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Real-time PCR optimization to identify environmental *Vibrio* spp. strains

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Abstract :

Aims

To identify *Vibrio vulnificus*,*Vibrio cholerae* and *Vibrio alginolyticus* using standardized DNA extraction method and real-time PCR assays, among a large number of bacterial strains isolated from marine environment.

Methods and Results

Methods for DNA extraction and real-time PCR were standardized to identify a large number of *Vibrio* spp. strains isolated through regular collection campaigns of environmental samples. Three real-time PCR assays were developed from a multiplex PCR, targeting *V. vulnificus*,*V. cholerae* and *V. alginolyticus* on the *dnaJ* gene. After testing their specificity, these systems were applied for the identification of 961 strains isolated at 22°C (446 strains) and 37°C (515 strains) in September 2009. The predominance of *V. alginolyticus* (82·6%) among the *Vibrio* spp. strains isolated at 37°C was shown. At 22°C, only 1·6% of the strains were identified by PCR and they were *V. alginolyticus*.

Conclusions

Reproducible and specific real-time PCR assays combined to a DNA extraction method on microplates were used to constitute a large environmental *Vibrio* strains collection and to identify and detect potential human pathogenic *Vibrio* isolated at 37°C. For environmental strains isolated at 22°C, because of the higher species diversity, other approaches, like sequencing, should be chosen for identification.

Significance and Impact of the Study

The protocol developed in this study provides an appropriate and rapid screening tool to identify a large number of bacterial strains routinely isolated from the environment in long-term studies.

Keywords : *dnaJ* ; Identification ; real-time PCR ; *Vibrio alginolyticus* ; *Vibrio cholerae* ; *Vibrio vulnificus*

Introduction

The *Vibrio* genus encompasses gram negative bacteria species indigenous of marine and estuarine waters. To date, one hundred and thirty species of vibrios have been described and twelve were classified as human pathogens implicated mostly in food or water-borne diseases (Thompson *et al.* 2006). The sanitary consequences on human health and the socio-economic impacts of these pathogens, particularly of *V. cholerae* (Colwell *et al.* 1977; Colwell 1996), and of *V. parahaemolyticus* (Kaneko and Colwell 1973), *V. vulnificus* (Oliver *et al.* 1982) and *V. cholerae* non-O1/non-O139, are well documented. In Europe, the risk of *Vibrio*–associated infectious diseases is limited to a few cases each year but it is expected to increase in the future due to raw shellfish consumption, the increase of immune-compromised people, and also the impact of anthropogenic activities and global warming on the marine environment (Baker-Austin *et al.* 2010). The presence of potentially pathogenic *Vibrio* spp. for human was reported in French coastal waters (Hervio-Heath *et al.* 2002; Deter *et al.* 2010b) but little is known about the ecology and virulence of these species in these areas.

Understanding of the dynamics and evaluation of the diversity of *Vibrio* populations appeared necessary to develop prediction models to monitor the coastal environments, and to anticipate the microbiological health risks linked to the presence of *Vibrio* spp. as some authors started to explore (De Magny *et al.* 2009; Baker-Austin *et al.* 2010; Johnson *et al.* 2010; Rodriguez-Castro *et al.* 2010; Collin and Rehnstam-Holm 2011). Thus, determining the ecological drivers of *Vibrio* seasonality, abundance and diversity is important. For this purpose, long term studies are necessary particularly in coastal areas under the influence of anthropogenic activities. Generally, these studies have coupled the dynamics and diversity analyses of total bacterial community and associated pathogens (Oberbeckmann *et al.* 2010; Oberbeckmann *et al.* 2011). Thus, there is a need to adapt high screening methods for microbiological analyses.

For a long time, the identification of *Vibrio* spp. during epidemiological inquiries was mainly based on phenotypic markers for strains isolated at 37°C. This identification can be performed with normalized methods based on phenotypic characters description and biochemical tests (Alsina and Blanch 1994; ISO 2007a; b). However, they do have some limitations. Firstly, these assays are not reliable enough to identify strains collected from environmental samples, *i.e.*, seawater, seafood or marine sediment samples mainly because of the adaptation and subsequent phenotypic changes to varying environmental conditions (Abbott *et al.* 1998; O'Hara *et al.* 2003). Secondly, the classical methods used for bacterial identification are incapable with distinguishing pathogenic strains from non pathogenic strains. Furthermore, they are not adapted for the analysis of total bacterial population diversity. Thus, molecular methods were developed to improve the speed of detection and identification of culturable strains (Brauns *et al.* 1991; Thompson *et al.* 2003). To date, several molecular-based methods are used for bacterial identification such as DNA-DNA hybridization (Reichelt *et al.* 1976), ribotyping (Kumar and Nair 2007), Multi Locus Sequence Analysis or MLSA (Thompson *et al.* 2008; Pascual *et al.* 2009). For bacterial detection these methods are mainly based on PCR. Real-time PCR is widely used as a rapid and less time consuming method for *Vibrio* species detection and identification (Fukushima *et al.* 2003; Takahashia *et al.* 2005; Gubala 2006; Blackstone *et al.* 2007; Nordstrom *et al.* 2007), which is useful for screening a high number of strains.

The present study describes standardization and validation of real-time PCR protocols targeting the species *V. vulnificus, V. cholerae* and *V. alginolyticus*. These species are responsible for the majority of the 134 vibriosis cases reported in France between 1995 and 2009 (Quilici and Robert-Pillot 2011). We have standardized a high throughput DNA extraction method and have optimized and validated the specificity of real-time PCR with previously published primers for the species *V. cholerae, V. alginolyticus* and *V. vulnificus*. These developments were validated

and applied to a high number of strains isolated from the environment during one sampling campaign (September 2009) of a two-year study regarding the ecology of *Vibrio* populations and the presence of pathogenic species in French coastal waters.

