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ORIGINAL PAPER

Prevalence of Biofilm Formation and Wide Distribution of Virulence Associated Genes among *Vibrio* spp. Strains Isolated from the Monastir Lagoon, Tunisia

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Abstract

In the current study, 65 *Vibrio* spp. were isolated from the Monastir lagoon water, were characterized phenotypically and genotypically. In addition, we looked for the presence of three *Vibrio parahaemolyticus* virulence genes (*tlh*, *trh* and *tdh*) and ten *Vibrio cholerae* virulence genes (*ctx*A, *vpi*, *zot*, *ace*, *tox*R, *tox*T, *tos*S, *tox*RS, *tcp*A and *cp*P). We also investigated the antibiotic susceptibilities and the adherence ability of the identified strains to abiotic material and to biotic surfaces. The cytotoxicity activity against HeLa and Vero cell lines were also carried out for all tested strains. All *Vibrio* isolates were identified to the species level and produced several hydrolytic exoenzymes. The results also revealed that all strains were expressing high rates of resistance to tested antibiotics. The minimum inhibitory concentration (MIC) values showed that tetracycline and chloramphenicol were the most effective antibiotics against the tested bacteria. *Vibrio alginolyticus* and *V. cholerae* species were the most adhesive strains to both biotic and abiotic surfaces. Besides, *V. alginolyticus* isolates has the high levels of recombination of genes encoding *V. cholerae* and *V. parahaemolyticus* virulence factors. *In vitro* cytotoxic activities of several *Vibrio* extracellular product were also observed among HeLa and Vero cells.

Key words: *Vibrio* spp., antibiotic susceptibility, biofilm, Monastir lagoon, virulence genes

Introduction

Vibrio species are widely distributed in marine environments, estuarine waters, sediments and hatcheries microbiota (Costa *et al.*, 2010; Mechri *et al.*, 2012). They have been associated with some human infections (Barton and Acton, 2009; Reilly *et al.*, 2011) and can cause several epizootics in many aquatic animals, especially in fish, shellfish and crustaceans (Ben Kahla-Nakbi *et al.*, 2006; Rebouças *et al.*, 2011).

The basis of pathogenicity of *Vibrio parahaemolyticus* depends on three major virulence factors having several biological activities, the thermostable direct haemolysin (*tdh*); the TDH-related haemolysin (*trh*); and the thermolabile haemolysin (*tlh*) (Matsumoto *et al.*, 2000; Nair and Hormazabal, 2005). *Vibrio cholerae* carries a wealth of pathogenic determinants encoded by two separate genetic elements; the cholera toxin genes encoded by the filamentous phage, *CTX*φ and the putative prophage *VPI*φ, which encodes several genes clusters required for toxin co-regulated pilus (*TCP*) production, accessory colonization factors (ACF) and the *tox*T, *tcp*P, *tcp*H and *tcp*I regulatory proteins (Peterson, 2002). Other factors have been associated with enteropathogenicity including two membrane regulatory proteins (*tox*R and *tox*S) (Miller *et al.*, 1987; Miller *et al.*, 1989), a zonula occludens toxin (*zot*) (Fasano *et al.*, 1991) and an accessory cholera enterotoxin (*ace*) (Trucksis *et al.*, 1993).

In most ecosystems, bacterial communities often adopt a sessile biofilm lifestyle in the target to increase their surviving chances by protecting themselves from adverse environmental stressful conditions (Hall-Stoodley *et al.*, 2004; Hoffman *et al.*, 2005). Biofilms exhibits complex spatial organization composed by capillary water channels allowing the flow of nutrients and oxygen into the interior of the biofilm-associated bacteria and allow toxic metabolites to diffuse out of the biofilm (Costerton *et al.*, 1995).

The present study was aimed for isolation and identification of three *Vibrio* species (*Vibrio alginolyticus*, *V. cholerae* and *V. paraheamolyticus*) from the Monastir lagoon water, for detection of biofilm formation and

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for investigation of the presence of three *V. parahaemolyticus* virulence genes (*tlh*, *trh* and *tdh*) and ten *V. cholerae* virulence genes (*ctx*A, *vpi*, *zot*, *ace*, *tox*R, *tox*T, *tos*S, *tox*RS, *tcp*A and *tcp*P). The isolates were also tested for their cytotoxic activity towards two epithelial cells. Their pattern of resistance to antibiotics was also carried out.

Experimental

Materials and Methods

Study area and sample collection. The lagoon of Monastir is situated on the eastern littoral of Tunisia, between the experimental fish and shellfish hatcheries of the National Institute of Marine Sciences and Technologies and a private hatchery of *Sparus aurata* and *Dicentrarchus labrax*. This part of the lagoon is used for supplying the fish and clam hatcheries with rearing water and also used for clam (*Ruditapes decussatus*) farming. The water samples were collected every ten days for a period of 12 months (January to December 2009). All the samples were collected in sterile glass containers (500 ml) and transported in isothermal condition to the laboratory for analysis within 2 h.

Isolation and bacterial characterization. *Vibrio* species were isolated using the membrane filtration technique. The water samples were filtrated through a sterile 0.45 µm pore size cellulose nitrate membrane filter (Millipore, Germany). These filters were transferred in alkaline peptone water (pH 8.6, 1% NaCl) and incubated at 37°C for 24 h. The enrichments were streaked onto Thiosulfate Citrate Bile Salts Sucrose agar (TCBS agar) supplemented with 2% NaCl to increase the detection of *Vibrio* species and incubated at 37°C for 24 h.

