polymyxin B.

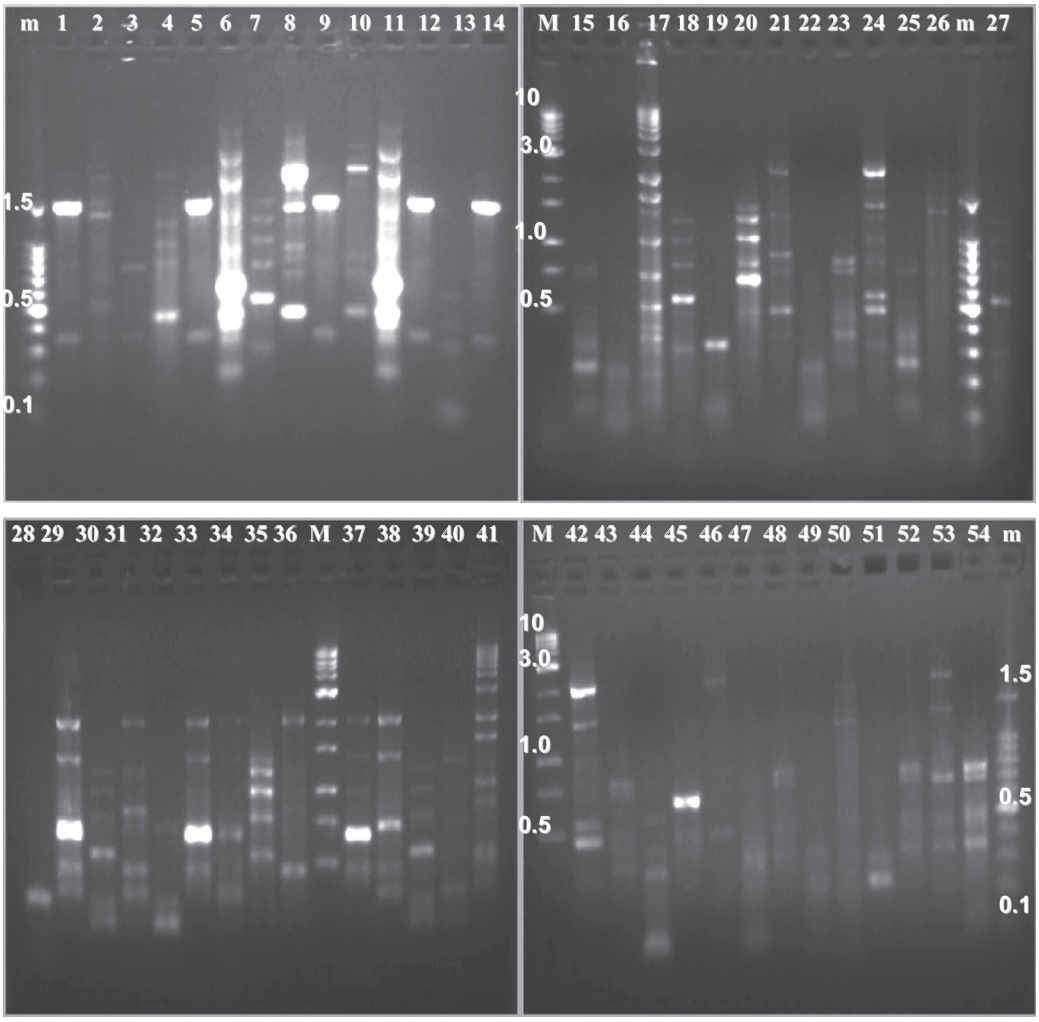

Fig. 3. Fingerprint patterns for the ERIC PCR of ***V. alginolyticus*** isolates run on 1.5% agarose gel.