Materials and methods

Study area and sample collection

The study area was located in Northern France on the English Channel coast (Figure S1). This area is characterized by important industrial and recreational activities. The sampling of seawater and superficial sediments was performed along two transects. Each transect was composed of 4 sampling points, one at the coast and the others at 300, 1.300 and 3.300 m from the first point with mean depths of 4, 6, 9 and 23 m, respectively. The first transect (T1) was under the influence of industrial activities and the second transect (T2), used as a reference, was located in a protected natural area (Platier d'Oye, 59 Nord, France). Two liters of surface and bottom seawater were sampled per point using a Van Dorn bottle and conditioned into 2 liter-sterile flasks to perform bacteriological analyses. The superficial sediments were sampled for each point using a metallic grab sampler and conditioned into 1 liter sterile flasks. The samples were transported in isothermal condition to the laboratory for analysis within 6h.

Bacterial strains

Fifty six strains of *Vibrio* species including *V. alginolyticus* (n=11), *V. cholerae* (n=12), *V. parahaemolyticus* (n=10), *V. vulnificus* (n=10), *V. mimicus* (n=4) and 9 other *Vibrio* species and six non-*Vibrio* species were used to perform gradient thermal PCR and inclusivity/exclusivity real-time PCR assays (Table 1). The six non-*Vibrio* species were

Escherichia coli, Shewanella putrefaciens, Proteus vulgaris, Salmonella enteridis, Klebsiella pneumoniae and *Citrobacter freundii*.

Isolation of *Vibrio* strains from seawater and superficial sediments samples

Water volumes of 0.1, 1, 10, 50 and 100 ml were filtered through 0.45μm-pore-size nitrocellulose filters (Millipore™ SA, ST Quentin Les Yvelines, France). All the membranes were plated onto thiosulphate–citrate-bile-salts–sucrose agar plates (TCBS, Difco™, Becton Dickinson and Company, Le Pont de Claix, France) and incubated for 24h and 48h at 37 °C and 22°C, respectively. After 24h or 48h of incubation, the colonies were enumerated and the number of total *Vibrio* was estimated as CFU l^{-1} of water (data not shown). Twenty colonies showing phenotypic appearance of *Vibrio* were selected and streaked onto TCBS. After incubation at 22°C or 37°C for 24h, single colonies were isolated onto Bacto™ Heart Infusion 2% NaCl agar medium pH 7.4 (HI agar, Becton Dickinson and Company, Le Pont de Claix, France) to control purity and enriched in 1.5 ml HI 2% NaCl liquid medium before conservation. Due to the high number of strains, the bacterial colonies were sub-cultured on 96-well plates (Masterblock 2 ml 96-well sterile, Fisher Scientific, Germany). After incubation at 22°C or 37°C for 24h, volumes of 1.5 ml cultures were split into two 96-well plates (750 μl per well). Volumes of 250 μl HI 40% glycerol solution were added to each well for a final concentration HI 10% glycerol and the plates were immediately stored at -80°C (cryoconservation).

Ten grams of sediment diluted in 90 ml of tryptone salt (0.1% Peptone from Casein; 0.85% of NaCl; 0.2% Tween 80 and MilliQ water 988 ml; pH : 7,0 ± 0,2) were mixed during 10 min. The supernatant was streaked onto TCBS plate directly (0.1 ml and 0.2 ml) or filtered (10 ml and 25 ml) on 0.45μm-pore-size nitrocellulose filters before being plated onto TCBS. The number of *Vibrio* isolated was estimated as CFU l^{-1} of wet sediment (data not shown). The isolation,

142 culture and cryoconservation of the presumptive *Vibrio* strains were performed as described
143 above for water samples.
144
145 **DNA extraction**
146 The DNA extraction protocol was adapted to be performed on 96-well plates. The strains
147 stored at -80°C were cultured in a new 96-well plate with 1.5 ml of HI 2% NaCl liquid medium
148 per well and incubated 24h at 22°C or 37°C, depending on the isolation temperature of the
149 strains. The plate was centrifuged at 2000 g for 10 min at room temperature (RT). Pellets were
150 washed two times with 1.5 ml and 1 ml of sterile buffered saline (0.45% Na_2HPO_4, 12 H_2O;
151 0.04% of K_2HPO_4; 0.72% NaCl and MilliQ water 988 ml) at 2000 g for 10 min. Pellets were
152 suspended in 100 μL of RNase DNase Protease free water (5 PRIME, Hamburg, Germany) and
153 transferred onto a 96-well PCR plate (Agilent Technologies France, Massy, France) and lysed by
154 heating at 100°C for 15 min with a Peltier Thermal Cycler (PTC-200, Biorad, France). After 10
155 min of centrifugation at 3500 g, the supernatants containing the DNA extracts were transferred to
156 a new 96-well PCR plate and a subsample was diluted to obtain a final concentration of 50 ng μl^{-1}
157 before storing at -20°C.
158
159 **Primer selection**
160 All the DNA extracts from presumptive *Vibrio* strains were tested by real-time PCR
161 SYBR Green (Invitrogen Kit, Fischer Scientific SAS, Ilkirch Graffenstaden, FRANCE) using
162 primers selected in the 16S rDNA region specific for the *Vibrio* genus (Thompson *et al.* 2004)
163 (Table 2). All the strains positive for *Vibrio* spp. were further tested to determine the species
164 level. *Vibrio parahaemolyticus* was identified using a TaqMan real-time PCR targeting the *toxR*
165 gene developed by Hervio-Heath *et al* (in process of publication). *Vibrio cholerae*, *V. vulnificus*

