Characterization of Vibrio Isolates from Carpet Shell Clam (*Ruditapes Decussatus*) Suffering from Brown Ring Disease (BRD) on Tunisian Coasts

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Abstract   A total of 13 pathogenic *Vibrio spp*. bacteria were isolated from carpet shell clams (*Ruditapes decussatus*) with Brown Ring Disease (BRD) in Tunisia. Organisms were identified based on a combination of phenotypic and molecular methods (SSP-PCR and 16SrDNA sequencing). Virulence effects were determined by in vivo testing on *R. decussatus* and in vitro testing on the Manila clam (*Ruditapes philippinarum*). All isolates demonstrated biochemical profiles typical of *Vibrio spp*; nine different biotypes. Three isolates were identified as *V.splendidus* biovar II TA E2 but the remaining isolates fell into eight biotypes different from each other and from the *V.tapetis* biotype. Species delineation based on 16S rDNA sequencing indicated that the isolated *Vibrio sp*p closely resembled *V.chagasii* (eight isolates), *V.splendidus* (two isolates), *V.alginolyticus*(one isolates), *Psychrobacter spp*. and *Pseudoalteromonas mariniglutinosa* (one isolates for each). In vitro cytotoxicity effects and mortalities could be induced by the isolates of *V.chagasii, V.splendidus* and *V.alginolyticus* at lower dosages than induced by *V.tapetis* (CECT 4600).

Keywords   *Vibrio*, Brown Ring Disease, Biochemical Profiles, Sequencing, Toxicity, *Ruditapes decussatus*, *Ruditapes Philippinarum*

1. Introduction

Many bacterial diseases in adult bivalves are caused by organisms in the genus *Vibrio* (Gram-negative, rod-shaped bacteria) that are widespread in coastal and estuarine environments ([6],[20],[23],[33]). Recent data have shown an expansion in the host range of aquatic organisms affected by pathogenic *Vibrio* species; these are primarily bivalve invertebrates ([7],[11],[27]). In addition to bivalve bacteriosis, Brown Ring Disease (BRD), a disruption of the periostracal lamina with abnormal conchiolin deposition, which affects mainly the Manila clam *Ruditapes philippinarum* and the carpet shell clam *Ruditapes decussatus*. Infections of BRD have been documented in wild populations and cultured clams isolated from waters around France, England, Ireland, Spain, occasionally in Italy and even in Korea ([2],[33],[35],[37]).

In most reports of BRD contaminations, *Vibrio tapetis* bacteria have been identified ([2],[10],[30]). *V.tapetis* – like

organisms, such as those identified in[35], have also been reported as causing BRD in Manila clams in Korea. BRD could be induced by exposing stocks of Manila clams to *V. tapetis*[13], resulting in high mortality. However, the number of *Vibrio* species negatively impacting *R. decussatus* and *R. philippinarum* has increased to more than 12 species, including *Vibrio alginoyticus* and the *Vibrio splendidus* clade ([7],[8],[9]). *Vibrio splendidus* and related species *Vibrio celticus*, *Vibrio tasmaniensis*, *Vibrio neptunius* and *Vibrio furnissii* have been characterized as pathogen inducing mortalities for a number of bivalve species including *R. phillipinarum*, *Venerupis pullastra*, *Mya arenaria* and *Meretrix meretrix* ([5],[23],[39],[40]).

The carpet shell clam represents the most economically important bivalve in Tunisia and is widely distributed along the Tunisian shoreline where it is extensively harvested for local consumption and export to Europe. However, very few studies have been published documenting the health status of this species. In 2003 we reported the occurrence of BRD in several wild populations of carpet shell clam on the Tunisian coast (Southern Mediterranean Sea) with prevalence rates ranging between 1 and 40%, but with no mortalities[14]. More recently[17], we described the results of a 6-year surveillance of BRD in natural populations of carpet shell

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clams in 14 zones along the Gulf of Gabes (southern coast of Tunisia) with BRD prevalence ranging from 35% to 100% for all sites and mortalities observed at several sites. A positive correlation was demonstrated between BRD prevalence and concentration of *Vibrio spp*. in clams. *Vibrio tapetis* was not identified among the bacterial isolates, suggesting that other marine *Vibrio* species are capable of causing BRD-like illness in local carpet shell clams. Here, we present the first results of identification, characterization, and pathogenicity of *Vibrio* isolates from BRD affected carpet shell clams sampled in Tunisian natural clam beds.

2. Materials and Methods

Bacterial isolation and cultivation 13 isolates were recovered from carpet shell clams with signs of BRD (Table 1). We also included three other strains: a *V. tapetis* strain CECT 4600 previously isolated from a Manilla clam by Paillard and Maes[30] and by[10-32], *V. tapetis* strain LP2 isolated from the corkwing wrasse *Symphodus melops* by Jensen[21], and *V. splendidus* strain ATCC25914 isolated and kindly provided by[20] from a moribund carpet shell clam from Spain

Bacterial isolation All the isolates were obtained during surveillance for BRD from 2004 to 2007 in natural beds of carpet shell clams in Tunisia and purified from infected shells showing BRD signs of damaged periostracum[17]. All of the bacterial isolates were obtained from extrapallial fluids withdrawn from affected clams and cultured on differential media (TCBS, Zobell – mannitol and marine agar (Difco) at 20℃ as described by Paillard et al[33]. Isolates were maintained on marine agar 2216 (Difco) at 4℃ or stored frozen in marine broth 2216 (Difco) supplemented with 50 % (v/v) glycerol (Sigma, St. Louis, MO, USA) at -80℃. The whole collection of strains is maintained as part of the national collection of the National Institute of Sea Sciences and Technologies (INSTM), Salammbô, in Tunisia.