Lanes: m – molecular weight (Promega, 100 pb), M – molecular weight (Biolabs, 1 kb); 1 to 14, post-larva samples ($PL_1$15, $PLW_1$19, $PL_1$22, PL_1 27, $PL_1$39, $PL_2$18, PL_2 24, PLW_2 32, $PL_3$48, PL_3 25, $PLW_3$32, $PL_4$34, $PLW_4$54, $PL_5$24); 15 to 26, larva samples ($L_1$2, $L_1$6, $LW_1$6, $L_2$4, $LW_2$8, $L_3$6, $L_3$12, $L_4$2, $L_4$8, $LW_4$12, $L_5$4, $L_5$10); 27 to 36, Broodstock ($B_1$1, $B_1$3, $B_2$6, $BW_2$4, $B_2$4, $B_3$2, $B_3$4, $BW_3$2, $B_4$1, $B_5$2); 37 to 39, Broodstock food ($BF_2$6, $BF_4$3, $BF_2$4); 40 to 49, Water inside the hatchery (W4, W9, W17, W2 8, W33, W35, W46, W52, W58, W66); 50 to 54, Larva food ($LF_2$6, $LF_3$4, $LF_3$8, $LF_4$2, $LF_5$4).

Table II
Biotypes, MIC patterns, virulence genes and cytotoxic effects of Vibrio alginolyticus isolates

Origins	Strains	Biotypes	MIC (mg/l)				Virulence genes			Cytotoxicity
			E	S	T	AM	*toxR*	*toxT*	*toxS*	
Post-larva	$PL_1$15	4045124	64	32	0.5	128	–	+	–	–
(n=14)	$PLW_1$19	4046124	32	16	4	32	+	–	+	–
	$PL_1$22	4144125	128	64	32	128	+	–	+	–
	PL_1 27	4146125	16	4	8	64	+	–	+	–
	$PL_1$39	4366124	8	4	16	16	–	+	–	+
	$PL_2$18	4366124	32	8	0.5	128	+	+	+	++
	PL_2 24	4746524	32	32	2	64	+	–	+	+
	PLW_2 32	4144124	8	64	16	256	+	+	+	+
	$PL_2$48	4045125	32	8	0.5	8	+	+	+	++
	$PL_3$25	0146124	0.5	4	8	64	+	+	+	+++
	$PLW_3$32	4146124	4	2	16	128	+	–	+	–
	$PL_4$34	4046124	64	16	4	64	+	+	+	++
	$PLW_4$54	4146125	0.5	1	0.5	16	–	–	–	–
	$PL_5$24	4146124	128	4	8	64	+	–	+	–
Larva	$L_1$2	4146125	4	8	0.5	8	+	–	–	–
(n=12)	$L_1$6	4045125	256	16	8	256	+	–	+	+
	$LW_1$6	4045125	0.5	1	16	64	+	–	–	–
	$L_2$4	4146125	16	2	8	16	+	+	+	++
	$LW_2$8	4046124	32	64	32	16	+	+	+	+
	$L_3$6	4046125	128	1	0.5	32	+	+	+	++
	$L_3$12	0146124	32	4	4	128	+	–	+	+
	$L_4$2	4064125	64	4	16	8	+	–	–	–
	$L_4$8	4046024	8	16	2	4	+	–	–	–
	$LW_4$12	4746524	8	64	0.5	32	+	+	+	++
	$L_5$4	4247124	64	8	1	64	+	+	+	++
	$L_5$10	4247124	64	4	4	256	+	+	+	++
Broodstock	$B_1$1	4044124	2	2	8	4	+	–	+	–
(n=10)	$B_1$3	4064125	128	16	2	32	+	–	–	–
	$B_2$6	4064125	4	8	1	8	+	+	+	+++
	$BW_2$4	0146124	32	32	16	128	–	–	+	+
	$B_2$4	4044125	8	8	0.5	4	+	+	+	+
	$B_3$2	4046124	64	1	8	128	+	–	+	+
	$B_3$4	0046124	32	16	4	16	+	–	+	–
	$BW_3$2	4046024	32	4	32	64	+	–	+	–
	$B_4$1	4044125	4	16	0.5	8	–	+	+	+
	$B_5$2	4146124	32	2	8	128	+	+	+	+
Seawater	W4	4045124	32	32	64	32	+	–	+	–
(n=10)	W9	4045125	2	4	16	16	+	–	+	–
	W17	4064125	64	16	2	128	+	–	+	–
	W28	0146124	16	4	4	64	+	–	+	–
	W33	4044525	32	8	0.5	16	+	–	+	–
	W35	4046124	32	4	4	64	+	+	+	+
	W46	4046124	128	1	1	128	+	–	+	+
	W52	4064125	128	4	0.5	32	+	–	+	+
	W58	4147124	32	1	2	256	+	+	+	++
	W66	4146125	256	2	0.5	64	+	–	+	–