and *V. alginolyticus* were identified using primers selected on the *dnaJ* gene (a housekeeping gene encoding for a heat shock protein 40) designed by Nhung *et al.* (2007a) for a multiplex PCR to identify clinical *Vibrio* strains. In a previous study, Nhung *et al.* determined this gene as being a powerful phylogenetic marker for *Vibrio* species identification (Nhung *et al.* 2007b). The universal forward primer VM-F (5'-GGCGTAAAGCGCATGCAGGT-3') was common to the three species and the reverse primer was designed specifically for each species. They were named respectively as VC-Rmm (5'-AGCAGCTTATGACCAATACGCC-3'), VV-Rmm (5'-GTACGAAATTCTGACCGATCAA-3') and V.al2-MmR (5'-GATCGAAGTRCCRACACTMGGA-3'). The multiplex PCR assays were performed according to the conditions described by Nhung *et al* (2007a). The amplification reactions contained 1X PCR buffer (Roche Diagnostics, Meylan, France), 0.2 mmol l^{-1} of each deoxyribonucleotide triphosphate, 0.1 U Taq polymerase (Roche Diagnostics, Meylan, France), 0.4 μmol l^{-1} of each primer (Eurogenetec, Seraing, Belgium) and 1 μl of the template in a final reaction volume of 20 μl. The thermal program consisted of a 3 min initial denaturation step at 94°C, followed by 35 cycles at 94°C for 30 s, 60°C for 30 s and 72°C for 1 min, and a final elongation step of 7 min at 72°C. In these conditions, the multiplex PCR did not work for the positive controls *V. alginolyticus* IF Va11, *V. vulnificus* IF Vv10, *V. cholerae* IF Vc10 or for any of the environmental strains isolated at 22°C and 37°C (data not shown). However, the three primer sets were selected and assay conditions were optimized towards standardisation of a real-time PCR assay for each target species.

Determination of the optimum annealing temperature by thermal gradient PCR for *V. cholerae, V. vulnificus* and *V. alginolyticus*

A specific adaptation has been performed from the initial protocol of multiplex PCR designed by Nhung *et al*. (2007a) including the thermal cycles and particularly, the annealing

190 temperature, in order to improve the sensitivity and the specificity of the amplification. For this
191 purpose, a thermal gradient PCR from 50 to 65°C was tested to determine the optimum annealing
192 temperature for each pair of primers using the gradient function of the Thermo Cycler (PTC-200,
193 Biorad, France). Four different *Vibrio* strains representing four different species including the
194 target (DNA concentration at 50 ng μl^{-1}) and a negative control (water used for the PCR mix)
195 were tested for each PCR. The PCR mix and the thermal cycle program were the same as the
196 ones described by Nhung *et al* (2007a) with the exception of annealing temperatures. The thermal
197 cycle program was as follows: a 3 min initial denaturation step at 94°C, followed by 35 cycles of
198 amplification, with a denaturation step at 94°C for 30 s followed by an annealing step with
199 temperature varying from 50°C to 65°C (through twelve wells per strain) for 30 s, and 72°C for 1
200 min, and a final elongation step of 7 min at 72°C. The amplified products were examined using
201 1.5% (w/v) agarose gel (Eurobio, Courtaboeuf, France) and 500 μg ml^{-1} ethidium bromide
202 (SIGMA-ALDRICH®, Saint-Quentin Fallavier France) staining in 1X Tris-EDTA-Borate
203 electrophoresis buffer (SIGMA-ALDRICH®, Saint-Quentin Fallavier, France). The optimal
204 annealing temperature was determined by selecting the temperature yielding the fragment length
205 expected for the target species only and no amplicon or a non-specific amplicon for others
206 species.
207
208 **Real-time PCR amplification conditions**
209 The transfer of the reaction conditions from the multiplex PCR described by Nhung *et al.*
210 (2007a) to the SYBR Green real-time PCR system induced to adapt the nature and the
211 composition of the PCR mix (primer and magnesium chloride concentrations, SYBR Green real-
212 time PCR SuperMix-UDG and buffer volume). Then, real-time PCR amplifications were run in a
213 25-μl volume containing 0.5 μmol l^{-1} of each primer (0.26 μmol l^{-1} for *VV*-Rmm); 2 mmol l^{-1}

214 $MgCl_2$; 2X Platinum® SYBR Green real-time PCR SuperMix-UDG (Invitrogen™, Life
215 Technologies, Carlsbad, CA, USA), 8.25 μl of RNase DNase Protease free water (8.5 μl for *V.*
216 *vulnificus* real-time PCR; 5 PRIME, Hamburg, Germany) and 2 μl of a 50 ng μl^{-1} DNA extract or
217 2 μl of RNase DNase Protease free water for negative control. Real-time PCR thermal cycling
218 was run using the Stratagene® MX3000P cycler (Agilent Technologies, Santa Clara, CA, USA).
219 The thermal program for *Vibrio* spp (Table 3) consisted in a 10 min denaturation step at 95°C,
220 followed by 40 cycles of amplification as follows : at 95°C for 5 s, 58°C for 5 s and 72°C for 4 s.
221 The program ended with a final dissociation curve analysis consisting in denaturation at 95°C for
222 1 min, annealing at 58°C for 30 s and a final denaturation at 95°C for 30 s. The thermal program
223 for *V. cholerae* and *V. vulnificus* (Table 3) consisted in a 3 min denaturation step at 95°C ,
224 followed by 40 cycles of amplification, at 95°C for 30 s, 63°C (61°C for *V. vulnificus*) for 30 s
225 and 72°C for 1 min. The program ended with a final dissociation curve analysis consisting in
226 denaturation at 95°C for 1 min, annealing at 63°C (61°C for *V. vulnificus*) for 30 s and a final
227 denaturation at 95°C for 30 s.

228 The fluorescence signal was measured in real time at the end of the elongation step of
229 every PCR cycle to monitor the amount of amplified DNA. A standard curve was calculated for
230 each real-time PCR using 100 ng of purified DNA extract from a representative strain of each
231 target species IF Vc14, IFVv22 and IF Va34, respectively, serially diluted in duplicate (ten-fold
232 dilution) six times. A negative control was prepared in duplicate for each real-time PCR assay
233 with 2 μl of the water used for the real-time PCR mix. Threshold was manually set using the
234 MxPro software (Stratagene 2007). Together with the dissociation curve analysis, the SYBR
235 Green system (Giglio *et al.* 2003) provides an excellent tool for specific product identification
236 and quantification. The dissociation curve led to discriminate the true positive from the false
237 positive amplifications. The latter were characterized by the presence of a fluorescence peak at a

different dissociation temperature (± 1°C) than that observed for the standard (see real-time PCR *V. cholerae* for September 2009 environmental strains as an example in Figure 1).

