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**Evaluation of different species-specific PCR protocols for the detection  
of *Vibrio tapetis***

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Submitted to: **Journal of Invertebrate Pathology**, January 2011

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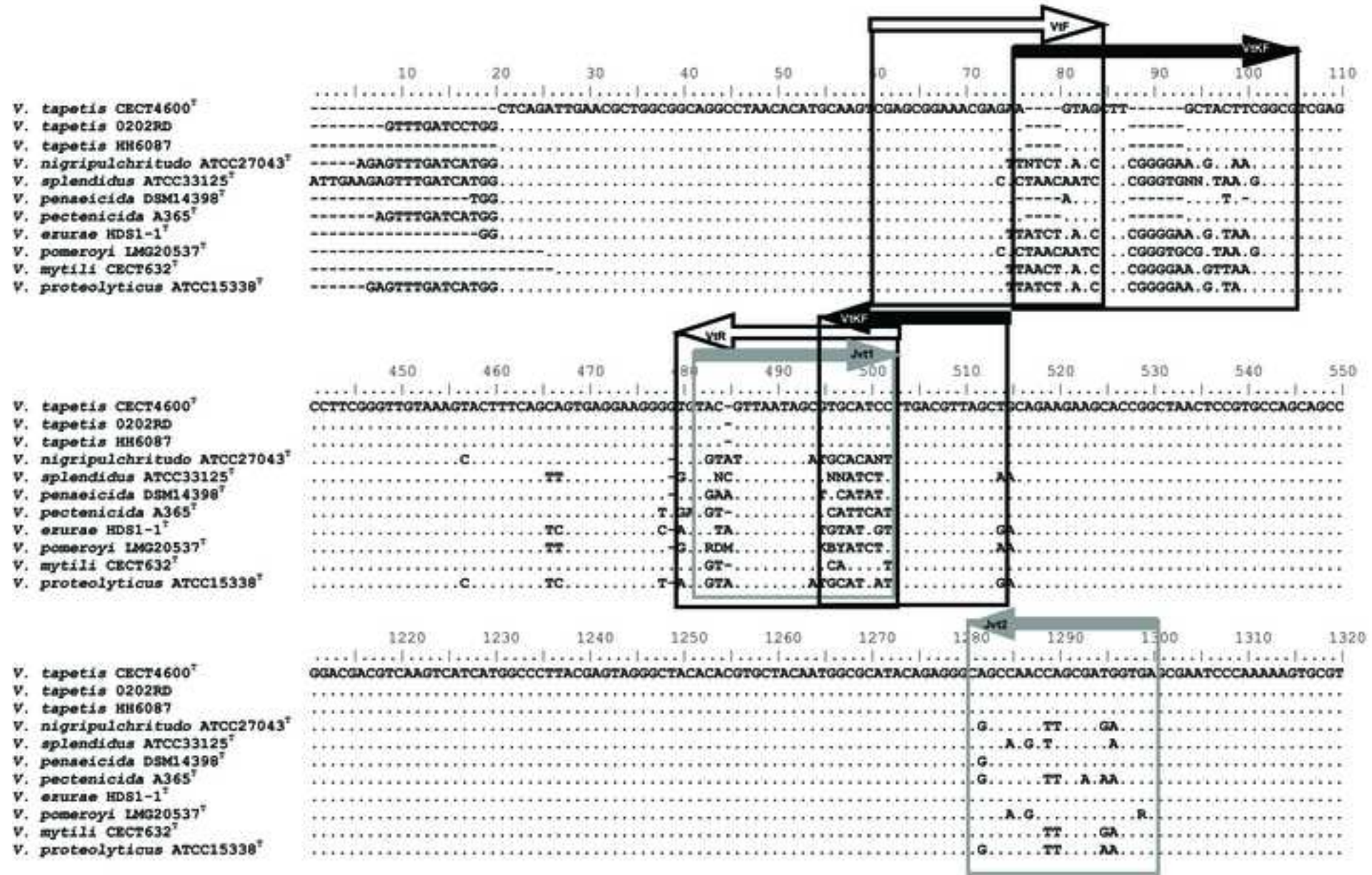
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### **Graphical abstract**

Alignment of the 16S rRNA gene sequences of the three strains of *V. tapetis*, CECT4600<sup>T</sup> (NR026361) GR0202RD (FR797810) and HH6087 (AY800101), representatives of the different genetic groups described within this bacterial species, and the sequences of the *Vibrio* species with cross-reactivity in the PCR protocols using primer pairs VtF-VtR and VtKF-VtKR. The correspondences among the sequences explain why these primer-pairs yield positive amplification with non-target *Vibrio* species.