Preliminary identification of the strains had been performed on the bases of colony morphology on TCBS (Scharlau Microbiology, Spain) supplemented with 2% NaCl, Gram nonstaining (KOH) method, cytochrome oxidase activity, motility (Mannitol-Motility agar; Pronadisa, Madrid, Spain), resistance to vibriostatic O129 (10 and 150 µg), salt requirement (growth on 0%, 2%, 4%, 8% and 10% NaCl medium) and growth at 23 and 37°C. All of the isolates were processed using API 20E strips (bioMerieux), following the manufacturer's instructions. Ability of *Vibrio* isolates to produce extracellular enzymes such as lipase, amylase, lecithinase, caseinase and Dnase was performed as described previously (Liu *et al*., 1996). *Vibrio* strains were assessed for hemolytic activity on blood base agar supplemented with 5% (v/v) human blood. The strains were conserved as frozen stocks at –80°C in tryptic soy broth (TSB; Bio-Rad, France) with 2% NaCl plus 15% (v/v) glycerol.

Antibacterial susceptibility. Antibiotic susceptibility tests were performed using the disk diffusion method on Mueller-Hinton agar (bioMérieux, France) plates supplemented with 1% NaCl as described by Ottaviani *et al.* (2001). The commercial disks (Bio-Rad, France) containing the following antibiotics were used: ampicillin (10 µg), chloramphenicol (30 µg), co-trimoxazole (25 µg), gentamicin (10 µg), nalidixic acid (30 µg), streptomycin (10 µg), tetracyclin (30 µg), erythromycin (15 µg), kanamycin (30 µg) and carbenicillin (100 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 followed 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 quality control strain.

Determination of minimum inhibitory concentration (MIC). Minimum inhibitory concentration of six antibiotics (Sigma-Aldrich, USA): ampicillin sodium salt, erythromycin, tetracycline hydrochloride, streptomycin sulfate, gentamycin sulfate and chloramphenicol against *Vibrio* isolates were carried out using the broth microdilution method in Muller Hinton broth (bioMérieux, France) supplemented with 2% NaCl (M7-A7; CLSI, 2006). All *Vibrio* strains were cultured on Trypticase Soy Agar plates (TSA) supplemented with 2% NaCl and incubated at 30°C for 24–48 h. The tested isolates were suspended in 0.85% saline to a turbidity equivalent to a 0.5 McFarland standard (1×10^8 CFU/ml) and serially diluted to obtain a concentration of 10^5 CFU/ml in sterile U shaped bottom 96-well microtiter plates containing the test concentrations of antibiotics (0.125–256 mg/l). The plates were incubated at 35°C for 18–20 h after which they were examined for the presence or absence of growth. *E. coli* ATCC 25922 was used as a control microorganism.

Chromosomal DNA preparation. *Vibrio* isolates were grown aerobically on TSA plates containing 1% NaCl at 37°C overnight. Genomic DNA was extracted using Wizard genomic DNA purification kit (Promega, France) according to the manufacturer's instructions.

Molecular characterization. *Vibrio* strains identified by microbiological methods were subjected to polymerase chain reaction assays to assess the presence of genes encoding the heat shocking protein 40 (Hsp-40) specific to *V. alginolyticus*, the outer membrane protein (Omp W) specific to *V. cholerae* and the regulatory toxin protein (ToxR) specific to *V. parahaemolyticus* (Table I). Amplification reactions contained 5 × 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 in a final reaction volume of 25 µl. PCR amplifications were carried out in a thermal cycler (Eppendorf, Mastercycler per-

Table I
PCR primers used in this study

Target genes	PCR primer sequences (5'–3')	Product size (bp)	Reference
hsp-40	VM-F, 5'-CAGGTTTGYTGCACGGCGAAGA-3' V.al2-MmR, 5'-GATCGAAGTRCCRACACTMGGA-3'	144	Nhung *et al*., 2007
toxR-Vp	toxR-Vp1, 5'-GTCTTCTGACGCAATCGTTG-3' toxR-Vp1, 5'-ATACGAGTGGTTGCTGTCATG-3'	678	Lin *et al*., 1993
omp-W	ompW1, 5'-CACCAAGAAGGTGACTTTATTGTG-3' ompW2, 5'-GAACTTATAACCACCCGCG-3'	588	Nandi *et al*., 2000
*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	Fields *et al*., 1992
tlh	tlhf1, AGC GGA TTA TGC AGA AGC AC tlhr2, ATC TCA AGC ACT TTC GCA CG	150	Xie *et al*., 2005
trh	trhf1, TTG GCT TCG ATA TTT TCA GTA TCT trhr1, CAT AAC AAA CAT ATG CCC ATT TCC G	500	Bej *et al*., 1999
tdh	tdhf1, CCA TTC TGG CAA AGT TAT T tdhr1, TTC ATA TGC TTC TAC ATT AAC	534	Xie *et al*., 2005

sonal). The reaction mixture was subjected to an amplification of 35 cycles. Apart from the primer annealing temperature, each cycle consisted of denaturation at 94°C for 30 sec, annealing for 30 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 60°C for *hsp-40* and 64°C for *omp*W and *tox*R. PCR products were electrophoresed through 1.5% agarose gel to resolve the amplified products which were visualized under UV light after ethidium bromide staining.