Table II continued

Origins	Strains	Biotypes	MIC (mg/l)				Virulence genes			Cytotoxicity
			E	S	T	AM	*toxR*	*toxT*	*toxS*	
Larva food (n = 5)	$LF_2$6	4144124	16	8	8	4	+	–	+	–
	$LF_3$4	4046124	0.5	8	8	16	+	–	+	–
	$LF_3$8	4044525	32	2	0.5	64	+	+	+	+
	$LF_4$2	4146125	4	4	32	8	+	–	–	–
	$LF_5$4	4044125	16	16	0.5	32	+	–	+	+
Breeder food (n = 3)	$BF_2$6	0146124	8	8	0.5	16	+	–	+	–
	$BF_2$4	4346125	2	1	4	32	+	–	+	–
	$BF_4$3	4045124	16	4	4	64	+	–	+	+

Antibiotics: AM – ampicillin, E – erythromycin, CB – carbenicillin, S – streptomycin, T – tetracyclin.
Cytotoxicity: – none, + weak, ++ moderate, +++ strong.

phylogenetic dendrogram and to be able to estimate the relationship between isolates. Some strains isolated from the same source but from different ponds were classified into one genotype type. In fact, the *V. alginolyticus* strains $PL_1$15 and $PL_1$39 (lanes 1 and 5) were isolated from post-larva samples in the first pond, while strain $PL_2$48 (lane 9) was recovered from the second pond, the isolate $PL_4$34 (lane 12) from the