*Highlights:*

*V. tapetis* is a fastidious bacterium difficult to detect and/or isolate.

A comparative evaluation of the performance of three PCR protocols developed for the detection of this pathogen.

Only one protocol showed to be specific for *V. tapetis*, yielding also a good limit of detection (2-20 cells), and was therefore, proposed as the more adequate for diagnosis of Brown Ring Diseases in clams.

The low specificity of the other two protocols can be explained on the basis of primer design.

34 **Abstract**

35 In this study the specificity and sensitivity of three primer pairs, Jvt1-Jvt2, VtF-  
36 VtR and VtKF-VtKR, for the detection of *Vibrio tapetis* were evaluated in parallel  
37 using 23 *V. tapetis* strains isolated from different mollusc and fish species and with  
38 different geographical origin, as well as 29 representatives of related *Vibrio* species. The  
39 three primer pairs amplified all the *V. tapetis* strains, regardless their host or  
40 geographical origin. However, with primer sets VtF-VtR and VtKF-VtKR amplification  
41 products of the expected size were obtained from chromosomal DNA of some of the  
42 non-*V. tapetis* bacteria tested. The sensitivity of the three PCR detection methods was  
43 also different. The detection limit obtained with primer pairs Jvt1-Jvt2 and VtF-VtR  
44 was between 1 and 10 pg DNA /PCR tube (2-20 bacterial cells per reaction). The primer  
45 set VtKF-VtKR showed a reduction of sensitivity in at least one order of magnitude.  
46 The results were highly reproducibly with all primer sets when using the same thermal  
47 cyclers, although some differences were observed in the results obtained in different  
48 PCR machines. Based on the findings reported here, we propose the Jvt1-Jvt2 PCR  
49 protocol as the most adequate for an accurate detection of *V. tapetis* in diagnostic  
50 pathology as well as in epidemiological studies of this clam pathogen.

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52

53 *Keywords:* Brown ring disease (BRD); *Vibrio tapetis*; PCR-detection; PCR  
54 performance.

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## 57 **1. Introduction**

58 Brown Ring Disease (BRD), caused by *Vibrio tapetis* (Borrego et al., 1996), is an  
59 epizootic infection described in adult clams. The main sign characterizing the disease is  
60 a brown conchiolin deposit on the inner surface of the valves, typically located between  
61 the pallial line and the edge of the shell. This organic deposit perturbs the calcification  
62 process (Paillard et al, 1994, Paillard, 2004) causing severe deformations of the clam's  
63 shell and subsequently the death of the animal.

64 Identification of this shellfish pathogen is based on the study of its phenotypical and  
65 antigenic characteristics. However, biochemical identification of *V. tapetis* strains  
66 involve the isolation of the microorganism from affected clams. This methodology is  
67 timeconsuming given the very fact that incubation periods range from 24h to 7 days.  
68 (Bergh et al., 2007; Castro et al., 1997; Jensen et al., 2003; Novoa et al., 1998; Reid et  
69 al., 2003). Once pure cultures are achieved, identification of the pathogen is based  
70 mainly on four biochemical characteristics: growth on TCBS, non utilization of sucrose,  
71 inability to grow above 27 to 30°C (varying among authors), and lack of acid production  
72 from mannitol (Paillard, 2004). It has been often described that classical methods may  
73 fail in the detection of *V. tapetis* or that, although being detected by indirect procedures  
74 (i.e. immunofluorescence), the pathogen could not be isolated on culture media (Castro et  
75 al., 1992, 1995). This methodology has been proved to be unsuccessful in some  
76 geographical areas as in Southwest of Spain where, although the incidence of the  
77 disease is nearly 40%, the isolation of the etiological agent was impossible (Castro et al,  
78 1992, 1997)