Phenotypic characterization

Morphological, physiological and biochemical tests used included: Gram staining, motility and sporulation features, sensitivity to the vibriostatic agent O129, oxidase and catalase tests, temperature growth, and utilization of substrates such as carbon and other energy sources as determined using API 20NE test strips (Bio Merieux, Marcy l'Etoile, France).

Agglutination test This assay was performed as described by Paillard et al. (2006). Five milliliters of an overnight liquid culture of each isolate were centrifuged at 13,000xg. Bacteria were re-suspended in 1ml phosphate buffered saline (PBS) and 5µl were placed on a Teflon glass slide with addition of 5µl of anti *V. tapetis* (CECT 4600) polyclonal antibody solution (IUEM – Brest). Anti *V. cholerae* (Pasteur Institute, Paris, France) antibody was used as a negative control and all isolates were tested twice. The agglutination test was performed only for *Vibrio* species as clarified by Paillard et al[33].

Extraction of genomic DNA Whole genomic DNA was extracted from overnight cultures of each isolate cultured in LBS medium at 25℃, using a genomic DNA extraction Kit (Sigma); DNA was quantified using a Nanodrop apparatus (Labtech, France).

Species-specific primer PCR Identification of *V. tapetis* species among all isolates was initially made using the species-specific primer-PCR (SSP-PCR) method developed by Paillard et al[33] using *V. tapetis* specific primers (SSPVtF and SSPVtR; Table 2). A positive identification was inferred if a 416bp fragment of the 16SrDNA was amplified. As a positive control we used extracted *V. tapetis* CECT 4600 DNA, while two negative controls were used: T1) deionized water as template (no DNA) and T2) PCR negative DNA tube (CECT 4600 DNA without polymerase enzyme).

Table1.   Sources of Vibrio isolates used in this study

Isolate	Source and location	Accession numbers	Year
TN1	*R. decussatus* (Tunisia)	2J12	June 2007
TN3	*R. decussatus* (Tunisia)	LMG 21354	June 2007
TN5	*R. decussatus* (Tunisia)	LMG 21354	June 2007
TN7	*R. decussatus* (Tunisia)	LMG 21354	June 2007
TN9	*R. decussatus* (Tunisia)	LMG 21354	June 2007
TN11	*R. decussatus* (Tunisia)	LMG 21354	June 2007
TN13	*R. decussatus* (Tunisia)	LMG 21354	June 2007
TN15	*R. decussatus* (Tunisia)	LMG 21354	June 2007
TN17	*R. decussatus* (Tunisia)	LMG 21354	June 2007
TN22	*R. decussatus* (Tunisia)	2J36	June 2007
V2	*R. decussatus* (Tunisia)	*Pseudoalteromonas mariniglutinosa*	January 2004
V3a	*R. decussatus* (Tunisia)	CHNDP34	January 2004
V3b	*R. decussatus* Tunisia)	YJ06167B	January 2004
V. tapetis CECT4600	*R. philippinarum* (Landeda, France)		October 1990
V. tapetis LP2	*Symphodus melops* (Bergen, Norvège)		September 1999

Table 2. Oligonucleotid primer sequences used for PCR

Primer	Sequence 5'- 3'	References	Size of the Product
Ent16S-F	AGAGTTTGATCATGGCTCAG	William et al. (1991)	ADNr 16S of *Enterobacteria* (1500 pb)
Ent16S-R	ACGGTTACCTTGTTACGACTT		
SSP VtF	CGAGCGGAAACGAGAAGTAG	Paillard et al. (2004)	ADNr 16S of *V. tapetis* (400 pb)
SSP VtR	GGATGCACGCTATTAACGTACA		

Amplification and sequencing of genomic DNA

The 16SrDNA was amplified, using the universal primers Ent16S-F and Ent16S-R (Table 2), according to Williams et al[41]. Amplification conditions were as follows: DNA was denatured at 94 ℃ for 5 min prior to 35 cycles of denaturation (94℃ for 60 sec), annealing (50℃ for 60 sec) and extension step (72℃ for 90 sec); a final cycle in which the extension step was extended to 10 min provided efficient amplification of target DNA. All PCR assays utilized 20 ng template DNA, 25 µl of a master mix (BioMix, Bioline Ltd., London, UK), including reaction buffer, dNTPs, $MgCl_2$ and *Taq* DNA polymerase, PCR-grade water (Genaxis, Montigny le Bretonneaux, France), and 5 µl of each oligonucleotide primer (Table 2) to achieve a final volume of 50 µl. All PCR assays were carried out on a My Cycler Thermal Cycler (BioRad Laboratories, Hercules, CA, USA). PCR products were resolved by electrophoresis through a 1% horizontal agarose gel. Amplified fragments were sequenced and compared with sequences of known Enterobacteria and *V. tapetis* strains located in GenBank.

