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Adhesive properties and antibacterial susceptibility of *Vibrio alginolyticus* strains isolated from a Tunisian *Ruditapes decussatus* hatchery

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***Vibrio alginolyticus* has been reported to cause several damages to reared bivalve molluscan larva. In the present work, we characterized fifty four phenotypic and genetic *V. alginolyticus* strains isolated from a *Ruditapes decussatus* hatchery in the National Institute of Marine Sciences and Technologies, Monastir, Tunisia, then we studied the antibiotics susceptibility and the antibacterial activity of clam hemolymph against the tested isolates. We also investigated the adherence ability of these strains to abiotic materials (glass, polyethylene, polystyrene, polyvinyl-chloride and stainless steel) and to biotic surfaces (epithelial cell lines: Hep-2 and Vero). *Vibrio* cells showed high levels of resistance to many antibiotics and produced several hydrolytic exoenzymes. *Vibrio* strains isolated from broodstock showed the highest resistance to clam hemolymph. The majority of *V. alginolyticus* strains were able to adhere to all abiotic surfaces tested to varying degrees. The highest cell density was observed on polyvinyl-chloride surface followed by that on the glass slides, polyethylene, polystyrene and stainless steel materials. Adherence properties were found in 64% of the analyzed strains in Hep-2 cells and in 73% when using Vero cells. These results explain how *V. alginolyticus* manage to persist and proliferate in the entire hatchery.**

Key words: *Vibrio alginolyticus*, *Ruditapes decussatus*, hatchery, biofilm, antimicrobials.

INTRODUCTION

Vibrio alginolyticus is one of the most important opportunistic pathogens causing vibriosis in molluscan larva (Tubiash and Otto, 1986; Anguiano-Beltrán et al., 1998; Luna-González et al., 2002). It is well known that *Vibrio* pathogenesis is associated with some virulence factors such as toxins and lytic enzymes (Iyer et al., 2000; Ottaviani et al., 2001; Masini et al., 2007).

Biofilm production is a complex phenomenon in which physical, chemical and biological factors are involved (Characklis and Marshall, 1990; Lappin-Scott and Costerton, 1995). The development of biofilms can occur through at least three mechanisms: (i) redistribution of attached cells (O'Toole and Kolter, 1998; Watnick et al., 2001), (ii) binary division of attached cells (Tolker-Nielson et al., 2000) and (iii) recruitment of cells from the bulk fluid to the developing biofilm (Stoodley et al., 2000). It has been extensively reported that *V. alginolyticus* species were able to adhere to epithelial cell lines (Vero, Hep-2 and Caco-2) and fish mucus (Snoussi et al., 2008; Ben Abdallah et al., 2009). These adhesive properties

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may represent the first step in the development of diseases for stressed aquatic animals (Zorrilla et al., 2003; Baffone et al., 2006).

Biofilms are notoriously difficult to eradicate and are often resistant to systemic antibiotic therapy (Costerton et al., 1999; Lewis, 2001). It is now well documented that the increased resistance of biofilm to antimicrobial agent is due to several factors: penetration of antimicrobials, decreased growth rate and expression of resistance genes. These factors can explain biofilm persistence in several cases (Gilbert et al., 1997; Costerton et al., 1999; Maira-Litran et al., 2000).

The purpose of this study was to investigate *V. alginolyticus* survival and persistence in the different compartments of a *Ruditapes decussatus* hatchery even after antibiotic treatments and to study biofilm formation on both biotic and abiotic surfaces including Vero and Hep-2 cell lines, glass, polyvinyl chloride (PVC), polystyrene, polyethylene (PE) and stainless steel materials. Bacterial characterization, exoenzyme production, antimicrobial susceptibility and antibacterial activity were also examined.

MATERIALS AND METHODS

Bacterial strains and exoenzyme production

Fifty four (54) *V. alginolyticus* strains were isolated from *R. decussatus* experimental hatchery in the National Institute of Marine Sciences and Technologies, Monastir, Tunisia as described by Mechri et al. (2011) and one *V. alginolyticus* ATCC 33787 (American Type Collection Culture) was kindly provided by Prof. Amina Bakhrouf. The bacterial strains were as follows: 14 from post-larva and rearing tank water, 12 from larva and rearing tank water, 10 from broodstock and from breeder tank water, 10 from sea water inside the hatchery, 5 from larva food and 3 from broodstock food.