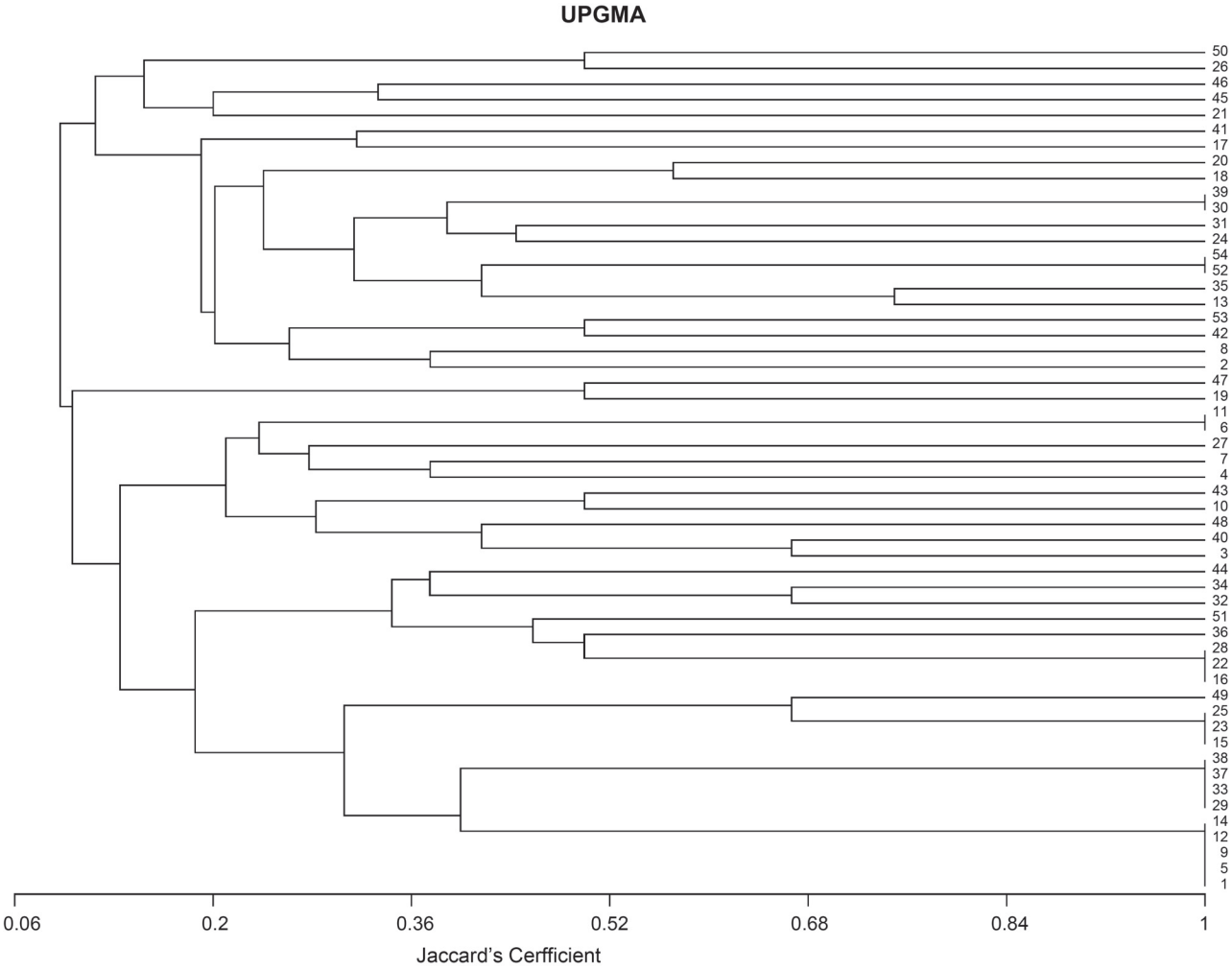

Fig. 4. Dendrogram showing genetic relationships between ***V. alginolyticus*** isolates based on ERIC-PCR fingerprints.

V. alginolyticus strains: 1 to 14, post-larva samples ($PL_1$15, $PLW_1$19, $PL_1$22, PL_1 27, $PL_1$39, $PL_2$18, PL_2 24, PLW_2 32, $PL_2$48, PL_3 25, $PLW_3$32, PL_4 34, $PLW_4$54, $PL_5$24); 15 to 26, larva samples ($L_1$2, $L_1$6, $LW_1$6, $L_2$4, $LW_2$8, $L_3$6, $L_3$12, $L_4$2, $L_4$8, $LW_4$12, $L_5$4, $L_5$10); 27 to 36, Broodstock ($B_1$1, $B_1$3, $B_2$6, $BW_2$4, $B_2$4, $B_3$2, $B_3$4, $BW_3$2, $B_4$1, $B_5$2); 37 to 39, Broodstock food ($BF_2$6, $BF_2$4, $BF_4$3); 40 to 49, Water inside the hatchery (W4, W9, W17, W28, W33, W35, W46, W52, W58, W66); 50 to 54, Larva food ($LF_2$6, $LF_3$4, $LF_3$8, $LF_4$2, $LF_5$4).

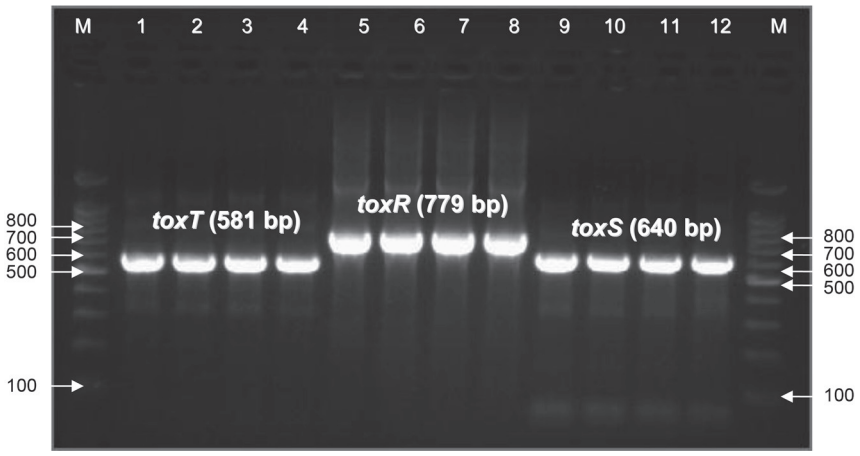

Fig. 5. Agarose gel electrophoresis (1.6% agarose) of the *toxT* (lanes 1 to 4); *toxR* (lanes 5 to 8) and *toxS* (lanes 9 to 12) amplification products of different ***Vibrio alginolyticus*** isolates.
M: molecular weight marker 100 bp ladder (Pro mega, France); 1, $PL_2$48; 2, $L_2$4; 3, $B_2$6; 4, W35; 5, PL_4 34; 6, $L_3$6; 7, $B_3$2; 8, W9; 9, $PL_2$48; 10, $L_3$6; 11, W58; 12, $LF_3$8.