Phylogenetic analyses: 16SrDNA sequences for all isolates were aligned and phylogenetic analyses were performed using the Muscle program (Phylogeny.fr site). Sequences were aligned using the Gblocks program, and complete phylogenetic analyses were performed by the PhyML-aLRT program. Sequences were submitted to GenBank (see Table 1 for accession numbers of all isolates).

Cellular effect toward clam hemocytes using an *in vitro* cytotoxicity test BRD was first observed in the Manila clam and was attributed to the causal agent *V. tapetis*. Thus, we tested and compared cytotoxicity induced by local isolates and *V. tapetis* in Manila clams using the *in vitro* cytotoxicity test. In addition, we compared the cytotoxicity of local isolates to those of controls *V. splendidus* strain (ATCC 25914), and *V.tapetis* strain LP2 (isolated from *S. melops*). Cytotoxicity effects of isolates against Manila clam hemocytes were quantified using flow-cytometry and direct microscopic observations for the detection of eventual modifications in hemocyte structure after incubation with *Vibrio* isolates as performed by[12]. Manila clams (length 25 to 35 mm) were maintained in aquaria with aerated seawater. Clams were supplied by the SATMAR Company (Marennes, France). Hemolymph was obtained from the posterior adductor muscle using a 1ml syringe fitted with a 25-gauge needle inserted through the hinge. Prior to use, individual hemolymph samples were observed by microscopy to verify

that they were free of any contaminating particles (ciliates or large pieces of debris). Hemolymph samples were pooled to provide the final volume required for the experiment and pooled hemolymph samples were passed through an 80mm mesh screen to eliminate cell aggregates. A subsample of each pooled sample was fixed with 6% formalin solution (v/v) in Filtered Sterilized Sea Water (FSSW) to determine the cell concentration. These concentrations were used to prepare the various bacterial dilutions required. The cytotoxicity of bacteria was tested using (bacteria: 25-clam hemocyte) ratio with 3-hour incubation. The cytotoxicity assay was performed in 24 – well microplates by addition of bacteria to hemocytes. Sub samples of each hemocyte pool were exposed to both washed bacteria and FSSW at 18℃. After 3h incubation, 6% formalin (v/v) was added to stop the interactions, and supernatants were transferred to cytometry tubes. Hemocyte numbers present in bacteria or FSSW-exposed supernatants was determined by flow cytometry (FACS-Calibur^{TM} flow cytometer, Becton Dickinson, Cockeysville, MD, USA). Cells were detected with forward scatter as the primary parameter, since this parameter is related to the size of cells. Cells were stained with Syber Green I (final conc. = 10^{-3} of commercial solution obtained from Molecular Probes (Life Technologies), a DNA-binding fluorochrome, to verify that counted events corresponded to cells[28]. Indeed, only DNA carrying particles have to be counted. Results are expressed as a non-adherent cell ratio, i.e. the number of non-adherent cells incubated with bacteria divided by the number of cells incubated with FSSW[12]. A non-adherent cell ratio above 1 represents a cytotoxic effect of the tested bacteria.

Lethal virulence *in vivo* test The lethal effect of *Vibrio* isolates (TN22, TN3 and TN5)were compared to the same effect of *V. tapetis* (CECT 4600) using the direct injection into the posterior adductor muscle of carpet shell clams as described by[3]. Isolates to be tested were cultured on marine agar (1L distilled water, 15g agar, 20g sea salts[Sigma], 4g peptone, 0.1 g Fe(PO4)2) until bacterial growth reached exponential phase (typically 72h at 20℃). Bacteria were suspended in sterile FSSW and adjusted spectrophotometrically to approximately 5×10^8 colony forming units (CFU) ml^{-1}. Adult (34.2 +/- 0.8mm) carpet shell clams with no clinical signs of BRD were provided by the SATMAR Company and maintained in an aerated marine aquaria at 14℃ throughout the experiments. Clams were fed cultured algae daily during the period of study. Five different

batches of carpet shell clams (n = 180 clams per batch) were separately inoculated within the posterior adductor muscle with 0.1ml (5×10^7 CFU) of different bacteria (*V. tapetis* CECT4600, *V. splendidus* TN22 and two isolates of *V. chagasaii* (TN3 and TN5). The fifth batch of clams was injected with sterile sea water (SSW) as a control. Each batch of clams was maintained at 14°C in a separate aquarium at about 180 clams m^{-2}. Dead clams were counted, removed and recorded each day. Cumulative mortality was calculated over the 2-week experiment from the number of remaining clams after each sampling.

3. Results

Although all 13 isolates characterized as a part of this study were recovered from carpet shell clams with apparent BRD, the majority of the isolates could be distinguished from one another based on a series of phenotypic assays (Table 3). Growth on TCBS agar, mannitol and sucrose oxidation/fermentation, production of acetoin, arginine dihydrolase, ornithine decarboxylase and growth at temperature of 30°C primarily separated the isolates from one another.