Bacterial strains were subjected to standard morphological, physiological and biochemical tests using the procedures described in Bergey's Manual of Systematic Bacteriology (Krieg and Holt, 1984). *V. alginolyticus* strains were identified by the API 20E system (BioMerieux, Marcy l'Etoile, France). The enzymes lipase, amylase, lecithinase and caseinase were tested as described by Liu et al. (1996). DNA hydrolysis and hemolytic activities using 5% (v/v) human blood were tested as described by Snoussi et al. (2006).

Antibacterial sensitivity

Antibiogram tests for the isolate were performed using the disc diffusion technique on Mueller Hinton agar plates (Biomérieux, France) supplemented with 1% NaCl as described by Ottaviani et al. (2001). The following antibiotic discs (Bio-Rad, France) were used: 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), tetracycline (30 µg) and erythromycin (15 µg). After incubation at 37°C for 18 to 24 h, the diameter of the inhibition zone was measured according to the "Comité de la Société Française de l'Antibiogramme" (Cavallo et al., 2006) and the recommendations of the National Committee for Clinical Laboratory Standards (NCCLS, 2002). Also, the ATB-Vet (Biomerieux, France) strip system was used to obtain the antibiotic sensitivity of the isolated strains. After inoculation with *V. alginolyticus* strains, the strips were incubated for 18 to 24 h at 37°C in aerobic conditions. *Escherichia coli* ATCC 25922 was used as a control throughout all the experiments.

PCR confirmation of *V. alginolytitcus* strains

For the identification of *V. alginolyticus* strains, we targeted dnaJ sequences, a housekeeping gene that encodes heat shock protein 40 (Hsp40) as described by Nhung et al. (2007). The following primers were used to amplify the target sequence: VM-F (5'-CAGGTTTGYTGCACGGCGAAGA-3') and V.al2-MmR (5'-GATCGAAGTRCCRACACTMGGA-3'). Amplification reactions were carried out with mixtures containing 5x PCR buffer (Promega, France), 200 µmol/L of each desoxyribonucleotide triphosphate, 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 amplification was 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. Amplified products were examined with agarose gel electrophoresis (2%) and ethidium bromide staining.

Quantitative assays with clam hemolymph

Hemolymph collection and enzymatic characterization

Hemolymph was collected from the pericardial cavity of adult *R. decussatus* using a sterile syringe. A free cell fraction was obtained by filtering the crude hemolymph using a 0.22 µm sterile filter (Millipore, Germany). The filtered material was stored in aliquots at -80°C until used as described by Olicard et al. (2005). For the enzymatic characterization, we used the Api Zym system at 25°C for 24 h.

Anti-vibrio alginolyticus activity

The antibacterial effect of clam hemolymph was evaluated using a modification of the method described by Chung and Secombes (1987). *V. alginolyticus* colonies were harvested with sterile PBS and diluted to a standard concentration equal to an optical density (OD) of 1.0 at 540 nm. This standard suspension of bacteria contained approximately 1 x 10^9 colony forming units/mL (CFU/mL) as determined by a standard dilution and plating method. Three dilutions were prepared in tryptone soya broth (TSB, Bio-Rad, France): 10^8, 10^6 and 10^4 bacteria/mL. 75 µL of each suspension was added to 25 µL of clam hemolymph in triplicate wells of a 96-well plate. Bacteria were also added to wells containing 25 µL of TSB as controls. Plates were shaken and incubated at 20°C for 24 h and centrifuged for 10 min at 150 *g* and supernatants were discarded. One hundred microliters of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (Sigma), diluted 10-fold in TSB from a stock solution of 5 mg/ mL in distilled water, was added to each well. Plates were incubated at 18°C for 15 min and optical density was measured at 600 nm in a multiscan spectrophotometer (Multiskan Ascent 96/384 Plate Reader, Finland).

Qualitative and quantitative detection of biofilm formation by *V. alginolyticus* strains

Biofilm formation on glass surfaces

The qualitative biofilm formation of *V. alginolyticus* strains on glass

surfaces was tested on glass tubes as described by Wolfe et al. (2004). Bacteria which form purple pellicule at the air liquid interface were considered glass-biofilm positives. Experiments were performed in triplicate.

The quantitative assessment of biofilm formation of *Vibrio* strains on glass surfaces was performed as described by Krovacek et al. (1987) and Snoussi et al. (2008). All strains were tested in triplicate, and the bacteria were classified according to Stepanovic et al. (2000) as follows (0): OD ≤ ODc; weakly adherent (+): ODc < OD ≤ 2x ODc; moderately adherent (++): 2 x ODc < OD ≤4 x ODc; strongly adherent (+++): 4 x ODc ≤ 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.