Virulence gene. Oligonucleotide primers used in this study were listed in Table I. Amplification was carried out in a thermal cycler (eppendorf, Mastercycler personal) with a standard PCR reaction mixture that contained 10 µl of 5 × PCR reaction buffer (Promega, France), 200 µmol/l of each of the four dNTPs, 1.5 µmol/l $MgCl_2$ (Promega, France), 1 µmol/l of each primer, 1 µl extracted DNA (50 ng), 1.25 U Taq polymerase (Promega, France) and sterile ultrapure water to make the volume to 50 µl. The mixtures were incubated for 5 min at 94°C, followed by 35 cycles of amplification. Except for 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 and the mixtures were kept at 72°C for 10 min. The annealing temperature was 48°C for *tdh*, 54°C was used for *tox*RS, *tox*R, *tox*T and *tlh*, 58°C was used for *tcp*P, *tcp*A, *tox*S, *trh* and *ace* whereas the temperature was 60°C for *vpi*, *zot* and *ctx*A. The amplified products were electrophoresed in a 1.6% agarose gel at 90 V for 30 min, stained with ethidium bromide then visualized and photographed using Gel Doc XR apparatus (Bio-Rad, Milan, Italy).

Adherence to PE and PVC surfaces. The quantitative estimate of biofilm formation of *V. alginolyticus* strains on PE and PVC surfaces was determinate using the protocol described by Cerca *et al.* (2006). *Vibrio* strains from fresh agar plates were harvested with sterile

PBS and diluted to a standard concentration equal to an OD of 1.0 at 540 nm (1×10^9 CFU/ml). The 1 cm PE and PVC squares were inserted in the bottom of 24-well microtitre plates (Greiner Bio-One Cellstar, Germany) and 2 ml of each cell suspension was added to each well. Adhesion to each material was allowed to occur for 2 h at room temperature, with gentle shaking.

Negative control wells without bacterial cells were filled with PBS. At the end of the experiment, each well was washed twice with PBS to remove non-adherent or loosely adherent bacteria. After the last wash the pieces were removed from each well and immersed in a new microtiter plate with 1 ml of 98% (w/v) methanol in each well (Henriques *et al*., 2005). The methanol was discarded after 15 min of contact and the pieces were allowed to dry at room temperature. Aliquots of crystal violet were added to each well and incubated for 5 min. After the pieces were washed in water, they were left to dry, then immersed in 1 ml of 33% acetic acid to release and dissolve the stain. The OD of the obtained solution was measured at 570 nm using a spectrophotometer (Jenway 6405 uv/vis). All strains were tested in triplicate, and the bacteria were classified according to Stepanovic *et al.* (2000) as follows (0): $OD \leq ODc$; weakly adherent (+): $ODc < OD \leq 2 \times ODc$; moderately adherent (++): $2 \times ODc < OD \leq 4 \times ODc$; and strongly adherent (+++): $4 \times ODc \leq OD$. This classification was based on the cut-off OD (ODc) value defined as three standard deviation values above the OD of the negative control.

Cell culture conditions. Two cell monolayers were used to examine the adhesive properties of *Vibrio* strains: Hep-2 (human larynx carcinoma) and Vero (kidney epithelial cells of African Green Monkey). For the cytotoxicity assay, we used Vero cells and HeLa (human cervical epitheloid carcinoma) cells.

The 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 (Baffone *et al*., 2005).

Adherence assay. Bacterial adherence was performed as described previously by Snoussi *et al.* (2008). Briefly, 100 µl of 10^7 cells /ml was added to Vero and Hep-2 cells and the 24-well plates were incubated at 37°C for 3 h in 5% CO_2. The cells were washed three times in sterile PBS to remove non-adherent bacteria, fixed in methanol and stained with Giemsa for microscopic examination under oil immersion. Uninoculated cell lines served as negative controls. All organisms were tested twice. The adhesion index was assayed as NA = no adhesive (0–10 bacteria/cells); W = weak adhesion (10–20 bacteria/cells); M = medium adhesion (20–50 bacteria/cells); S = strong adhesion (50–100 bacteria/cells).

Cytotoxicty assay. *In vitro* cytotoxicity was examined on HeLa and Vero cell lines as performed by Baffone *et al.* (2005). *Vibrio* isolates were 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, each flask contents were transferred to sterile tubes (50 ml) and centrifuged at 3000 rpm for 15 min. The supernatant was filtered through a 0.22 µm pore size filter membrane (Millipore, Germany). The bacterial cell-free filtrates were serially diluted (dilutions of 1:10, 1:50 and 1:100), were added to HeLa and Vero cells, previously washed in PBS, and incubated at 37°C in 5% CO2 for 24 h. At the end of incubation, cells were observed under light inverted microscopy and checked for cytotoxic effect (rounding and shrinking to ≥ 50% of cells). All tests were performed in duplicate. The filtrates showing cytotoxic activity 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 test. All data were analyzed with SPSS for Windows, version 16.0. The correlation between presence and absence of the virulence genes was studied by the Crosstabs method. For all test P-values < 0.05 were considered statistically significant.