Results

Determination of the optimum annealing temperature

The optimization step through the temperature gradient assays led to select an annealing temperature of 63°C for *V. alginolyticus* and *V. cholerae* and of 61°C for *V. vulnificus* with an expected amplicon size at 144 bp, 375 bp and 412 bp, for each target species, respectively. At these temperatures, no amplification was observed for the *Vibrio* species other than the target ones (see example given for *V. cholerae* in Figure 2).

Specificity of the real-time PCR

The inclusivity and exclusivity tests for the real-time PCR targeting *V. alginolyticus*, *V. vulnificus* and *V. cholerae* proved to be specific for each target species (Table 4). For the inclusivity test, all the *V. alginolyticus* strains (11/11) generated a unique dissociation curve peak at 85°C (± 1°C), the *V. vulnificus* strains (10/10) a unique dissociation curve peak at 89°C (± 1°C) and the *V. cholerae* strains (12/12) a unique dissociation curve peak at 88.5°C (± 1°C). Concerning the exclusivity test, no Ct values were recorded or false positives were determined according to the final dissociation curve analysis for the non target *Vibrio* species or the non-*Vibrio* strains. The real-time PCR systems validated for *V. alginolyticus*, *V. vulnificus* and *V. cholerae* were then used to identify the natural diversity of a high panel of environmental strains.

Identification of the environmental bacterial strains

For strain identification, the real-time PCR were performed successively, starting with 16S rDNA, to confirm the genus *Vibrio*, and following with specific real-time PCR for *V. alginolyticus* (Va), *V. parahaemolyticus* (Vp), *V. vulnificus* (Vv) and *V. cholerae* (Vc). In September 2009, 961 bacterial strains presenting typical phenotypic traits of the *Vibrio* genus on TCBS (saccharose-positive/yellow colonies or saccharose-negative/green colonies) were isolated and conserved. Among them, 446 (46%) had been isolated at 22°C (381 from seawater and 65 from superficial sediments) and 515 (54%) at 37°C (375 from seawater and 140 from superficial sediments). The majority of the strains (99.6%, 957/961) was confirmed as belonging to the genus *Vibrio* (Table 5). Previous data obtained in the same sampling area showed that *V. alginolyticus* was the most frequently isolated *Vibrio* species at 37°C (Antajan *et al*. 2010). Based on these data, bacterial strains were further identified starting with the real-time PCR targeting *V. alginolyticus*. Among the isolated strains, *V. alginolyticus* was also the most frequently detected *Vibrio* species at 37°C in this study with 423/512 of the *Vibrio* spp (82.6%), *i.e*. 76.6% and 89.4% in T1 and T2, respectively (Table 5). At 22°C, only 7 of the 445 *Vibrio* spp. strains (1.6%) were identified as *V. alginolyticus*. The species *V. parahaemolyticus* and *V. cholerae* were detected at 37°C, 2 strains on T1 and 2 strains on T2, respectively. *V. vulnificus* was not detected in any of the transects. The real-time PCR methodology including the four most detected *Vibrio* species isolated at 37°C in French coastal waters, led to rapidly identify almost all the strains isolated at this temperature with 427/512 *Vibrio* spp strains identified (83.4%). On the contrary, the majority of the strains isolated at 22°C (98.4%) was not identified when using the same methodology.

Discussion

Since the 1980s, a significant warming was highlighted in the Northern seas (Southward *et al.* 2005; Saulquin and Gohin 2010; Smyth *et al.* 2010; Lima and Wethey 2012). The open systems of the English Channel and the North Sea, with large available datasets of measurements, represent areas of interest to study the impact of the global changes in marine ecosystems and the emergent issues for human (Southward *et al.* 1995; Hawkins *et al.* 2003; Philippart *et al.* 2011; Vezzulli *et al.* 2012). As recent cases of vibriosis have been described (Vezzulli 2012) and *Vibrio* spp. isolated in North European seas (Eiler *et al.* 2006; Oberbeckmann 2011), studies of *Vibrio* populations in these areas will provide a better view of their evolution and adaptation face to global changes. Moreover, the incidence of vibriosis on human health implies the need to better understand the dynamics of co-occurring *Vibrio* population and their diversity, and not only to focus on a single member (Thompson *et al.* 2004). This means to regularly characterize the *Vibrio* populations in the environment, through a sufficient amount of strains isolated and associated with environmental parameters measurement, to determine the *Vibrio* ecology drivers. Most of the ecological studies on *Vibrio* populations follow this approach (Harriague *et al.* 2008; Oberbeckmann *et al.* 2010; Vezzulli *et al.* 2010). However, in most ecological studies (1-, 2- or 3-year study) involving evaluation of vibrios diversity and occurrence, the amount of strains isolated and identified is limited (below 300 strains per year) and this is primarily because of the use of labour intensive bacteriological methods (Barbieri Elena *et al.* 1999; Eilers *et al.* 2000; Croci *et al.* 2001; Hervio-Heath *et al.* 2002; Deter *et al.* 2010a; Rodriguez-Castro *et al.* 2010). This could be questionable concerning the representation of the *Vibrio* diversity in the environment considering the number of strains isolated especially for studies focusing not only on the potentially pathogenic *Vibrio* but also on the total *Vibrio* community. In the perspective of an accurate monitoring of the vibriosis risk, the direct detection and quantification of potential pathogenic *Vibrio* species on environmental DNA through real-time PCR assays would constitute