Biofilm formation on polystyrene surface

The quantitative estimate of biofilm formation of *V. alginolyticus* strains on polystyrene surface was determined using the protocol described by Mathur et al. (2006) with slight modifications. Isolates from fresh agar plates were inoculated in BHI with 1% glucose and incubated for 24 h at 37°C in stationary condition and diluted 1 in 100 with fresh medium. 0.2 ml aliquots of the diluted cultures were used to inoculate sterile polystyrene, U-bottomed 96-well microtitre plates (Evergreen, Los Angeles, US). Wells with sterile broth alone served as control to check sterility and non-specific binding of media. The microtitre plates were incubated for 24 h at 37°C. After incubation, the contents of each well were gently removed by tapping the plates. The wells were washed four times with 0.2 ml of phosphate buffer saline (PBS, pH 7.2) to remove the non-adherent bacteria. Adherent cells were stained with 1% crystal violet for 15 mn. The wells were rinsed once more and the crystal violet was dissolved in 200 µl of ethanol-acetone (80:20 v/v).

Optical density (OD_{595}) was measured in a multiscan spectrophotometer (Multiskan Ascent 96/384 Plate Reader, Finland). The following values were assigned: non-biofilm forming (-): $OD_{595} \leq 1$; weak biofilm (+): forming $1 < OD_{595} \leq 2$; medium biofilm forming (++): $2 < OD_{595} \leq 3$; strong biofilm forming (+++): $OD_{595} \geq 3$. Each test was performed in triplicate (Snoussi et al., 2008).

Adherence to polyethylene (PE) and polyvinyl chloride (PVC) surfaces

The quantitative study of biofilm formation of *V. alginolyticus* strains on PE and PVC surfaces was performed as described by Cerca et al. (2006). Cells were harvested with sterile PBS and diluted to a standard concentration equal to an OD of 1.0 at 540 nm. This standard suspension of bacteria contained approximately 1 x 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 controls were obtained by placing materials in PBS without bacterial cells. The experiments were performed in triplicate. At the end of the experiment, each well was washed twice with PBS, carefully pipetting only the liquid above the pieces. 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). The bacteria were classified according to Stepanovic et al. (2000).

Adherence on stainless surfaces

Stainless steel was cut in 1.5 x 1.5 cm squares, which were then disinfected and sterilized. First, the squares were cleaned with 100% acetone, and washed by immersion in alkaline detergent (NaOH 1% (w/v), pH 13.2) for 1 h. Then they were rinsed with sterilized distilled water, dried and cleaned with alcohol 70% (v/v). After the disinfection, they were washed with sterilized distilled water, dried for 2 h at 60°C and autoclaved at 121°C for 15 min (Rossoni et al., 2000).

V. alginolyticus strains were inoculated in TSB + 1% NaCl and incubated for 24 h at 37°C. Sterile stainless steel (308 – 18/8) coupons (1.5 x 1.5 cm) were inserted in the bottom of 6-well microtitre plates (Greiner Bio-One, Cellstar, Germany); 2 mL of each cell suspension was added to each well. Negative controls were obtained by placing coupons in sterile TSB. The number of bacterial cells adhering to the stainless steel was determined after 4 h of cultivation. The coupons were first immersed three times in saline solution to remove planktonic cells, then the adhering cells were removed using previously sterilized standardized swabs. The swabs were transferred to test tubes containing 10 mL of saline solution and stirred in vortex for one minute. Serial dilutions of up to 10^{-6} were made in test tubes containing 9 mL of saline solution. Aliquots of 100 µL of each dilution were inoculated in Petri dishes containing TSA + 1% NaCl, using the spread plate technique. Afterwards, the Petri dishes were incubated at 37°C for 24 h. Three repetitions of the experiments were conducted, and the results were shown as an average per coupon in Log CFU/ cm^2 (De Oliveira et al., 2010).

Adherence to human epithelial cells: Vero and *Hep-2*

For the quantitative adherence assays, two cell lines, Vero (kidney epithelial cells of the African Green Monkey) and Hep-2 (human larynx carcinoma) have been used. 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 cells/ mL), and incubated at 37°C in 5% CO_2 for 24 h. Bacterial strains were grown on TCBS agar (Scharlau Microbiogy, Spain), at 37°C for 18 to 24 h. *V. alginolyticus* strains were drawn and inoculated in TSB (Bio-Rad, France) + 1% of NaCl, and incubated at 37°C for 18 to 24 h (Baffone et al., 2006).