All isolates were sensitive to the vibriostatic agent O129 and none of the isolates displayed the hallmark characteristics of *V. tapetis* (failure to ferment mannitol and sucrose, inability to replicate at 30°C). In addition, none of the isolates agglutinated when tested against *V. tapetis* antisera. On the basis of the phenotypic features, we distinguished the following groups: Group N°1 phenotypically resembling *V. splendidus* biovar II TA2, isolated from *R. decussatus* larvae, which included isolates TN5, TN7 and TN9,. Group N°2 including isolates TN1, TN11, TN15 and TN17, displaying a phenotype unique from both *V. tapetis* CECT 4600 and *V. splendidus* biovar II TAE2. The remaining isolates TN3, TN13, TN22, V2, V3a and V3b each presented a phenotypic profile that is unique.

Molecular identification using the SSP-PCR method showed that only one isolate (V2) amplified a band indistinguishable in size from *V. Tapetis* (previous data[17]).

Phylogenetic analysis based on 16S rDNA. 16SrDNA genes were amplified for all isolates using universal *Enterobacteriaceae* bacterial primers, the 1540 bp products were sequenced and most similar matches were sought using the program Blast from GenBank. A dendrogram derived from the sequence homology comparisons of 16SrRNA gene sequences of isolates with respect to reference sequences of *V. tapetis* (CECT strains 4600and LP2) and *V. splendidus* is shown in Fig.1. BlastN identified TN1 and TN22 as *V. splendidus* with high sequence homology (respectively 99% and 98%) and eight strains: TN3, TN5, TN7, TN9, TN11, TN13, TN15, and TN17 as *V. chagasii* with sequence homology of 99%, very close to *V. splendidus* cluster, and V2 as *V. alginolyticus* with sequence homology of 97%. All sequences were different from those of *V. tapetis* (CECT4600 and LP2) as shown by the phylogenetic tree.The two isolates V3a and V3b were identified respectively as *Psychrobacter* and *Pseudoalteromonas mariniglutinosa* with sequence homology of 99%.

Table 3.   Selected API20E characteristics of bacterial strains studied

Test	TN1	TN3	TN5	TN7	TN9	TN11	TN13	TN15	TN17	TN22	V2	V3a	CECT 4600	LP2	TA2
Gram	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
oxydase	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Catalase	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
indole	+	-	+	+	+	+	-	+	+	-	-	-	+	+	+
Acetoin	-	-	-	-	-	-	-	-	-	-	+	+	-	-	-
Glucose fermentation	-	+	+	+	+	-	-	-	-	-	+	+	+	+	+
Arginine dihydrolase	-	-	-	-	-	-	-	-	-	-	+	-	+	-	-
Ornithine decarboxylase	-	-	-	-	-	-	-	-	-	-	+	+	-	-	-
βgalactosidase	-	-	-	-	-	-	+	-	-	-	-	-	+	+	-
Gelatinase	+	-	+	+	+	+	-	+	+	-	+	+	+	+	+
Amylase	+	+	+	+	+	+	-	+	+	+	+	+	+	+	+
Mannitol fermentation	+	+	+	+	+	+	-	+	+	+	+	+	-	-	NT
Saccharose fermentation	-	-	-	-	-	-	-	-	-	-	+	-	-	+	NT
Growth at 30°C	+	+	+	+	+	+	+	+	+	+	+	+	-	-	+
Sensitivity to agent O/129	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S
Growth on TCBS	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+

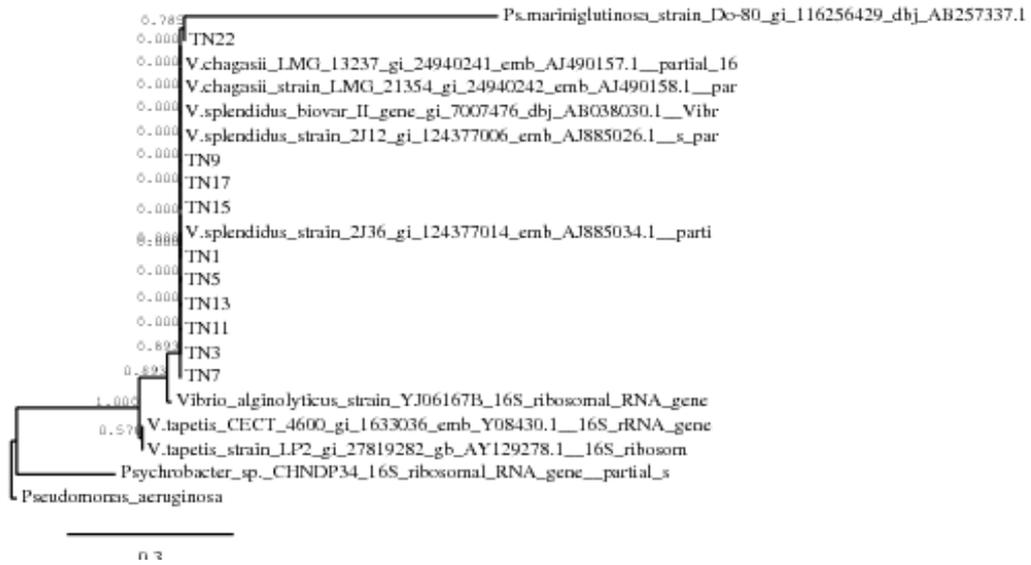**Legend:** (+): positive reaction; (-): negative reaction; (N.T): non typed, (S): sensitive to O/129