308 a fast and efficient tool. However, in such studies the isolation of bacterial strains from the
309 environment remained necessary to determine first the *Vibrio* culturable diversity and the
310 potentially pathogenic strains associated. In the present study, we proposed an analysis strategy
311 to isolate and identify a large number of *Vibrio* strains, including potentially pathogenic isolates
312 within long-term ecological studies. In the perspective of a large number of strains to be
313 identified from each sampling campaign (4274 strains were isolated from September 2009 to
314 September 2011), it was necessary to use a reliable and least labor-intensive sample treatment
315 and identification method to rapidly screen strains isolated from the environment. For that
316 purpose, we firstly optimized a DNA extraction method in a 96-well format that proved to be
317 reliable and robust for high-throughput studies. Secondly, we needed to design real-time PCR
318 assays to identify these strains at the species level. The multiplex PCR published by Nhung *et al.*
319 (2007a) and targeting five human pathogenic vibrios was particularly interesting in this
320 perspective. The *dnaJ* gene was shown to be a promising phylogenetic marker for the
321 identification of *Vibrio* species, particularly for human pathogenic *Vibrio* species (Nhung *et al.*
322 2007b). Nhung and coworkers (2007a) designed this multiplex PCR in order to get a fast
323 identification tool for strains isolated from clinical stool samples. However, when tested, it failed
324 to identify the bacterial strains isolated from the environment. Hence, each pair of primers
325 designed on the *dnaJ* gene for *V. alginolyticus*, *V. vulnificus* and *V. cholerae* was used separately
326 to develop real-time PCR targeting each of these species. The real-time PCR assays designed for
327 *V. alginolyticus*, *V. vulnificus* and *V. cholerae* were found to demonstrate specificity. This real-
328 time PCR methodology optimized in this study and the real-time PCR developed previously for
329 *V. parahaemolyticus* (Hervio-Heath *et al*, work unpublished yet) led to identify the great majority
330 of the strains isolated at 37°C with 427 strains identified among the 512 isolated *Vibrio* spp
331 strains (83.4% *Vibrio* spp identified). *Vibrio alginolyticus*, an opportunistic human pathogen

frequently involved in ear and wound infections, was the most frequently detected species from the strains isolated at 37°C with 423 strains among 512 *Vibrio* spp strains isolated (82.6%). The reported cases of vibriosis due to *V. alginolyticus* are sporadic and concern mostly susceptible population with an immune system compromised by various pathologies. Extracellular protease and collagenase have been implicated as possible factors eliciting diseases in humans, yet their precise role in pathogenicity is unclear (Tantillo *et al.* 2004). Previous studies reported possible pathogenic gene transfers between *V. cholerae*, *V. parahaemolyticus* and *V. alginolyticus* (Xie *et al.* 2005; Gonzalez-Escalona *et al.* 2006). This highlights the interest to study *V. alginolyticus* in the environment even if, to date, these strains have not been associated to strains isolated from clinical cases. The results obtained in this study confirmed previous studies performed in European coastal waters, from the Adriatic Sea to the North Sea, which shown the predominance of this species among isolates at 37°C (Carli *et al.* 1993; Barbieri Elena *et al.* 1999; Hervio-Heath *et al.* 2002; Schets *et al.* 2006; Cooke and Shapiro 2007; Antajan *et al.* 2010; Oberbeckmann 2011). The protocol developed and standardized in this study was reliable and suitable for strains isolated at this temperature. However, this methodology has shown its limits for the analysis of the diversity at 22°C. Only 1.6% of the strains isolated at 22°C in September 2009 were characterised by real-time PCR and they were all identified as *V. alginolyticus*. This indicates that species other than *V. alginolyticus*, *V. parahaemolyticus*, *V. vulnificus* and *V. cholerae* might be predominant at this temperature and that the potentially pathogenic vibrios for human may represent a small part of the total *Vibrio* community in the area. Some authors highlighted the predominance of the *Vibrio* species belonging to the *V. splendidus* group on coastal seawater and sediment particularly in temperate regions (Sobecky *et al.* 1998; Urakawa *et al.* 1999; Radjasa *et al.* 2001; Le Roux and Austin 2006; Hunt *et al.* 2008; Vezzulli *et al.* 2010). In the perspective of this two-year ecological study starting in September 2009, we will perform a partial sequencing

of housekeeping genes, as described by many authors, to further identify the remaining non-identified strains isolated at 22°C or at 37°C during contrasting sampling campaigns. Moreover, simultaneous recording of environmental parameters would allow to determine any potential links between these parameters and occurrence of potentially pathogenic environmental *Vibrio* spp.. Such field approaches and the further exploration of the strains collected in our study could lead to better understand the real distribution of potentially pathogenic isolates in the environment and their connections with their habitat parameters and the others *Vibrio* species.

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Table 1 List of bacterial strains used for real-time PCR tests of specificity

Strains	Source	Code	Origin
IF Vp1	CIP Paris	CIP 73.30	Oyster
IF Vp2	CIP Paris	CIP 75.2T	Type
IF Vp18, 22, 69, 143	Ifremer/RBE/EMP/MIC	*	Mussel
IF Vp27, 31, 48, 71	Ifremer/RBE/EMP/MIC	*	Water
IF Va5, 22, 23, 30	Ifremer/RBE/EMP/MIC	*	Water
IF Va11, 24, 25, 26, 27	Ifremer/RBE/EMP/MIC	*	Mussel
IF Va33	IPIMAR	*	*
IF Va34	CEFAS	V05/007	NCTC Reference
IF Vv1	CNRVC Paris	970061	Water
IF Vv8	CNRVC Paris	ATCC 27562	Type
IF Vv10	Ifremer/RBE/EMP/MIC	*	Mussel
IF Vv13,14,18, 22, 35, 39, 41	Ifremer/RBE/EMP/MIC	*	Water
IF Vc1	CNRVC Paris	IP930177	Environment
IF Vc10, 14	Ifremer/RBE/EMP/MIC	*	Mussel
IF Vc15, 16, 17, 19, 20, 22, 23	Ifremer/RBE/EMP/MIC	*	Water
IF Vc29	EQA	*	*
IF Vc33	Ifremer/RBE/EMP/MIC	*	Cockle
IF Vm2, 3, 4	Ifremer/RBE/EMP/MIC	*	Water
IF Vm5	EQA	*	*
V. harveyi	CIP Paris	CIP 103192	*
V. natriegens	CIP Paris	CIP 103193	*
V. orientalis	CIP Paris	CIP 102891	*
V. pomeroyi	*	LMG 21352	*
V. crassosstreae	*	*	*
V. tubiashii	CIP Paris	CIP 102760	*
V. corallilyticus	CIP Paris	ATCC CIP 107925	*
V. gigantis	*	ATCC LGP13T	*
V. campbelli	CIP Paris	CIP 75.1	*
Escherichia coli	Ifremer/RBE/EMP/MIC	ATCC 76.24	*
Shewanella putrefaciens	Ifremer/RBE/EMP/MIC	*	Water
Proteus vulgaris	IUT Brest	*	*
Salmonella enteridis	Ifremer/RBE/EMP/MIC	68	Oyster
Klebsiella pneumoniae	CIP Paris	82.91T	*
Citrobacter frundii	Ifremer/RBE/EMP/MIC	Cf1	*