The assay was performed as described by Snoussi et al. (2008). 100 µL of 10^7 cells/ mL were 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. The number of bacteria adhering to each of the cell lines was counted. All organisms were tested twice. The adhesion index was set as follows: NA = non-adhesion (0-10 bacteria/ cell); W = weak adhesion (10-20 bacteria/ cell); M = medium adhesion (20 to 50 bacteria/ cell); and S = strong adhesion (50 to 100 bacteria/cell).

Statistical analysis

Each analysis was performed using the SPSS 16.0 statistics package for Windows. All data from quantitative adhesion assays were expressed as means ± standard deviation (S.D.). The differences in the degree of biofilm formation were examined by the

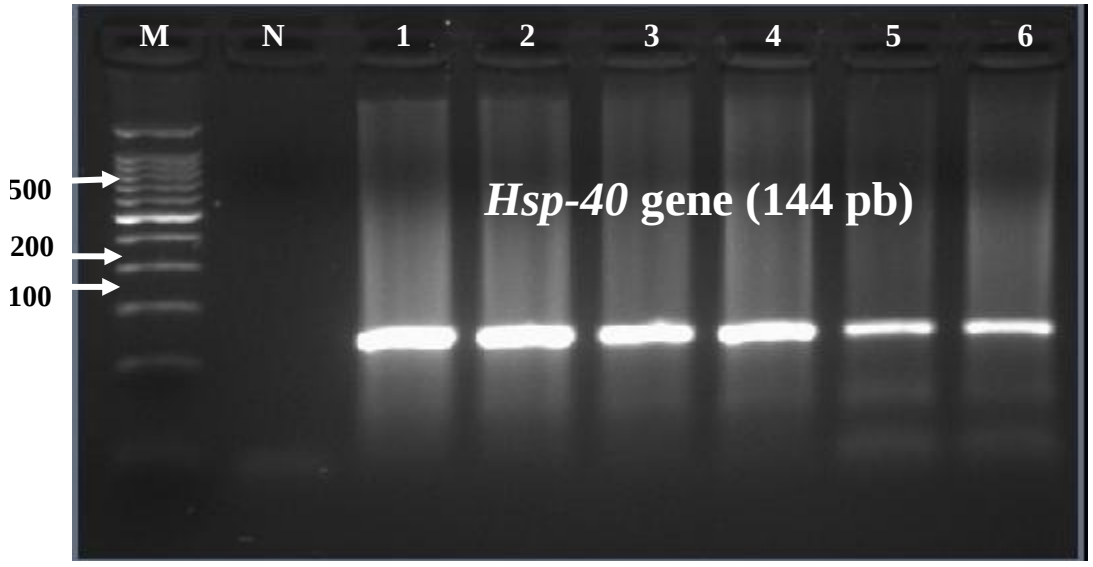

Figure 1. Agarose gel electrophoresis (2% agarose) of the amplification products obtained for heat shock protein 40 gene. M, Molecular weight marker (100-pb DNA ladder, Promega); N, negative control; lane (1 to 5), *V. alginolyticus* (strains: L_{12}, PL_{48}, B_5, W_9, LF_4); Lane 6, *V. alginolyticus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

Friedman test, followed by the Wilcoxon signed ranks test. P-values <0.05 were considered significant. For the antibacterial activity of *R. decussates* hemolymph, we used nonparametric comparisons (Kruskal–Wallis' test) to determine whether the difference between the averages of K independent samples was significant ($p < 0.05$) or not. In addition, the Mann-Whitney test was used to test whether the difference between the averages of two independent samples was significant ($p < 0.05$).

RESULTS

Phenotypic and molecular characterization

Fifty-four (54) strains of *V. alginolyticus* were characterized with a high level of identification (95 to 99%) using the API 20E Strep system. The majority of *Vibrio* strains produced many enzymes such as lecithinase (77%) and amylase (80%). While all strains were gelatinase and lipase positives and had hydrolyzed DNA, 36 of the *V. alginolyticus* strains (67%) were β-hemolytic. All strains tested amplified a 144-pb size fragment corresponding to the heat shock protein 40 gene specific for this species (Figure 1).