Results

A total of 65 *Vibrio* isolates were obtained on the selective TCBS agar plates and then they were characterized through the API 20E miniaturized system. Three environmental *Vibrio* species were identified on the basis of their biochemical profile as *V. alginolyticus* (n = 48), *V. cholerae* (n = 12) and *V. parahaemolyticus* (n = 5). The majority of *Vibrio* isolates were positive for lysine decarboxylase, indole production, glucose fermentation and mannitol fermentation. *V. cholerae* and *V. parahaemolyticus* strains gave positive results with ornithine decarboxylase and gelatinase. The five *V. parahaemolyticus* isolates were able to utilize citrate and to assimilate rhamnose (Table II). All *Vibrio* strains tolerated low concentrations of NaCl (2 and 4%). While only 5 (41.66%) *V. cholerae* strains and 4 (80%) *V. parahaemolyticus* strains grow in a nutrient broth prepared with 6% NaCl. Of the 48 *V. alginolyticus* isolates, 12 (25%) were capable of growing at 10% NaCl added to a nutrient broth. *Vibrio* isolates produced several hydrolytic exoenzymes such as amylase, lecithinase, lipase, caseinase, gelatinase and Dnase. Thirty seven of the forty-eight (77.08%) *V. alginolyticus* and 2/12 (16.66%) *V. cholerae* isolates were β-hemolytic. The PCR-based identification

Table II
Biochemical and enzymatic characterization of *Vibrio* isolates

Characteristic		*V. alginolyticus* no. (%)[a]	*V. cholerae* no. (%)[a]	*V. parahaemolyticus* no. (%)[a]
No. of tested strains		48	12	5
Gram		–	–	–
Motility		+	+	+
Oxydase		+	+	+
β-Galactosidase		0	12 (100)	3 (60)
Adenine dehydrolase		0	0	0
Lysine decarboxylase		47 (97.91)	12 (100)	5 (100)
Ornithine decarboxylase		24 (50)	12 (100)	5 (100)
Citrate utilization		8 (16.66)	10 (83.33)	5 (100)
H_2S production		4 (8.33)	2 (16.66)	0
Urea hydrolysis		0	0	1 (20)
tryptophan deaminase		6 (12.5)	0	0
Indole production		48 (100)	12 (100)	5 (100)
Voges Proskauer		10 (20.83)	3 (25)	0
Gelatinase		32 (66.66)	12 (100)	5 (100)
Fermentation of:	Glucose	48 (100)	12 (100)	5 (100)
	Mannitol	47 (97.91)	12 (100)	5 (100)
	Inositol	0	0	0
	Sorbitol	4 (8.33)	0	0
	Rhamnose	0	0	5 (100)
	Sucrose	48 (100)	12 (100)	0
	Melibiose	0	0	0
	Amygdalin	21 (43.75)	4 (33.33)	4 (80)
	Arabinose	0	0	3 (60)
O/129:	10 μg	R	R	R
	150 μg	S	S	S
Growth at:	0% NaCl	0	0	0
	2% NaCl	48 (100)	12 (100)	5 (100)
	4% NaCl	48 (100)	12 (100)	5 (100)
	6% NaCl	48 (100)	5 (41.66)	4 (80)
	8% NaCl	48 (100)	0	0
	10% NaCl	12 (25)	0	0
Growth at:	23°C	48 (100)	12 (100)	5 (100)
	37°C	48 (100)	12 (100)	5 (100)
Exoenzymes:	Amylase	37 (77.08)	9 (75)	3 (60)
	Lecithinase	41 (85.41)	12 (100)	4 (80)
	Lipase	48 (100)	12 (100)	5 (100)
	Caseinase	48 (100)	10 (83.33)	4 (80)
	Gelatinase	44 (91.66)	10 (83.33)	5 (100)
	Dnase	48 (100)	12 (100)	5 (100)
	β-hemolytic	37 (77.08)	2 (16.66)	0

[a] – Number and percentage of positive tests; S – sensitive; R – resistant.

of studied *Vibrio* strains yielded amplicon size of 144, 588 and 678 bp for *V. alginolyticus*, *V. parahaemolyticus* and *V. cholerae,* respectively (Fig. 1).

Antibiogram patterns obtained for the *Vibrio* spp. are presented in Table III. Tests for antimicrobial susceptibility revealed that bacterial strains belonging to different species of *Vibrio* genera exhibited some common pattern of antibiotic resistance or susceptibility. In fact, all strains displayed a total resistance to ampicillin and more than 70% of them showed a significant

Table III
Antibiotic resistance pattern expressed in (%) and minimum inhibitory concentration of *Vibrio* strains expressed in mg/L (%)