*not communicated; IF: Ifremer; Vp: *Vibrio parahaemolyticus*; Va: *Vibrio alginolyticus*; Vv: *Vibrio vulnificus*; Vc: *Vibrio cholerae*; Vm: *Vibrio mimicus*. Ifremer/RBE/EMP/MIC: Institut Français de Recherche sur l'Exploitation de la Mer (France)/Département Ressources Biologiques et Environnement/ Unité Environnement Microbiologie et Phycotoxines/ Laboratoire de Microbiologie ; IPIMAR/INRB : Portugese National Marine Fisheries and Aquaculture Research Institute and the National Institute of Biological Resources (Portugal);

CIP Paris: Collection de l'Institut Pasteur Paris (France); CEFAS : Center for Environment, Fisheries & Aquaculture Science (UK) ; CNRVC : Centre National de Référence des Vibrions et du Choléra, Institut Pasteur, Paris (France); EQA : External Quality Assessment Services (UK) ; IUT Brest : Institut Universitaire de Technologie Brest (France) ; Mussel: *Mytilus edulis* ; Oyster : *Crassostrea gigas* ; Cockle : *Cerastoderma edule*.

Table 2 Sequences of the primers used for *Vibrio* spp. and each targeted *Vibrio* species

Species	Target gene	Primers	Sequence (5′→ 3′)	Amplicon size (bp)
Vibrio spp.	*16S rDNA* [1]	567F 680R	GGCGTAAAGCGCATGCAGGT GAAATTCTACCCCCCTCTACAG	120
V. alginolyticus	*dnaJ* [2]	VM-F V.al2-MmR	CAGGTTTGYTGCACGGCGAAGA GATCGAAGTRCCRACACTMGGA	144
V. vulnificus	*dnaJ* [2]	VM-F VV-Rmm	CAGGTTTGYTGCACGGCGAAGA GTACGAAATTCTGACCGATCAA	412
V. cholerae	*dnaJ* [2]	VM-F VC-Rmm	CAGGTTTGYTGCACGGCGAAGA AGCAGCTTATGACCAATACGCC	375

[1] (Thompson *et al.* 2004) [2] (Nhung *et al.* 2007a)

Table 3 Real-time PCR programs for each target *Vibrio* species

Real-time PCR cycling conditions	*Vibrio* spp.	*V. alginolyticus* *V. cholerae*	*V. vulnificus*
Denaturation step	95°C 10 min	95°C 3 min	95°C 3 min
40 amplification cycles	95°C; 5 sec 58°C; 5 sec 72°C; 4 sec	95°C; 30 sec 63°C; 30 sec 72°C; 1 min	95°C ; 30 sec 61°C ; 30 sec 72°C ; 1 min
Dissociation curve cycle	95°C ; 1 min 58°C ; 30 sec 95°C ; 30 sec	95°C ; 1 min 63°C ; 30 sec 95°C ; 30 sec	95°C ; 1 min 61°C ; 30 sec 95°C ; 30 sec

Table 4 Specificity of real-time PCR tests developed for *V. alginolyticus*, *V. vulnificus* and *V. cholerae*

Test	Bacterial species (number of strains)	Real-time PCR (n/n) Va	Vv	Vc
Inclusivity	*V. alginolyticus* (11)	11/11		
	V. vulnificus (10)		10/10	
	V. cholerae (12)			12/12
Exclusivity	*V. alginolyticus* (11)	NT	-	-
	V. vulnificus (10)	-	NT	-
	V. cholerae (12)	-	-	NT
	V. parahaemolyticus (10)	-	-	-
	V. mimicus (4)	-	-	-
	V. harveyi (1)	-	-	-
	V. natriegens (1)	-	-	-
	V. orientalis (1)	-	-	-
	V. pomeroyi (1)	-	-	-
	V. crassosstreae (1)	-	-	-
	V. tubiashii (1)	-	-	-
	V. corallilyticus (1)	-	-	-
	V. gigantis (1)	-	-	-
	V. campbelli (1)	-	-	-
	Escherichia coli (1)	-	-	-
	Shewanella putrefaciens (1)	-	-	-
	Proteus vulgaris (1)	-	-	-
	Salmonella enteridis (1)	-	-	-
	Klebsiella pneumonia (1)	-	-	-
	Citrobacter frundii (1)	-	-	-
	Total number of strains tested	51	52	50

NT: Not Tested; n/n: number of strains positive in real-time PCR among the number of strains that were tested; -: negative in real-time PCR

Table 5 Strains identification using real-time PCR

Isolation temperature		22°C		37°C	
Transect		T1	T2	T1	T2
Vibrio spp.	Presumptive strains on TCBS	214	232	275	240
	Confirmed strains by rt PCR	213	232	274	238
Number of identified strains for each species	*V. alginolyticus*	0	7	210	213
	V. parahaemolyticus	NP	NP	2	0
	V. cholerae	NP	NP	0	2
	V. vulnificus	NP	NP	0	0
Non identified *Vibrio* spp		213	225	62	23
Mean % of non identified *Vibrio* spp		98.4%		16,6%	

NP: Not Performed

Figure 1 Example of a real-time PCR dissociation curve (*V. cholerae*) showing the case of true positive (9E7) and false positive strains (9A8, 9D7, 9E3, 9E12). A *V. cholerae* standard showed Ct values from 16 to 33 cycles for concentration ranging from 50 ng to 500 fg μl^{-1} respectively (data not shown). The dissociation temperature was estimated at 88.5°C ± 1°C (red bar) for the standard (IF Vc14). The environmental strain 9E7 showing a fluorescence peak at 87.7°C was determined as a true positive. Environmental strains 9A8, 9E3, 9D7, 9E12 showing fluorescence peaks at dissociation temperatures different from the standard (83, 80.5, 80 and 78°C respectively) were determined as false positives.