Antibacterial susceptibility

V. alginolyticus strains showed high resistance to ampicillin (100%), erythromycin (96%), streptomycin (88%), gentamicin (81%) and tetracycline (76%). Fifty two percent of tested strains were resistant to chloramphenicol and 35% to co-trimoxazole. The combined resistance to antimicrobials was very frequent among the *V. alginolyticus* isolates (90% showed at least four different resistances). The lowest percentages of resistance were observed with flumequine (4%), ciprofloxacin (5%) and nalidixic acid (17%). Variation in sensitivity was observed with ten antibiotics (Table 1): amoxicillin, chloramphenicol, colistin, erythromycin, flumequin, oxolinic acid, tetracyclin, streptomycin, gentamicin and kanamycin. In addition, the great majority of isolates were susceptible to amoxicillin-clavulanic acid, cefoperazone, apramycin, cephalothin, doxycyclin, enrofloxacin, lincomycin, metronidazole, fusidic acid, nitrofurantoin, penicillin, pristinamycin, sulfamethizole, rifampicin, oxacillin, trimethoprim-sulphamethoxazole, spectinomycin and tylosin.

Assays with clam hemolymph

The *R. decussatus* a-cellular hemolymph fraction contains ten enzymes: Leucine arylamidase, valine arylamidase, cystine arylamidase, trypsin, naphtol-AS-BI-phosphohydrolase, α-galactosidase, β-galactosidase, β-glucoronidase, α-glucosidase and α-fucosidase. All *V. alginolyticus* strains were able to survive in *R. decussatus* hemolymph. The OD values obtained with *V. alginolyticus* strains (broodstock, larva, post-larva and their respective rearing tanks) incubated in clam hemolymph were significantly higher ($p < 0.05$) at high bacterial dilution (10^8 bacteria/ mL) than the other dilutions and the control (TSB+ bacteria). Bacterial strains isolates from broodstock and breeder tanks samples showed better growth rates at 10^8, 10^6 and 10^4 bacteria/ mL than the other strains ($p < 0.05$) (Table 2).

Table 1. Antimicrobial susceptibility profile of *V. alginolyticus* isolates using the ATB-VET Kit (%).

Sample origin	N	AMO (4 mg/L)	CMP (8 mg/L)	COL (4 mg/L)	ERY (1 mg/L)	FLU (4 mg/L)	OXO (2 mg/L)	TET (4 mg/L)	STR (8 mg/L)	GEN (4 mg/L)	KAN (8 mg/L)
B	10	80	30	0	60	0	20	50	50	60	10
L	12	90	60	10	90	0	30	50	40	10	30
Pl	14	91	73	28	64	9	18	36	36	45	18
SWI	10	60	10	0	60	0	30	30	30	40	20
Lf	5	100	40	40	80	20	40	60	60	20	0
Bf	3	100	30	0	60	0	0	60	30	60	0

B, Broodstock and breeder tanks water; L, larva and tanks water; Pl, post-larva and tanks water; SWI, Sea water inside the hatchery; Lf, Larva food; Bf, Broodstock food. AMO, amoxicillin; CMP, chloramphenicol; COL, colistin; ERY, erythromycin; FLU, flumequine; OXO, oxolinic acid; TET, tetracycline; STR, streptomycin; GEN, gentamicin; KAN, kanamycin.

Table 2. Growth of *V. alginolyticus* strains in *R. decussatus* hemolymph.

Origin	Strain number	Average $OD_{600\ nm}$ ± SD		
		10^8 CFU/mL	10^6 CFU/mL	10^4 CFU/mL
B	10	5.81 ±0.2	2.62 ±0.14	1.10 ±0.1
L	12	5.58 ±0.15[a]	1.71 ±0.10[d]	0.72 ±0.04
Pl	14	5.63 ±0.16[a]	1.85 ±0.13[d]	0.87 ±0.01
SWI	10	5.38 ±0.25[b]	1.17 ±0.25	0.34 ±0.04
Lf	5	5.44 ±0.24[a]	1.30 ±0.1	0.29 ±0.03
Bf	3	5.33 ±0.14[b]	0.81 ±0.1[e]	0.20 ±0.01[f]
ATCC 33787	1	5.25 ±0.12[c]	0.75 ±0.22[e]	0.21 ±0.01[f]
Control	1	5.20 ±0.2[c]	4.88 ±0.1	4.31 ±0.2

B, Broodstock and breeder tanks water; L, larva and tanks water; Pl, post-larva and tanks water; SWI, Sea water inside the hatchery; Lf, Larva food; Bf, Broodstock food. Values in the same column with different superscript are not significantly different (*p>0.05*).