Figure 1. Phylogenetic tree of isolates based on partial 16Sr DNA sequences. The scale bar represents 0.03 substitutions per nucleotide
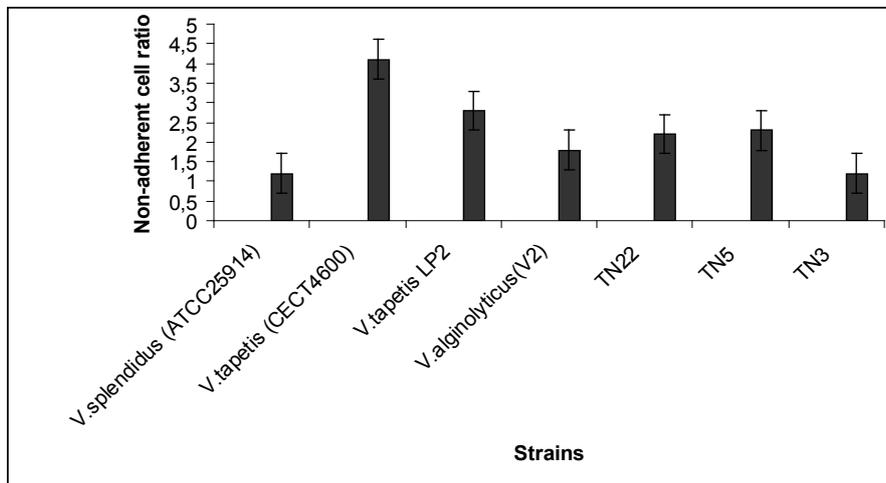

Figure 2. Effect of different strains of *Vibrio* on non-adherent cell ratio of clam hemocytes. Incubation time = 3h, 25 bacteria per hemocyte; T℃=18℃
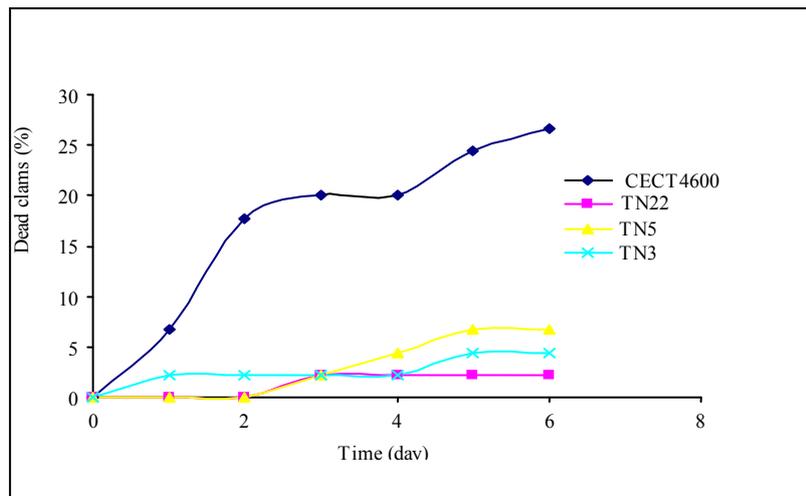

Figure 3. Mortality kinetics of *R.decussatus* induced by *Vibrio* isolates tested

For *in vitro* **cytotoxicity test** *V. tapetis* strain CECT 4600) showed the greatest capacity to inhibit hemocyte adhesion (Fig. 2); whereas *V. splendidus* strain ATCC25914 had no significant cytotoxicity. Within the Tunisian *Vibrio* isolates tested, significant cytototoxicity (i.e. the non-adherent cell ratio >1.2) was observed for *V. splendidus* (TN22), *V. chagasaii* (TN5 and TN3) and *V. alginolyticus* (V2), however, all had lower rates than that of *V. tapetis* strain CECT 4600.

***In vivo* mortality testing** Dead carpet shell clams appeared just one day post-inoculation in the group injected with *V. tapetis* strain CECT 4600 and the mortality reached 25% by day 6 (Fig. 3). All of the representative Tunisian Vibrio isolates tested[*V. chagasaii* (TN3 and TN5) or *V. splendidus* (TN22)], showed low mortality rates (< 5% after day 6 post-inoculation).

4. Discussion

Brown Ring Disease is designated as the main bacterial disease afflicting adult *R. philippinarum* and *R. decussatus* clams. BRD has been detected in natural carpet clam populations in coastal parts of Tunisia[14, 15, 16]. Globally clam production is often affected by vibriosis, which leads to high mortality rates as reported by[20] and[33] and. Recent studies have indicated that a wide variety of *Vibrio* species can cause disease in clams; these organisms can persist with lethal effects in the bivalve tissues despite the depuration process[26].

For several northern Mediterranean studies, *V. tapetis* was the principal agent involved in BRD ([2],[29],[33]). Data previously collected in Tunisia has indicated a strong correlation between BRD prevalence and the concentration of *Vibrio* spp. found in tissue or extrapallial fluids of infested clams but these studies failed to identify *V. tapetis* as a causative agent[17]. In this manuscript, we have identified and characterized 13 *Vibrio*-like isolates from affected carpet shell clams using a combination of phenotypic and molecular tools. We also evaluated their toxicity and lethality using *R. philippinarum* and *R. decussatus*.