Antibiotics	*V. alginolyticus* (n = 48)	*V. cholerae* (n = 12)	*V. parahaemolyticus* (n = 5)
Ampicillin (10 µg)	100	100	100
Chloramphenicol (30 µg)	62.5	33	0
Cotrimoxazole (25 µg)	58.33	0	0
Gentamicin (10 µg)	75	25	0
Nalidixic acid (30 µg)	70.8	75	80
Streptomycin (10 µg)	83.3	75	100
Tetracyclin (30 µg)	83.3	25	0
Erythromycin (15 µg)	100	75	80
Kanamycin (30 µg)	95.8	16.6	60
Carbenicillin (100 µg)	100	25	40
Ampicillin	16 (12.5)	16 (33.3)	16 (80)
	32 (35.4)	32 (41.6)	32 (20)
	64 (22.9)	64 (16.6)	–
	128 (20.8)	128 (8.3)	–
	256 (8.3)	–	–
Erythromycin	16 (20.8)	4 (33.3)	4 (40)
	32 (39.6)	8 (25)	8 (20)
	64 (22.9)	16 (25)	16 (20)
	128 (16.6)	32 (16.6)	–
	–	–	–
Tetracyclin	2 (10.4)	0.5 (16.6)	0.5 (20)
	4 (16.6)	1 (33.3)	1 (80)
	8 (41.6)	2 (16.6)	–
	16 (25)	4 (8.3)	–
	32 (6.2)	8 (8.3)	–
Streptomycin	4 (6.2)	2 (33.3)	4 (80)
	8 (10.4)	4 (41.6)	16 (20)
	16 (45.8)	8 (16.6)	–
	32 (27)	16 (8.3)	–
	64 (10.4)	–	–
Gentamycin	4 (12.5)	1 (25)	1 (20)
	8 (35.4)	2 (33.3)	2 (80)
	16 (43.7)	4 (16.6)	–
	32 (8.3)	8 (25)	–
Chloramphenicol	1 (18.7)	0.5 (16.6)	0.25 (20)
	2 (18.7)	1 (25)	0.5 (20)
	4 (31.2)	2 (50)	1 (60)
	8 (31.2)	4 (8.3)	–

resistance to streptomycin, nalidixic acid and erythromycin. *V. alginolyticus* strains had the highest multi-drug resistance showing a strong resistance to ampicillin, erythromycin, carbenicillin, streptomycin, kanamycin and tetracycline. The resistance to chloramphenicol was observed in 62.5% of the analyzed *V. alginolyticus* strains and in 33% of the *V. cholerae* isolates.

The MIC results for *Vibrio* isolates were summarized in the Table III. MIC values of antimicrobials observed throughout the study showed that all investigated isolates were highly susceptible to chloramphenicol (0.25–8 mg/l) and were moderately sensitive to both tetracyclin (0.5–32 mg/l) and gentamycin (0.5–32 mg/l). The MIC values of different tested anti-

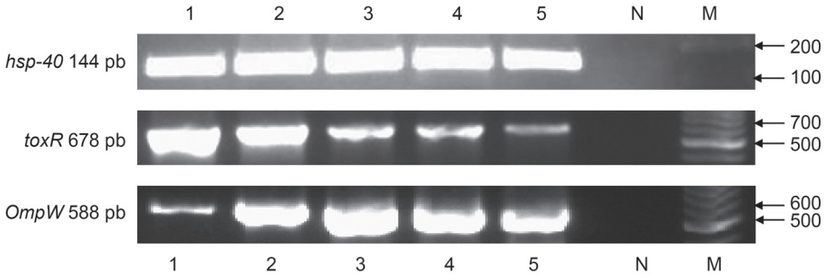

Fig. 1. Agarose gel electrophoresis of 1.5% agarose of the amplification products of isolates obtained with PCR for the *Hsp-40* (*V. alginolyticus*: 1, AMa_1, 2, BN_3, 3, CJ4, 5, CJ_3, 6, DS_3); PCR for the *toxR* (*V. parahaemolyticus*: 1, AA_2, 2, DM_4, 3, DJ_1, 4, CAt_4, 5, BJ_3) and PCR for the *OmpW* (*V. cholerae*: 1, BJ_1, 2, AM_1, 3, BN_2, 4, CF_3, 5, BJ_2). N, negative control, M, molecular weight marker 100 bp ladder (Promega, France).