Biofilm formation on abiotic and biotic surfaces

Results of the glass test tube adherence assay showed that all *V. alginolyticus* strains were able to adhere to the glass, giving a purple ring on the air surface of the glass tube. We noted the existence of three different phenotypes. Thirteen bacteria out of 55 tested (24 %) gave a large purple pellicle, 31 (56%) strains were moderately adherent and only 11 (20%) strains were weakly adherent (Table 3).

On glass slides, *V. alginolyticus* were able to adhere with different degrees among strains: 32 of 55 (55%) were strongly adhesive to glass surfaces, 15 (27%) strains were moderately adherent and 8 (14%) strains were weakly adherent (Table 3).

Twenty (20) of fifty five (55) *V. alginolyticus* strains (36%) were highly adhesive to polystyrene with a OD values ranging from 3.14 to 11.4 at 595

Table 3. Quantitative estimation of *V. alginolyticus* biofilm formation on biotic and abiotic materials.

Origin	Strain number	*Materials OD_{570}*									Polystyrene *OD_{595} (%)			Stainless steel average ± SD		Adherence					
		Glass surface (%)			PVC (%)			PE (%)								Hep-2 (%)			Vero (%)		
		W	M	S	W	M	S	W	M	S	W	M	S	Attached (LogCFU/cm^2)	Planktonic (LogCFU/mL)	W	M	S	W	M	S
B	10	20	30	50	20	20	60	20	50	30	20	30	40	4.19±0.26	8.58±0.43	20	20	40	50	30	10
L	12	17	33	50	0	44	66	14	50	33	0	30	40	5.27±0.4	8.70±0.43	33	17	17	33	25	7
Pl	14	15	28	57	7	43	50	0	36	64	14	14	50	4.49±0.24	8.28±0.8	43	28	7	50	35	14
SWI	10	20	40	40	0	40	60	20	30	40	30	10	40	3.83±0.52	8.23±0.54	30	10	10	30	20	10
Lf	5	20	20	60	20	20	60	0	60	40	40	0	40	3.28±0.34	8.17±0.69	20	20	0	60	0	0
Bf	3	0	33	67	0	33	67	33	67	0	33	67	0	2.80±0.50	8.50±0.73	0	0	0	33	33	0
ATCC 33787	1	-	-	100	-	-	100	-	-	100	-	100	-	3.80±0.14	8.34±0.52	100	-	-	-	100	-
Total strain (%)	55	14	27	59	7	33	60	13	42	42	18	23	36	-	-	31	20	14	40	24	9

B, Broodstock and breeder tanks; L, larva and tanks water; Pl, post-larva and tanks water; SWI, sea water inside the hatchery; Lf, larva food; Bf, broodstock food.

nm. About 50% of the *V. alginolyticus* strains obtained from post-larva samples were strongly adhesive to the polystyrene 96-well microtitre plate and 40% of them were highly adhesive to glass and polystyrene surfaces. Only 13 of 55 strains were non biofilm forming with an OD595 ≤1 (Table 3).

The results of the biofilm formation on PVC and PE surfaces showed that all strains were able to adhere strongly on both materials. In fact, 33 bacteria out of 55 (60%) were strongly adhesive to PVC pieces, whereas only 23 (42%) *V. alginolyticus* strains were able to adhere strongly to PE surfaces. Three strains obtained from seawater inside the hatchery were able to adhere strongly to both materials (Table 3).

All *V. alginolytitcus* strains were able to form biofilm on stainless steel surfaces of different degrees (Table 3). The population of *V. alginolyticus* attached to stainless steel coupons and the number of planktonic cells ranged from 2.8 to 5.27 and from 8.17 to 8.70 Log CFU/ mL.

Adherence was found in 36 of 55 (65%) of the analysed strains in Hep-2 cells while it was found in 40 of 55 strains (73%) when Vero cells were used. Weak (W) adherence was shown in 17 (31%) of the strains in Hep-2 cells and 23 strains (42%) in Vero cell lines. Moderate (M) adherence was detected in 20 and in 24% of the strains in Hep-2 and Vero monolayers. About 8 of 55 (14%) *Vibrio* strains were highly adhesive to Hep-2 cell lines, while only five (9%) strains were strongly adhesive to Vero cell lines (Table 3). Only three *V. alginolyticus* strains were able to adhere strongly to both epithelial cell lines (Figure 2).