Among the biochemical profiles identified (8 biotypes) one group, including isolates TN5, TN7 and TN19, closely resembled *V. splendidus* biovar IITA2; the remaining biotypes were different from *V. tapetis* and *V. splendidus* as well as each other. These findings highlight the great phenotypic variability associated with environmental isolates, as has been previously reported for of *Vibrio* species in bivalves from different marine areas ([4],[18],[19]). The diversity of *Vibrio* spp. associated with cultured clams was also demonstrated by Beaz-Hidalgo et al.[7]; 759 *Vibrio* isolates were recovered and from these clams more than 13 different species were observed with 3 potentially newly described species. The use of species-specific primer-PCR (SSP-PCR) analysis of a variable region in the present study also did not identify any of the isolates as *V. tapetis,* even though close band similarity was obtained for the strain V2

(identified as *V. alginolyticus* by 16Sr DNA sequencing); The application of 16S rDNA sequencing was able to discriminate the *Vibrio*-like isolates in to 5 clusters: *V. chagasaii*, *V. splendidus, V. alginolyticus, Pseudo-alteromonas mariniglutinosa* and *Psychrobacter spp.*); the majority of isolates appearing to be either *V. chagasaii* or *V. splendidus*. These findings demonstrate for the first time that BRD associated with adult carpet shell clams in Tunisia should be caused similarly by *V. splendidus*. This result is similar to previous findings in the Mediterranean region of Spain where carpet shell and manila clams, oyster *Crassostrea gigas*, *Mya arenaria* and fish (*Scophtalmus maximus*; *Symphodus melops*) also adversely affected by *V. splendidus* group, including *V. pomeroyi*, *V. kannalloae* and *V. aestuarianus* ([1],[20],[21]). Besides 16S rDNA sequencing, additional molecular methods such as DNA/DNA hybridization, polymerization of toxin genes and *rpoA, pyrH* and *recA* sequencing can be used to increase the differentiation between closely related *Vibrio* species ([7],[24],[38]). In light of our results, a fast detection PCR test for *Vibrio* species[23]; mainly for *V. splendidus* – related group) could potentially greatly benefit surveillance for pathogenic organisms.

Most of the isolates tested demonstrated some degree of cytotoxicity to carpet shell clams (i.e. the non-adherent cell ratio >1,2) although the observed cytotoxicity was lower than that of *V. tapetis* strain CECT 4600.

According to Allam et al[2], the *Vibrio* cell wall contains smooth lipopolysaccharides that play an important role in virulence. They help in the penetration of the pathogen into host tissues and allow bacteria to better resist the defense systems of clams, particularly phagocytosis by hemocytes. The viability of clam hemocytes was significantly affected by incubation with both *V. splendidus, V. alginolyticus,* or the extra cellular products of both species as described by Gomez-Leon et al. (2005). The ability of pathogenic *Vibrios* to proliferate rapidly in the tissues of *R. philippinarum* is considerable when compared with *R. decussatus* which is more resistant to pathogens ([3],[25]). Nevertheless, Prado-Alvarez[36], demonstrated a negative effect on cell viability after treating *R. decussatus* clams with either living or dead *V. splendidus*; this result suggests that for bacterial extracellular products might play a role in modulating the bivalve immune response.

According to present data, direct injection of *Vibrio* isolates (*V. splendidus* or *V. chagasii*) in to the adductor muscle of *R. decussatus* clams induced lower rates of mortalities in as compared to *V.tapetis*. Similar results were obtained by[3] who showed that development of BRD in *R. decussatus* injected in the pallial cavity is much less pronounced than in *R. philippinarum*. The resistance of *R. decussatus* to BRD and therefore lack of animal death is primarily explained as the result of a strong barrier to infection at the perisotracal membrane level and efficient neutralization of any *V. tapetis* that enter the extrapallial cavity by[31]. According to Labreuche[22], for *V.aestruarianus,* a pathogen of *C. gigas*, lethality was

modulated by incubation temperature. Present results were obtained for *Vibrio* isolates cultured at 18°C (the optimal incubation temperature of *V. tapetis*). Further experiments will be conducted for these local cultures incubated at 30℃ in order to determine eventual temperature effect on lethality of *Vibrio* isolates.

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REFERENCES

[1] Azandégbé A, Garnier M, Andrieux-Loyer F, Kérouel R, Philippon X, Nicolas JL. Occurrence and seasonality of *V.aesturianus* in sediment and *Crassostrea gigas* heamolymph at two oyster farms in France. Diseases of Aquatic Organisms, vol. 91, pp.213-221, 2010.

[2] Allam B, Paillard C, Howard A, Le Pennec M. Isolation of the pathogen *Vibrio tapetis* and defense parameters in brown ring diseased Manilla clams *Ruditapes philippinarum* cultivated in England. Diseases of Aquatic Organisms, vol. 41, pp.106-113, 2000.

[3] Allam B, Ashton-Alcox KA, Ford SE. Flow cytometric comparison of haemocytes from three species of bivalves molluscs. Fish and Shellfish Immunology, vol.13, pp. 141-158, 2002.