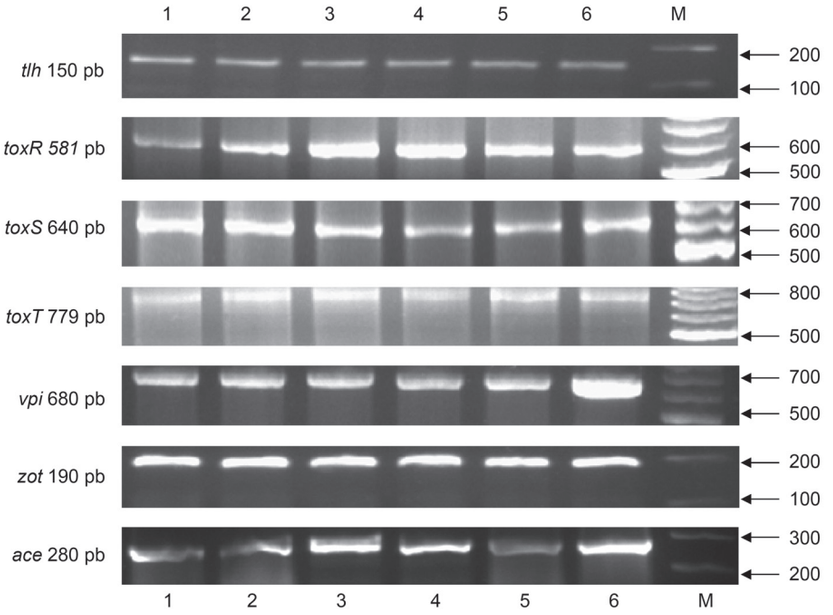

Fig. 2. Virulence genes espression of *Vibrio* strains isolated from Monastir lagoon. Agarose gel electrophoresis (1.6% agarose) of the *ace* (*V. alginolyticus*: 1, AMa_1, 2, BN_3, 3, CJ_4, 4, DMa_3, 5, BJt_1, 6, CO_1), the *zot* (*V. alginolyticus*: 1, AJ_3, 2, BAt_3, 3, CAt_3, 4, DS_3, 5, AAt_2, 6, DAt_3), the *vpi* (*V. alginolyticus*: 1, AAt_1, 2, AAt_2, 3, BJ_2, 4, CJ_3, 5, DJt_4, 6, DS_3). the *toxT* (*V. cholera*: 1, BJ_1, 2, AJt_3, 3, BN_2 and *V. aliginolyticus*: 4, AMa_1, 5, CJ_3, 6, DS_3), the *toxS* (*V. cholera*: 1, CF_3, 2, AM_1, 3, BJ_2, *V. parahaemolyticus*: 4, CAt_4 and *V. alginolyticus*: 5, CAt_3, 6, DMa_1), the *toxR* (*V. cholera*: 1, CF_3, 2, AM_1, 3, BJ_2, *V. parahaemolyticus*: 4, CAt_4 and *V. alginolyticus*: 5, BJt_3; 6, CMa_4) and the *tlh V. parahaemolyticus*:1, AA_2, 2, DM_4, 3, DJ_1, 4, CAt_4, *V. alginolyticus*: 5, BM_2, 6, AJ_3). M, molecular weight marker 100 bp ladder (Promega, France).

biotics for *V. parahaemolyticus* strains were lower than those found among other *Vibrio* species. In other hand, the vast majority of *V. alginolyticus* isolates showed a strong resistance to ampicillin (87.4% ≥ 32 mg/l); erythromycin (79% ≥ 32 mg/l); tetracyclin (31.5% ≥ ≥ 16 mg/l); streptomycin (37.4% ≥ 32 mg/l) and gentamycin (52% ≥ 16 mg/l).

The distribution of *V. cholera*e and *V. parahaemolyticus* virulence-associated genes among the tested *Vibrio* strains was presented in the Table IV. The presence of the *tox*R and the *tox*S genes was detected in the majority of *V. cholerae* (100% and 83%, respectively) strains and *V. alginolyticus* (73% and 58%, respectively) strains, while only one *V. parahaemolyticus* isolates was positive to these genes (Fig. 2). The *tox*T fragment was amplified from the chromosome of 10/12 (83%) *V. cholerae* strains whereas 13/48 (27%) *V. alginolyticus* isolates gave a positive result to this gene. Only the *V. alginolyticus* strains exhibited the presence of three *V. cholerae* virulence genes: *vpi* (25%), *ace* (19%) and *zot* (29%).

Table IV
Biofilm formation on biotic and abiotic materials, virulence genes distribution and cytotoxic activity of *Vibrio* isolates

Strain	Strain number	Virulence genes (%)						Adherence										Cytotoxic effect					
								Materials OD_{570}						Hep-2 (%)			Vero (%)	HeLa (%)			Vero (%)		
								PVC (%)			PE (%)												
		*tox*R	*tox*S	*tox*T	*vpi*	*ace*	*zot*	*tlh*	**W**	**M**	**S**	**W**	**M**	**S**	**W**	**M**	**S**	**W**	**M**	**S**	**W**	**M**	**S**
VA	48	73	58	27	25	19	29	37	20	37	41	41	39	19	37	14	8	31	24	10	42	21	6	31	25	10
VC	12	100	83	83	–	–	–	–	17	33	50	33	25	42	42	33	8	50	25	17	33	17	8	42	33	–
VP	5	20	20	–	–	–	–	100	60	20	–	20	20	–	60	–	–	60	20	–	20	–	–	60	20	–

VA – *V. alginolyticus*; **VC** – *V. cholerae*; **VP** – *V. Parahaemolyticus*; **PVC** – polyvinyl-chloride; **PE** – polyethylene; **W** – weak; **M** – moderate; **S** – strong.

All *V. parahaemolyticus* isolates were positive to the *tlh* virulence gene, while 18/48 *V. alginolyticus* strains possessed this gene. The crosstabs method revealed a significant relationship ($P < 0.05$) between the presence of the *tox*R gene and the *tox*S gene. On other hand, a positive correlation was observed between the presence of the *vpi* gene and the *tox*R gene ($P = 0.039$), *tox*S gene ($P = 0.007$) and the *tox*T gene ($P = 0.005$). However, no significant relationship was observed between the presence of *V. cholera*e and *V. parahaemolyticus* virulence genes. All isolates gave negative results for the amplification of *tox*RS*I tcp*P, *tcp*A, *tdh* and *trh.*

The results of the biofilm formation by *Vibrio* species on PVC and PE surfaces showed that *V. cholerae* and *V. alginolyticus* strains were strongly adhesive to both abiotic materials than other isolates. In fact, 50% (6/12) of *V. cholerae* isolates and 41% (20/48) of *V. alginolyticus* exhibited high adherence ability to PVC pieces. *V. cholerae* isolates presented better adherence ability on PE surface than *V. alginolyticus* strains (42 and 19%, respectively).