79 In the recent years there has been much interest in the development of specific PCR  
80 protocols based, most of them, on the amplification of 16S rRNA genes for detection  
81 bacterial fish and shellfish pathogens (Beaz-Hidalgo et al., 2008; Brown et al., 1994;  
82 Del Cerro et al., 2002; Gonzalez et al., 2003; Lee et al., 1998; Nhung et al., 2007;  
83 Osorio et al., 1999; Romalde and Toranzo 2002; Romalde et al., 2004; Saulnier et al.,  
84 2000). These methods have proved to be very useful to improve the detection, not only  
85 in acute cases of infection but also from asymptomatic carrier organisms.

86 In the last years three PCR protocols for detection of *V. tapetis* have been developed  
87 (Paillard et al., 2006, Park et al., 2006; Romalde et al., 2007) based on a variable area of  
88 16S rDNA. In this work, we tested the specificity as well as the sensitivity of these three  
89 PCR protocols.

## 90 **2. Material and methods**

### 91 *2.1. Bacterial strains*

92 Bacterial strains used in the primer specificity studies are listed in Table 1 and 2. This  
93 collection comprises 23 *Vibrio tapetis* strains with different host and geographical  
94 origin, including representative strains of the three major genetic groups described for  
95 this pathogen (Rodríguez et al., 2006). In addition, 29 *Vibrio* species selected on the  
96 basis of 16S rRNA gene similarity with *V. tapetis* were also analyzed.

97 All the bacteria were routinely cultured on Marine Agar (MA) (Pronadisa, Madrid,  
98 Spain) and incubated for 24 hours at 25°C except for *V. tapetis* strains that were grown  
99 for 72h at 15°C. Stock cultures were stored at -70°C in Marine Broth (MB)(Pronadisa,  
100 Madrid, Spain) supplemented with 15% glycerol.

101

### 102 *2.2. DNA extraction*

103 Chromosomal DNA was extracted using InstaGene Matrix (BioRad, Madrid, Spain) as  
104 previously described by Romalde et al. (1999). Strains of *V. tapetis* and other *Vibrio*  
105 species were resuspended from the plates in 1 ml of tri-sterile distilled water,  
106 centrifuged at 12000 rpm for 1 min and the supernatant was removed. The pellets were  
107 resuspended in 200 µl of InstaGene Matrix and incubated for 30 min at 56°C. Then, the  
108 cell suspensions were vigorously vortexed and boiled in a water bath for 8 min. The  
109 lysates were mixed again at high speed and then centrifuged at 12000 rpm for 3 min.

110 The DNA concentration of each sample was spectrophotometrically (Lambda2 UV/VIS  
111 Spectrophotometer, Perkin Elmer, Überlingen, Germany) measured at 580 nm and  
112 adjusted to 1000 ng/µl. All DNA was maintained at -20°C until used for PCR reactions.  
113 All the experiments were carried out with DNA obtained from 3 different extractions  
114 for each bacterial strain.

115

### 116 *2.3. PCR amplification*

117 All PCR amplifications were performed with the commercial kit Ready-To-Go™PCR  
118 beads (Amersham Pharmacia Biotech, Little Chalfont, Buckinghamshire, England, UK),  
119 which included all the reagents needed for the PCR reactions except the specific primer  
120 pairs and DNA.

121 Primer pairs used for the comparison VtF-VtR, VtKf-VtKr and Jvt1-Jvt2, were  
122 previously described by Paillard et al. (2006), Park et al. (2006) and Romalde et al.  
123 (2007) respectively. All the primer pairs were designed on the basis of the 16S rRNA

124 gene, yielding amplification products of 416, 413 and 816 bp respectively. All PCR  
125 reactions were carried out in parallel in a T-Professional basic (Biometra, Goettingen,  
126 Germany) and an Uno Cycler (VWR, Barcelona, Spain) thermocyclers. PCR conditions  
127 and amplification cycles used for denaturation, primer annealing and extension were  
128 carried out according to each published PCR protocol.