[4] Alsina M, Blanch AR. A set of keys for biochemical identification of environment *Vibrio* species. Journal of Applied Bacteriology, vol.76, pp. 79-85, 1994.

[5] Araya MT, Markhan F, Mateo DR, McKenna P, Johnson JR, Berthe FCJ, Siah A. Identification and expression of immune–related genes in hemocytes of soft shell clams, *Mya arenaria,* challenged with *V. splendidus*. Fish and Shellfish Immulology, vol. 29, pp. 557-562, 2010.

[6] Austin B. *Vibrios* as causal agents of zoonoses. Veterinarian Microbiology, vol.14, pp. 310-317, 2010.

[7] Beaz-Hidaldgo R, Cleenwerck I, Balboa S, Dewachter M., Thompson FL, Swings J, Devos P, Romalde JL. Diversity of *Vibrios* associated with reared clams in Galicia (NW Spain). Systematic and Applied Microbiology, vol. 31, pp. 215-222, 2008.

[8] BeazHidaldgo R, Doce A, Pascual J, Toranzo AE, Romalde JL. 2009. *V. gallaecicus sp* .nov isolated from cultured clams in north – western Spain. Systematic and Applied Microbiology, vol. 32, pp. 111-117, 2009.

[9] BeazHidaldgo R, Dieguez AL, Cleenwerck I, Balboa S, Doce A, Devos P, Romalde JL. *V. celtius sp* nov a new *Vibrios* species belonging to the *splendidus* clade with pathogenic potential for clams. Systematic and Applied Microbiology, vol. 33, pp. 311-315, 2010.

[10] Borrego JJ, Castro D, Luque A, Paillard C, Maes P, Garcia MT, Ventosa A .1996a. *Vibrio tapetis sp.nov*. The causative agent of the Brown ring disease affecting cultured clams.

[11] Cai JP, Li J, Thompson KD, Li, CX, Han, HC. Isolation and characterization of pathogenic *Vibrio parahaemolyticus* from diseased post-larvae of abalone *Haliotis diversicolor supertexta*. Journal of Basic Microbiology, vol. 47, pp. 84–86, 2007.

[12] Choquet G, Soudant P, Lambert C, Nicolas JL, Paillard C. Reduction of adhesion properties of *Ruditapes philippinarum* hemocytes exposed to *Vibrio tapetis*. Diseases of Aquatic Organisms, vol. 57, pp.109-116, 2003.

[13] Drummond LC, Balboa S, Beaz R, Mulcahy MF, Barja JL, Culloty SC, Romalde JL (2007) The susceptibility of irish grown and Galician grown Manila clams *R. philipinarum* to V. *tapetis* and BRD. Journal of Invertebrate Pathology, vol. 95, pp. 1-8. 2007.

[14] El Bour M, Attia H, Paillard C, Mraouna R, Ayari W, El Abed A. Etude de dépistage de la maladie de l'anneau brun et détermination des germes bactériens associés chez la palourde *Tapes decussatus* des côtes tunisiennes. Bulletin de l'ATSMer, pp.1-5, 2003

[15] El Bour M, Paillard C, Sintes C, Lakhal F, Attia H, Jacq A. Survey of brown ring disease in clams (*Ruditapes decussatus*) of northern lagoons in Tunisia. In proceedings of the 8th International Conference on Shellfish Restoration – ICSR05, 2005.

[16] El Bour M, Jaafar-kéfi F, Hamrouni M, Mahmoud N, Dellali M, Trigui-El-Menif N, Aloui-Bejaoui N. Etude de dépistage de la maladie de l'anneau brun chez des espèces de bivalves des côtes tunisiennes, Bulletin de la Société Zoologique de France, vol. 133, n°1-3, pp. 107-115, 2008.

[17] El Bour M, Dellali M, Boukef I, Lakhal F, Mraouna R, Hili H, Paillard C, Klena J. Occurrence of Brown Ring Disease in carpet shell clam *R. decussatus* from the gulf of Gabes (Tunisia, Central Mediterranean Sea) Journal of Shellfish Research, vol. 30, n°3, pp. 1-8. 2011.

[18] Farto R, Montes M, Perez MJ Nieto, TP, Larsen JL, and Pedersen K. Characterization by numerical taxonomy and ribotyping of *Vibrio splendidus* biovar I and *Vibrio scophthalmi* strains associated with turbot cultures. Journal of Applied Microbiology, vol. 86, pp. 796-804, 1999.

[19] Gauger EJ, and Gómez-Chiarri M. 16S ribosomal DNA sequencing confirms the synonymy of *Vibrio harveyi* and *V. carchariae*. Diseases of Aquatic Organisms, vol. 52, pp. 36-46, 2002.

[20] Gómez-Leòn J, Villamil L, Lemos ML, Novoa B, Figueras A. Isolation of *Vibrio alginolyticus* and Vibrio spelndidus from aquacultured Carpet Shell Clam (*Ruditapes decussatus*) Larvae associated with mass mortalities, Applied and Environmental Microbiologie, vol. 71, n°1, pp. 98-104, 2005.

[21] Jensen S, Samuelsen OB, Andersen K, Torkildsen L, Lambert C, Choquet G, Paillard C, Bergh O. Characterization of strains of *Vibrio splendidus* and *V. tapetis* isolated from corkwing wrasse *Symphodus melops* suffering vibriosis. Diseases of Aquatic Organisms, vol. 53, pp. 25-31, 2003.