Adherence ability was observed in 11 of 12 (92%) of the analyzed *V. cholerae* strains in Vero cells, while 10 (83%) isolates were found adhesive when Hep-2 cell line was used. The other tested *Vibrio* species revealed that were lower adhesive to both cell lines than *V. cholerae* isolates. We also noted that only *V. alginolyticus* strains showed a strong adherence to Hep-2 cells (Table IV). About 2 of 48 (4%) *V. alginolyticus* strains and one of 12 (8%) *V. cholerae* strains were able to adhere strongly to both epithelial cell lines (Fig. 3).

The cytotoxic activity of extracellular products (ECPs) of the three studied *Vibrio* species against HeLa and Vero cell lines showed that more than 60% of *V. alginolyticus* strains have cytotoxic effect with different degrees to both epithelial cell lines. About 5 of 48 (10%) *V. alginolyticus* isolates showed a strong cytotoxicity against Vero monolayer while only 3 strains gave the same results when Hep-2 cells were used. However, most strains of *V. cholera*e and *V. parahaemolyticus* exhibited essentially weak and moderate cytotoxic activities (Table IV).

Discussion

The past two decades have witnessed remarkable increasing frequency of *Vibrio* species isolated from diseased aquatic animals and from human infections. *V. alginolyticus* is recognized as one of the major causative agent of vibriosis in cultured fish and shellfish in Mediterranean coastal environment (Gomez-Leon *et al.*, 2005; Sonia and Lipton, 2012). Other studies reported that this specie is considered as an important human opportunistic pathogen usually associated with otitis

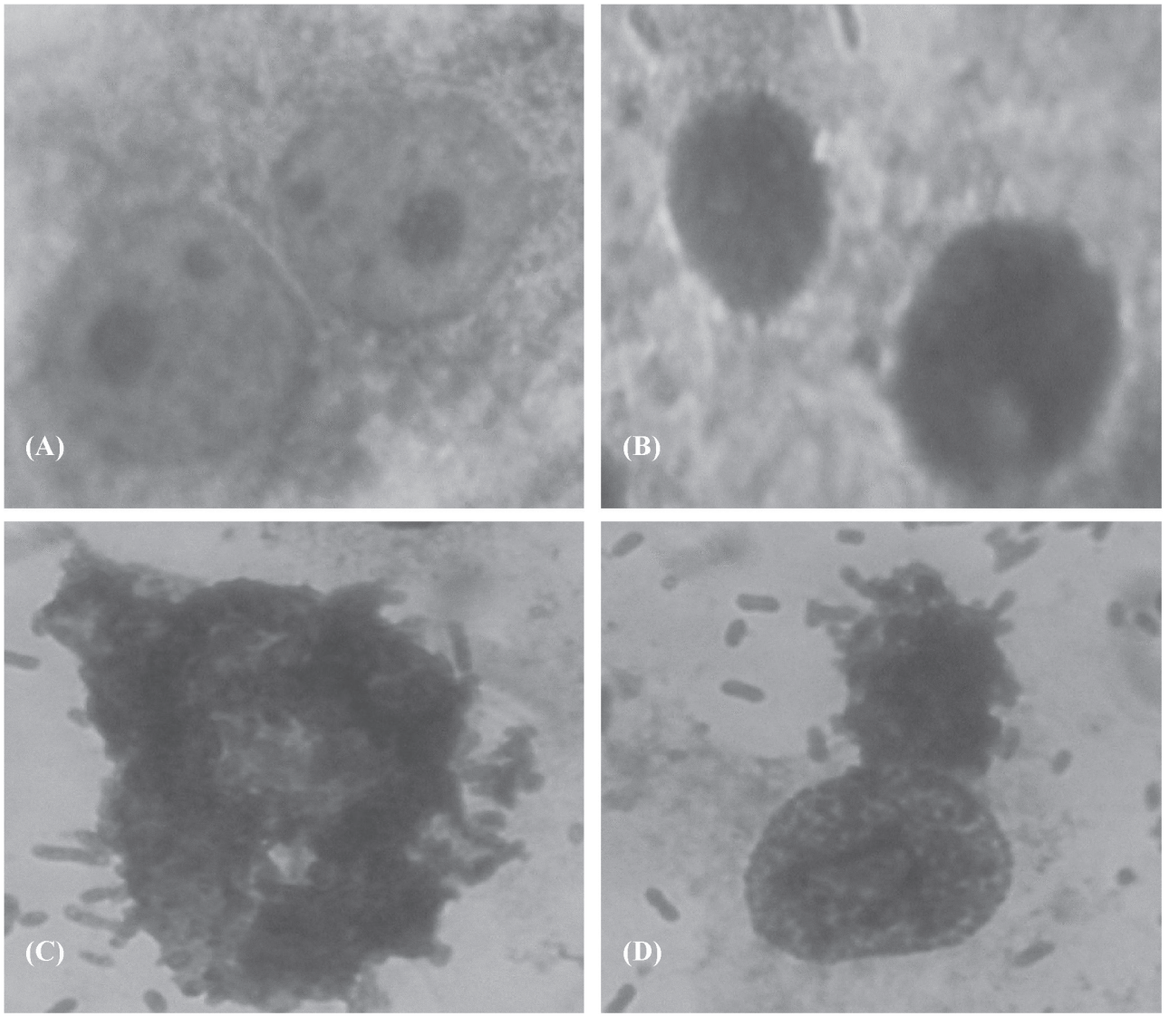

Fig. 3. Optic microscopy showing the high adherence ability of *Vibrio alginolyticus* (strain Bat4) to both Vero and Hep-2 cell lines. Giemsa stain: magnification (×1000). (a) and (B): Negative control for Vero and Hep-2 cells. (C) and (D): Vibrio alginolyticus strain Bat4 strongly adhesive to Vero and Hep-2 cells respecctively.

externa, endophthalmitis and wound infections (Li *et al*., 2009; Reilly *et al*., 2011). It's also well documented that *V. cholerae* and *V. parahaemolyticus* are most often incriminated in food-borne and waterborne gastroenteritis outbreaks (Nair *et al*., 2007; Yoder *et al*., 2008).