Statistical analysis showed a significant difference between adherence to PVC and glass surfaces *($p < 0.05$)* and adherence to PVC and polystyrene surfaces *($p < 0.05$).* In addition, the Wilcoxon test revealed a significant difference between adherence to PVC, PE, polystyrene, glass surfaces and adherence to both cell lines *($p < 0.05$)*.

DISCUSSION

The main inert materials used in the aquaculture installation were polyvinyl-chloride, glass, polystyrene and polyethylene (Snoussi et al., 2008). These materials were used to build rearing tanks, phytoplankton culture batches and instruments used in the *R. decussatus* hatchery. In this study, *V. alginolyticus* strains showed a high potential to adhere to glass and most of them were strongly adhesive to PVC, polystyrene and polyethylene.

It was also noted that adhesion ability depends on the strains and on the tested materials. These data were different from the results reported by Snoussi et al. (2008) when they studied the adherence of *V. alginolyticus* strains isolated from Tunisian fish hatchery. These researchers

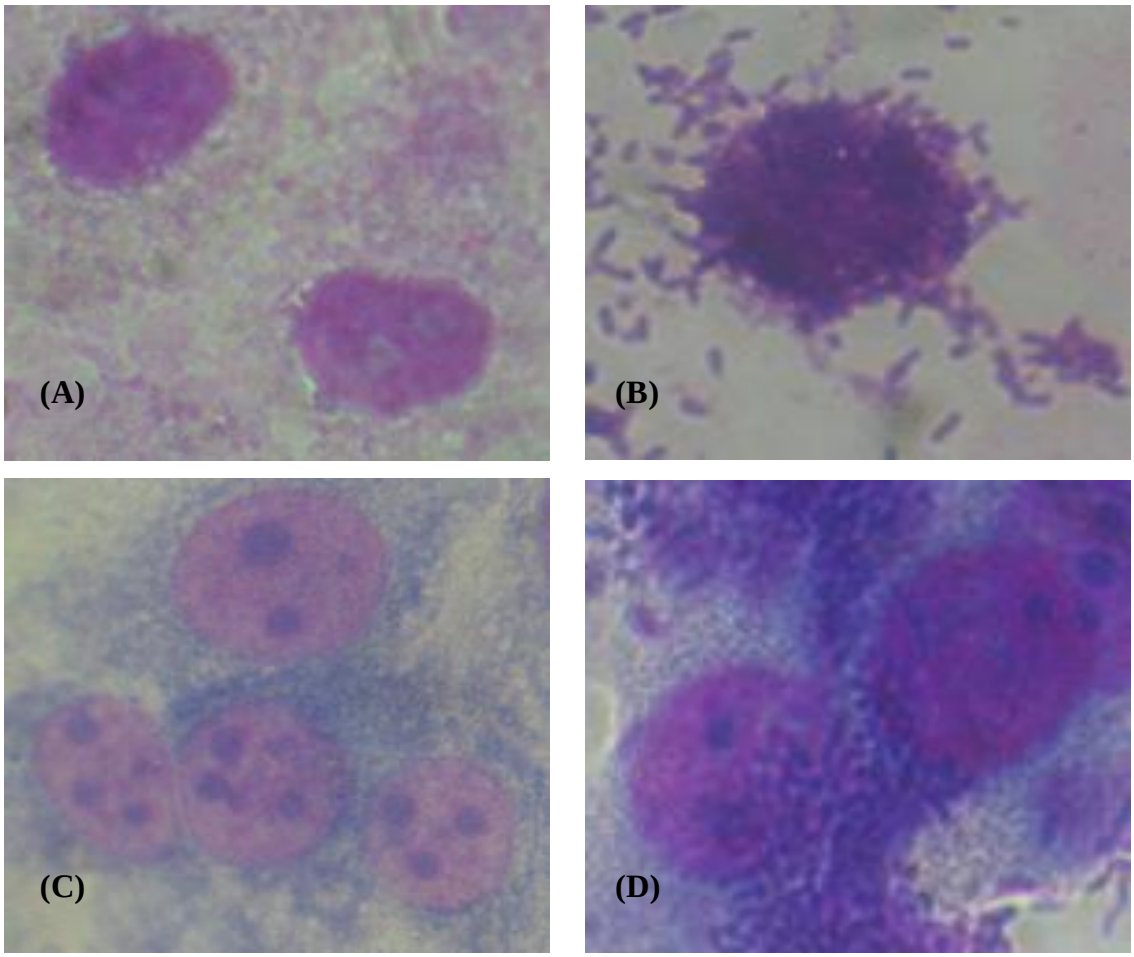

Figure 2. Optic microscopy showing the high adherence ability of *V. alginolyticus* (strain PL_{48}) to both Vero and Hep-2 cell lines. Giemsa stain: magnification (×1000). (A) and (C): Negative control for Vero and Hep-2 cells. (B) and (D): *Vibrio alginolyticus* strain PL_{48} strongly adhesive to Vero and Hep-2 cells respectively.

reported that 50% of *Vibrio* strains were strongly adhesive to PVC and only 14% were able to adhere strongly to PE material whereas in our study, these rates were 60 and 42%, respectively.