fourth pond and $PL_5$24 (lane 14) from the fifth pond. The strain $PL_2$18 (lane 6) was isolated from the second pond and $PLW_3$32 (lane 11) from the third pond. The *Vibrio* strains $L_1$2, $L_4$8 and $L_5$4 (lanes 15, 23 and 25) were recovered from larva samples (ponds 1, 4 and 5) presented the same genotype profile, whereas strains $L_1$6 (lane 16, pond 1) and $L_4$2 (lane 22, pond 4) showed identical patterns, indicating a high degree of similarity. Bacterial strains $LF_3$8 (lane 52, pond 3) and $LF_5$4 (lane 54, pond 5) were isolated from larva food samples belonged to the same genotype group (Fig. 4). However, other isolates collected from different origins showed an identical pattern, indicating that they belonged to the same clone (Fig. 4). The *V. alginolyticus* isolates $B_2$6 (lane 29, pond 2) and $BW_2$4 (lane 30, pond 2) recovered from broodstock and breeder tanks water samples presented the same genotype profile with $BF_2$6 (lane 37, pond 2) and $BF_2$4 (lane 39, pond 2) strains isolated from broodstock food samples (Fig. 4).

Virulence genes and cytotoxic activity. The results obtained in the PCR experiments of the *Vibrio cholerae* virulence genes demonstrated that 49/54 strains showed positive results in the amplification of the *toxS* gene. Additionally, the *toxR* fragment was amplified from the chromosome of 45/54 *V. alginolyticus* strains whereas the *toxT* gene was found in 22/54 strains (Fig. 5). The crosstabs method revealed a significant relationship ($P = 0.003$) between the presence of the *toxR* gene and the *toxS* gene. All isolates gave negative results for the amplification of *toxRS, tcpP*, *tcpA, vpi*, *zot* and *ctxA* (Table II).

A cytotoxic activity against human HeLa cell line was observed with different degrees in 51.85% of the assayed strains. Only two isolates (3.7%) gave a strong cytotoxic effect, nine (16.66%) strains were moderately cytotoxic and 17 of 54 (31.41%) strains were weakly cytotoxic (Table II).

Discussion

V. alginolyticus has often been isolated from molluscan hatcheries and has been considered to be responsible for epidemic mortalities in larval stages. As far as we know, this is the first time that this *Vibrio* species is isolated from a *R. decussatus* hatchery in the southern part of the Mediterranean. Yet, several studies conducted in Tunisia reported that *V. alginolyticus* causes damages and important economic loss in fish farms.

The phenotypic characterization of *V. alginolyticus* strains isolated from different compartments of *R. decussatus* hatchery showed that all isolates gives yellow colonies on TCBS agar and were able to grow in high salinity concentration. The biochemical characteristics of *Vibrio* strains were in accordance with those described previously by Ottaviani *et al.,* (2003). However, these findings are in disagreement with Ben Kahla-Nakbi *et al.* (2007) who showed that a majority of *V. alginolyticus* strains isolated from dead and moribund fish samples were negative to indole test. *V. alginolyticus* strains were genetically identified to the species level using the Hsp-40 gene as described previously by Nhung *et al.* (2007).

Several studies showed that the API ZYM system provides a simple method for the discrimination of related *Vibrio* species (Biosca and Amaro, 1996; Balebona *et al.*, 1998; Ben Kahla-Nakbi *et al.,* 2007). In fact, the enzyme activity profiles of *V. alginolyticus* strains ECPs obtained corroborate previous studies showing approximately the same enzymatic activities (Gómez-León *et al.*, 2005). On other hand, all the isolates examined gave positive results in tests for lipase, gelatinase and DNase. These data corroborated with the works of Mechri *et al.* (2011) and Snoussi *et al.* (2008a).