Sixty-five *Vibrio* spp. strains were isolated from water samples collected from the Monastir lagoon and biochemically characterized using the commercial miniaturized Api 20E kit. The phenotypic characteristics of *Vibrio* isolates were in accordance with those described previously by Snoussi *et al.* (2006). However, these findings are in discordance with Ben Kahla-Nakbi *et al.* (2007) who showed that a majority of *V. alginolyticus* isolates recovered from dead and moribund fish samples were negative to indole test. The environmental strains of *V. alginolyticus*, *V. cholerae* and *V. parahaemolyticus* were genetically identified to the species levels using the *hsp-40*, *omp*W and the *tox*R genes, respectively (Lin *et al*., 1993; Nandi *et al*., 2000).

In the present study, *Vibrio* isolates exhibited multidrug resistance to at least four antibiotics. Vaseerahan *et al.* (2005) carried a study of 80 *Vibrio* strains isolated from Indian shrimp culture ponds and hatcheries for determination of their susceptibility to the most used antibiotics in the shrimp farming, all tested isolates were resistant to ampicillin, which corroborate with our findings. Other studies, reported that *V. alginolyticus* strains showed resistance to erythromycin, streptomycin, gentamycin, tetracyclin and chloramphenicol (Gomathi *et al*., 2013; Mechri *et al*., 2013b). These data are in keeping with our results.

The MIC's obtained from the study showed that all *Vibrio* strains were sensitive to chloramphenicol (MIC's ≤ 8 mg/l), while most of them expressed high

rates of resistance to ampicillin (MIC's ≥ 32 mg/l) and erythromycin (MIC's ≥ 8 mg/l). In a previous study, Manjusha *et al.* (2005) reported strong resistance against amoxicillin, ampicillin, carbenicillin, cefuroxime, rifampicin and streptomycin in *Vibrio* spp. isolated from Indian coastal and brackish areas. Another study, showed that *Vibrio* isolates recovered from aquaculture structure expressed moderate resistance to chloramphenicol, gentamycin, tetracyclin and erythromycin (Akinbowale *et al*., 2006).

Previous work showed that *Vibrio* species represents an important recipient of some *V. cholerae* and *V. parahaemolyticus* virulence genes transfers (Xie *et al.*, 2005). Snoussi *et al.* (2008), reported the diffusion of six *V. cholerae* virulence genes among 28 *V. alginolyticus* strains isolated from the Mediterranean seawater. Our results corroborate with these findings and represents the first report describing higher frequencies of *V. cholerae* and *V. parahaemolyticus* virulence genes distribution, among environmental *V. alginolyticus* isolates, than observed previously (Ren *et al*., 2013; Khoudja *et al*., 2014). These data supports the evidence of genetic extensive exchange of virulence determinants between *V. alginolyticus* and other *Vibrio* species in marine and estuary environments.

Biofilm formation constitutes an efficient adaptive strategy utilized among numerous *Vibrio* species, which remarkably promotes bacterial persistence in the environment and/or colonization of eukaryotic hosts (Morris and Visick, 2010). In this study, *V. cholerae* and *V. alginolyticus* strains exhibited high capacity of adherence to both PVC and PE surfaces, while *V. parahaemolyticus* isolates showed low to moderate adhesion to the same materials. These data corroborate previous studies showing that environmental *Vibrio* species were able to form biofilm on abiotic surfaces of different degrees (Mechri *et al*., 2013a).

The attachment of bacterial pathogens to eukaryotic cells represents an essential first step in the colonization and the production of disease. This propriety seems to be diffused among *Vibrio* species (Scoglio *et al*., 2001; Mohammadi-Barzelighi *et al.*, 2011). Our data showed that *V. cholerae* and *V. alginolyticus* isolates exhibited an important adherence ability to both tested cell lines. These findings may explain a possible interaction between these strains and the epithelial cell lines used in this study.

Several studies reported cytotoxic effects of extracellular products of some *Vibrio* spp. against a variety of cell lines (Hiyoshi *et al*., 2010; Mechri *et al*., 2013b). Our investigation showed that *V. alginolyticus* isolates exhibited the most important cytotoxic activity against Vero and HeLa cell lines. Balebona *et al.* (1998), suggested that cytotoxicity in cell lines can be directly related to the virulence of *V. alginolyticus* strains.

Conclusions

This study highlights the incidence of multiple antibiotic resistance in three environmental *Vibrio* species and the wide distribution of some *V. cholerae* and *V. parahaemolyticus* virulence genes among the studied strains. Besides, it is clearly shown that tested bacteria present a high ability to adhere to biotic and abiotic surfaces though at varying levels. These isolates exhibited also a significant cytotoxicity against HeLa and Vero cell lines.

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