[22] Labreuche Y, Soudant P, Goncalves M, Lambert C, Nicolas JL. Effect of extra-cellular products from pathogenic *Vibrio aestuarinus* strain O1/O32 on lethality and cellular immune

International Journal of Systematic Bacteriology, vol.46, pp. 480-484, 1996a.

responses of the oyster *Crassostrea gigas*. Development and Comparative Immunology, vol.30, pp. 367-379, 2006.

[23] Lago EP, Nieto TP, Segruin RF. Fast detection of *Vibrio* species potentially pathogenic for mollusc. Veterinarian Microbiology, vol.139, pp. 339-346.2009.

[24] Le Roux F, Gay M, Lambert C, Nicolas JL, Gouy M, and Berthe, F. Phylogenetic study and identification of *Vibrio splendidus*-related strains based on gyrB gene sequences. Diseases of Aquatic Organisms, vol. 58, pp. 143-150. 2004.

[25] Lopez-Cortes L, Luque A, Martinez-Manzanares E, Castro D, Borrego JJ. Adhesion of *V. tapetis* to clam cells. Journal of Shellfish Research, vol. 18, pp. 91-9, 1999.

[26] Lopez-Joven C, Ruiz-Zarguela I, DeBlas I, Furones MD Roque A. Persistence of sucrose fermenting and non fermenting vibrios in tissues of Manila clam species R. *philippinarum*, depurated in sea water at two different temperatures. Food Microbiology, vol. 28, pp 951-956, 2011.

[27] Matte MH, Baldassi L, Barbosa ML, Malucelli MIC, Nitrini, SMOO, Matte GR. Virulence factors of *Vibrio metschnikovii* strains isolated from fish in Brazil. Food Control, vol.18, pp 747–751, 2007.

[28] Marie D, Partensky F, Jacquet S, Vaulot D. Enumeration and cell cycle analysis of natural populations of marine picoplankton by flow cytometry using nucleic acid strain SYBRGreen. I Applied and Environmental Microbiology, vol. 63, pp. 186-193, 1997.

[29] Novoa B, Luque A, Castro D, Borrego JJ, Figueras A. Characterization and infectivity of four bacterial strains isolated from Brown Ring Disease-affected clams. Journal of Invertebrate Pathology, vol. 71, pp 34-41, 1998.

[30] Paillard C, Maes P. Etiologie de la maladie de l'anneau brun chez *Tapes philippinarum* pathogénicité d'un *Vibrio sp*. Comptes Rendus de l'Académie des Sciences. Ser III life Sc 310, pp. 15-20, 1990.

[31] Paillard C, Maes P, Oubella R.1994. Brown ring disease in clams. Annual Review of Fish Diseases, vol. 4, pp 219-240, 1994.

[32] Paillard C, Maes P.The brown ring disease in manila clam, *Ruditapes philippinarum*. Part 1. Ultrastructural alterations of the periostracal lamina. Journal of Invertebrate Pathology, vol. 65, pp. 91-100, 1995.

[33] Paillard C. Rôle de l'environnement dans les interactions hôtes-pathogènes: développement d'un modèle de vibriose chez les bivalves. Habilitation à diriger des recherches (HDR), Université de Bretagne, Brest, 2004.

[34] Paillard C. A short-review of brown ring disease, a vibriosis affecting clams, *Ruditapes philippinarum* and *Ruditapes decussatus*. Aquatic Living Research, vol. 17, pp. 467-475, 2004b.

[35] Park KL, Paillard C, Le Chevalier P, Choi KS. Report on the occurrence of brown ring disease (BRD) in Manila clam, *Ruditapes philippinarum*, on the west coast of Korea. Aquaculture, vol. 255, pp. 610-613, 2006.

[36] Prado-Alvarez M, Romero A, Balseiro P, Dios S, Novoa B, Figueras A. Morphological characterization and fuctionnal immune responses of the carpet shell clam *R. decussatus* heamocytes after bacterial stimulation. Fish and Shellfish Immunology, vol. 32, pp. 69-78, 2012.

[37] Romalde JL, Castro D, Magarinos B, Lopez-Cortes L, Borrego, JJ. Comparison of ribotyping, randomly amplified polymorphic DNA, and pulsed-field gel electrophoresis for molecular typing of *Vibrio tapetis* Systematic and Applied Microbiology, vol. 25, pp. 544-550, 2002.

[38] Thompson FL, Iida T, Swings J. Biodiversity of vibrios. Microbiology and Molecular Biology Reviews, vol. 68, pp. 403-431, 2004.

[39] Yue X, Liu B, Xiang J, Jia J. Identification and characterization of the pathogenic effect of a *Vibrio paraheamolyticus* related bacterium isolated from clams (Meretrix meretrix) with mass mortality. Journal of Invertebrate Pathology, vol. 103, pp. 109-115, 2010.

[40] Yue X, Liu B, Sun L. Isolation and characterization of a virulent *Vibrio sp* bacterium from clams (*Meretrix meretrix*) with mass mortality. Journal of Invertebrate Pathology, vol. 106, pp. 242-249, 2011.