Epidemiological surveillance of antimicrobial resistance is essential for empirical treatment of infections

and for preventing the spread of antimicrobial resistant bacterial infections (Adeyemi *et al.*, 2008). The results from this study showed alarming resistance frequencies in *V. alginolyticus* strains isolated from *R. decussatus* hatchery to many antibacterial agents commonly used in aquaculture. *V. alginolyticus* has been reported in previous studies to be resistant to ampicillin, kanamycin, erythromycin, carbenicilin and nitrofurantoin (Lee *et al.*, 1996; Hörmansdorfer *et al.*, 2000 and Ottaviani *et al.*, 2001). In this study, 45/54 (83%) *Vibrio* isolates were resistant to at least 10 antibiotics; these results are in agreement with Snoussi *et al.* (2008a) and Mechri *et al.* (2011), who showed a huge resistance to the same antimicrobial agents.

In previous works, the ERIC-PCR fingerprinting technique was used to study the genetic distribution of *V. alginolyticus* strains isolated from Tunisian fish hatcheries. In fact, Ben Kahla-Nakbi *et al.* (2006) and Snoussi *et al.,* (2008a) reported that the ERIC2-PCR is a very efficient tool for the molecular typing of *V. alginolyticus* species. In this study, of all 54 isolates typed by ERIC-PCR, we recognized 21 strains generating seven profiles. Interestingly, seven *V. alginolyticus* isolates ($PL_1$15, $PL_1$39, $PL_2$48, $PL_4$34, $PL_5$24, $PL_2$18 and $PLW_3$32) recovered from different post-larva and tanks water samples belonged to two ERIC types. While two genotype profiles were found in clones originated from five *Vibrio* strains ($L_1$2, $L_4$8, $L_5$4, $L_1$6 and $L_4$2) isolated from different larva samples. Two isolates ($LF_3$8 and $LF_5$4) from different larva food samples showed identical pattern. Moreover, three *Vibrio* strains ($L_1$6, $L_4$2 and $B_1$3) isolated respectively from Larva and broodstock samples belonged to the same genotype group. While five *V. alginolyticus* strains ($B_2$6, $B_3$4, $BW_2$4, $BF_2$6 and $BF_2$4) recovered respectively from broodstock and broodstock food samples belonged to two genotype profiles. These data confirmed that some *Vibrio* strains, isolated from the same or different ponds, were able to colonize different compartments of the *R. decussatus* hatchery.

Several studies have demonstrated that *V. alginolyticus* has the particularity to acquire virulence genes from other *Vibrio* species. Indeed, Sechi *et al.,* (2000) have reported for the first time the dissemination of some *V. cholerae* virulence genes among related *Vibrio* species. Since there, Deriu *et al.* (2002); Xie *et al.* (2005) and Ben Kahla-Nakbi *et al.* (2009), reported the presence of DNA originating from *V. cholerae* and *V. parahaemolyticus* species in other *Vibrio* bacteria isolated from different marine ecosystems. Our findings corroborate with these results as the virulence gene *toxR*, *toxT* and *toxS* have been detected in most of *V. alginolyticus* isolates. However, this is the first report of the high frequencies of dissemination of the *toxT* gene (41%) among *V. alginolyticus* species.

The cytotoxic effects of extracellular products of *V. alginolyticus* species has been reported against CHSE-214, EPC, FHM cells (Balebona *et al.*, 1998) and HeLa cell lines (Baffone *et al.*, 2005). These authors have suggested that the presence of cytotoxic activities in cell lines can be related to the virulence of *V. alginolyticus* strains. Results of this study showed that 51.85% of the analyzed strains present a cytotoxic effect against the tested cell line.

In conclusion, the study of *V. alginolyticus* genotypes from different structures of *R. decussatus* hatchery is useful to determinate the relatedness of *Vibrio* isolates and to elucidate the source of the bacterial contamination. Besides, this work highlights the incidence of multiple antibiotic resistance in *V. alginolyticus* strains and the large diffusion of some *V. cholerae* virulence genes among the studied strains. Pronounced cytotoxicity was also exhibited by the tested *Vibrio* isolates on HeLa cells.

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