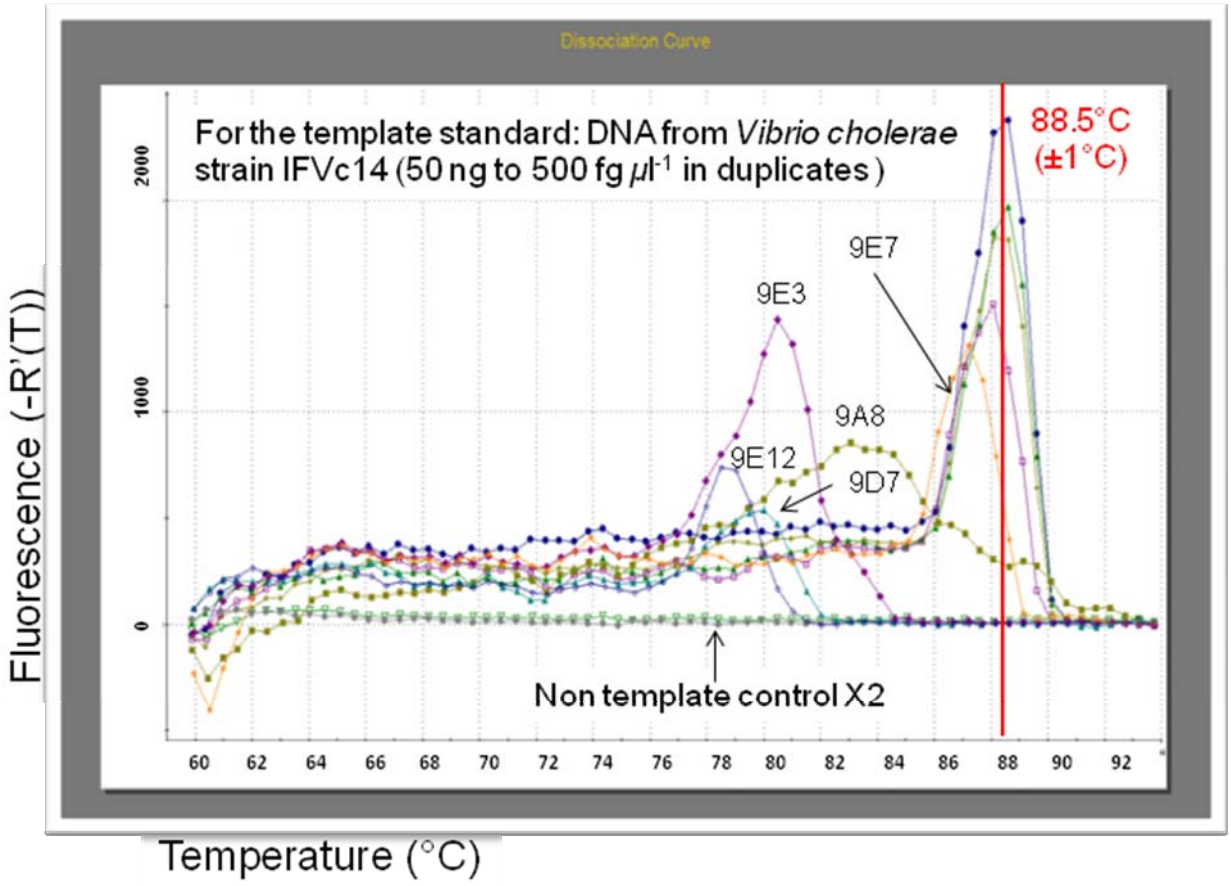
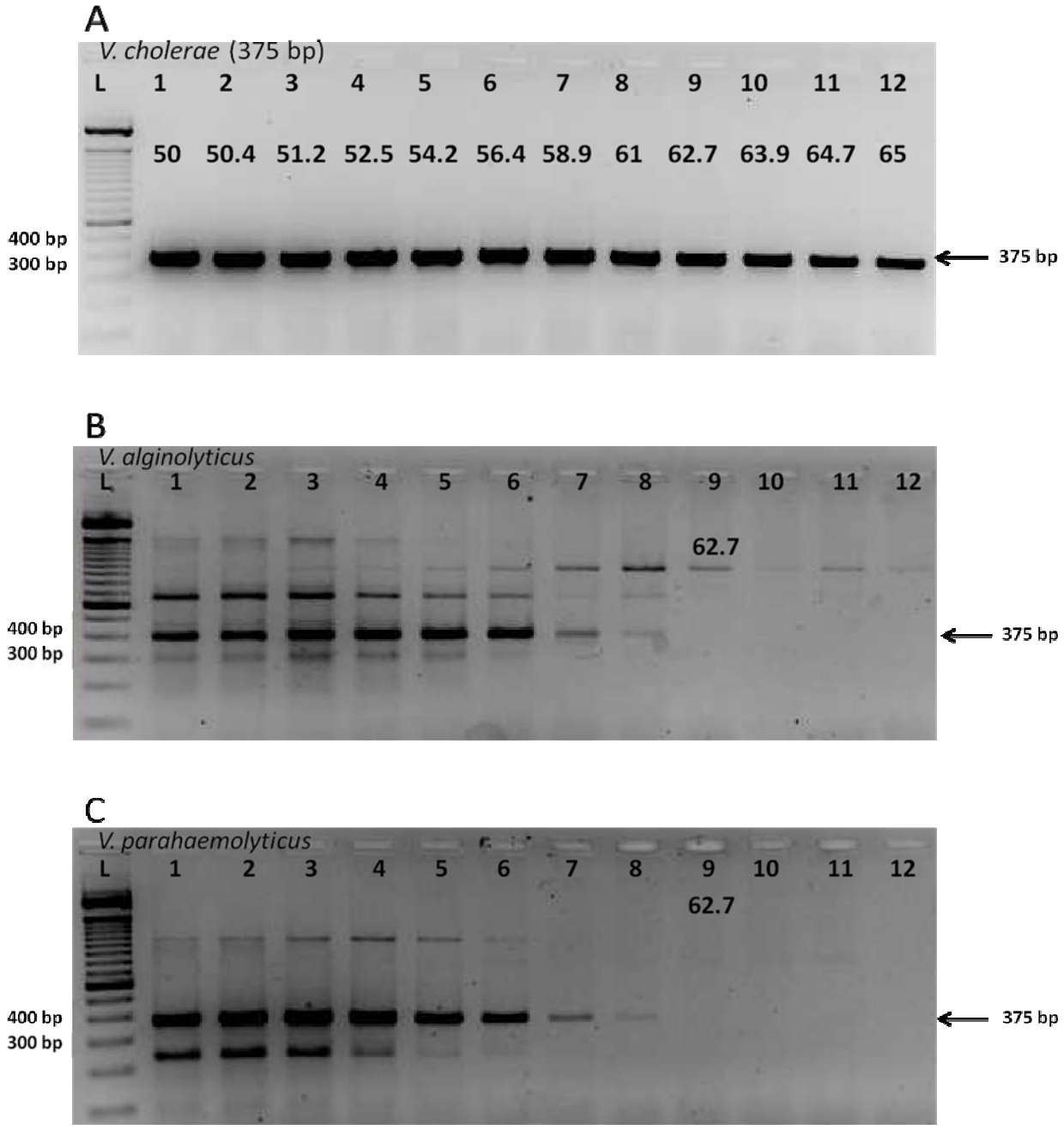
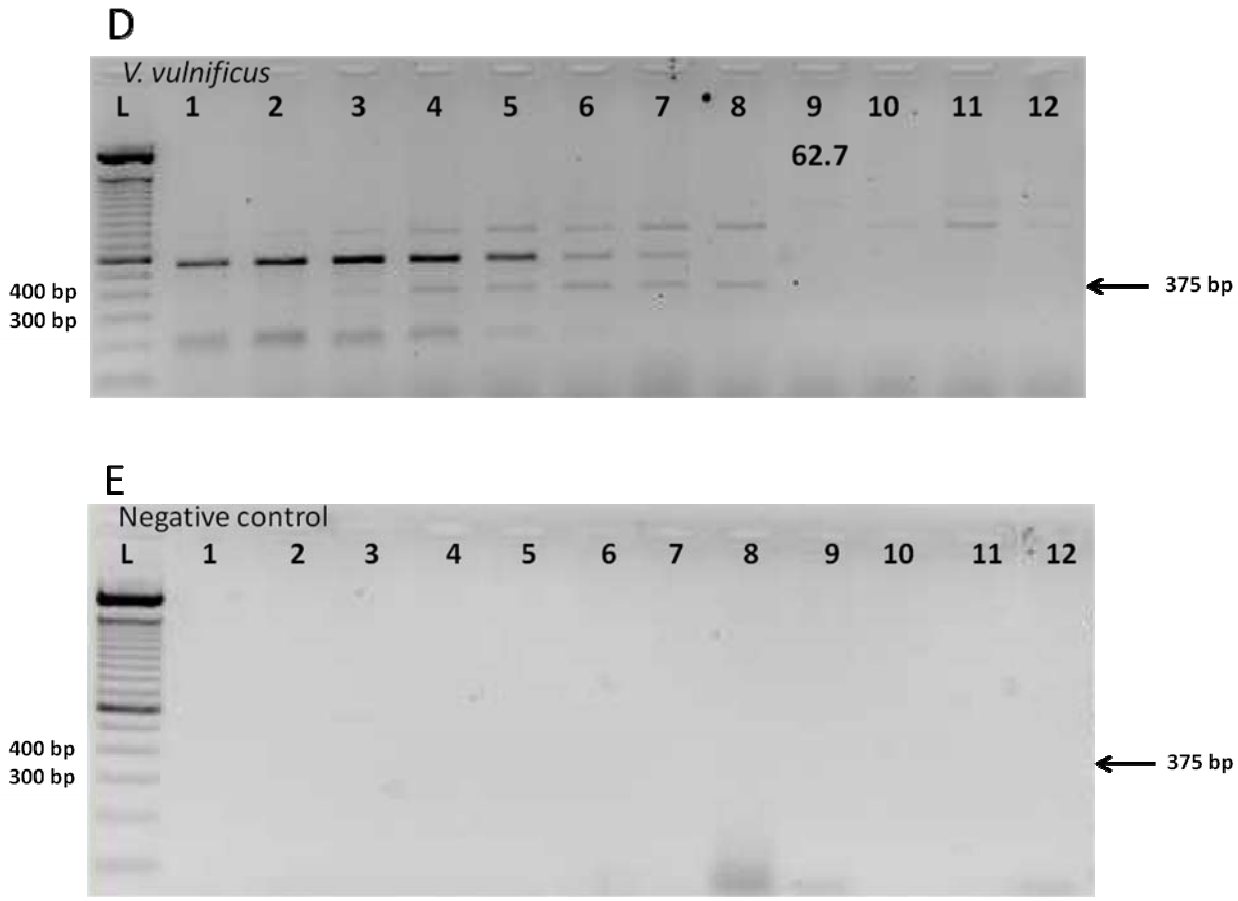


Figure 2 Experimental determination of optimal annealing temperature for *V. cholerae* PCR primers (*dnaJ* gene). Four strains were tested with *V. cholerae* primers *V. cholerae* IF Vc14 (A), *V. alginolyticus* IF Va34 (B), *V. parahaemolyticus* IF Vp2 (C), *V. vulnificus* IF Vv22 (D) and a negative control (E). Lanes 1 to 12 corresponded to annealing temperatures of 50, 50.4, 51.2, 52.5, 54.2, 56.4, 58.9, 61, 62.7, 63.9, 64.7 and 65°C, respectively; lane L, 100-bp DNA ladder (Invitrogen™, Life Technologies, Carlsbad, CA, USA).




D
V. vulnificus
L 1 2 3 4 5 6 7 8 9 10 11 12
62.7
400 bp
300 bp
375 bp
E
Negative control
L 1 2 3 4 5 6 7 8 9 10 11 12
400 bp
300 bp
375 bp