Gordon (1987) reported that the attachment density of *V. alginolyticus* strains on stainless steel strips was 6.14 Log CFU/cm^2. Overall, tested bacteria in our study were able to adhere to stainless steel surfaces to different degrees. *V. alginolyticus* strains isolated from larva and post-larva tanks showed the highest degree of adherence: 4.49 and 5.27 Log CFU/cm^2, respectively. This result demonstrated that stainless steel surfaces are less colonized by bacterial cells than other surfaces commonly used in the clam hatchery, which allows us to suggest that stainless steel material can be used in some hatchery equipment with the aim of reducing biofilm formation.

For many bacterial species, the ability to adhere to eukaryotic cells is the first step in the colonization and development of disease (Sechi et al., 2002). Attachment to culture cells has been studied in some *Vibrio* species especially with human epithelial cells (Paranjpye and Strom, 2005), human intestinal cells (Yamamoto and Yokota, 1989) and epithelial cells of the African Green Monkey (Ben Abdallah et al., 2009). The data reported in this study indicates that a considerable number of the examined strains exhibit adhesive properties in both epithelial cell lines with different degree. Our results were in accordance with the finding of Snoussi et al. (2008) and Ben Abdallah et al. (2009), in their studies of the adhesive proprieties of *V. alginolyticus* strains isolated from Tunisian fish hatcheries. These researchers showed, respectively, that adhesion to Hep-2 cells was found in 50% of analysed strains while all *V. alginolyticus* strains were able to adhere to Vero-cells to different degrees after 60 min of incubation. In this study, several strains tested showed adhesive properties in both epithelial cell lines. In fact, 60% of the analyzed *V. alginolyticus* strains were adhesive to Hep-2 cell lines while 72% of them were able to adhere to Vero cell lines.

The high adhesion ability to both cell lines suggested a possible interaction between these strains and the epithelial cell lines used in this study.

Bivalves immunity involves both humoral and cellular defense systems in a co-ordinated way to kill and eliminate infecting bacteria (Pruzzo et al., 2005). The mechanisms of action of these systems were mediated by lysosomal enzymes, anti-microbial peptides and soluble lectins (Tunkijjanukij et al., 1998; Hine, 1999; Mitta et al., 2000). In our study, we showed that overall tested bacteria were able to survive in *R. decussatus* hemolymph at a high concentration (10^8 CFU/mL). However, bacterial strains isolated from broodstock and breeder tanks presented a better growth rate than the other strains even at 10^6 and 10^4 CFU/mL. Survival of bacteria to the hemolymph microbicidal activity may depend on several factors such as the phagocytes attraction, the interaction with opsonizing molecules, the binding to hemocyte surfaces and the activation or the inhibition of the host cellular responses (Canesi et al., 2002).

The *V. alginolyticus* strains showed wide resistance to the majority of the antimicrobial agents tested. All *Vibrio* were resistant to ampicillin as reported by Snoussi et al. (2006). Zanetti et al. (2001) and Deriu et al. (2005) showed that a high percentage of resistance to this antibiotic is related primarily to the high production of β-lactamases. Overall, tested bacteria showed a high resistance to erythromycin, streptomycin, gentamicin and tetracycline as reported by Ottaviani et al. (2001). The high rate of resistance to those antibiotics may reflect the fact that these are the most commonly used antibiotics in the hatchery.

Conclusions

In this study, itis clearly shown that biofilm formation on tested surfaces allows the *V. alginolyticus* strains to persist in the different compartments of *R. decussatus* hatchery which represent a real threat for larval and post-larval rearing. However, in order to reduce biofilm formation, we suggested the use of stainless steel as substituent material in some hatchery equipments. Moreover, these strains exhibit a huge resistance to the most wildly used antibiotics in the hatchery. These results suggest that other studies are still necessary to find an effective way to eradicate *V. alginolyticus* biofilms and to find a possible correlation between virulence properties and adherence abilities.

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