129 Negative controls, consisting of the same reaction mixtures but with sterile distilled  
130 water instead of DNA template, were included in each batch of PCR reaction. The  
131 reproducibility of the results was assessed by repetition of the amplifications in at least  
132 3 independent PCR assays.

133

#### 134 *2.4. Analysis of PCR products*

135 Amplified products were detected by horizontal 1% (w/v) agarose gel electrophoresis  
136 for 60 min at 100 V in TAE 1x electrophoresis buffer, visualized using 0.06 µg ml<sup>-1</sup> of  
137 ethidium bromide (BioRad, Madrid, Spain) and photographed under UV light and  
138 computer digitized (Gel Doc 100, Bio-Rad). A 50 to 2000 bp ladder (Sigma-Aldrich,  
139 Saint Louis, MO, USA) was used as a molecular mass marker. The presence of a single  
140 product of the appropriate size, identical to the reference strains, was considered as a  
141 positive result.

142

#### 143 *2.5. Determination of PCRs sensitivity and specificity*

144 The detection limits of the three primer sets were evaluated using cultures of the type  
145 strain CECT 4600<sup>T</sup> grown until exponential phase on MB. Culture was centrifugated  
146 and resuspended on sterile saline (0.85% NaCl) adjusting turbidity to a OD=1 at 580 nm  
147 (Lambda2 UV/VIS Spectrophotometer. Perkin Elmer). DNA was extracted, adjusted to  
148 1000 ng/µl and serially diluted to 1 ag/µl. In each PCR tube, one µl of these dilutions  
149 was loaded.

150 For the study of the specificity with the 23 *V. tapetis* isolates and the representatives of  
151 the related 29 *Vibrio* species, DNA was adjusted to 1000 ng/µl and serially diluted to  
152 use 100 ng of DNA in each PCR reaction.

153 In both cases, PCR conditions and electrophoresis were the same as described above.

154

#### 155 *2.6. Sequence analysis*

156 In order to arise an explanation for the cross-reactivity observed for some primer sets,  
157 sequence analysis and multiple alignments were performed with the BioEdit package,

158 version 2.1, and the MEGA version 4.0 softwares (Tamura et al., 2007).  
159 Due to the lack of a 16S rRNA gene sequence for the strain GR0202RD in the  
160 GeneBank database, this gen was sequenced as previously described (Osorio et al.,  
161 1999) using a GenomeLab DTCS-Quick Start kit (Beckman Coulter). Sequence editing  
162 was performed with the DNASTAR Lasergene SEQMAN program. Ther obtained  
163 sequence was deposited in the GeneBank with accession number FR797810.

164

### 165 **3. Results**

#### 166 *3.1. Detection limit for the primer pairs*

167 The sensitivity of each species-specific PCR primer pairs were determined by  
168 amplification of different dilutions of DNA extracted from the *V. tapetis* type strain  
169 CECT 4600<sup>T</sup>.

170 With primer pairs VtF-VtR and Jvt1-Jvt2 and the T-Professional basic (Biometra)  
171 machine, the expected products of 416 and 816 bp respectively were obtained with  
172 samples containing as low as 1 pg of DNA per PCR tube (Fig. 1A and B), which  
173 corresponded to 2 to 20 cells per reaction (data not shown). When amplifications were  
174 performed in the Uno Cycler (VWR) apparatus, the same detection limit was achieved  
175 with the VtF-VtR primer set, being one log-unit less sensitive the primer pair Jvt1-Jvt2  
176 (Fig. 1A and B). The primer set VtKF-VtKR showed less sensitivity, being able to  
177 amplify 10 pg (Biometra apparatus) or 100 pg (VWR machine) of DNA (Fig. 1C),  
178 corresponding to 20 to 200 bacterial cells. The robustness of these results was  
179 determined by making these assays by triplicate, obtaining the same results in all cases.

180

#### 181 *3.2. Specificity study*

182 All *V. tapetis* isolates, regardless their geographical and host origin, were correctly  
183 identified by the three PCR protocols and primers pairs analyzed, rendering specific  
184 amplicons with the expected sizes of 816 (Jvt1-Jvt2), 416 (VtF-VtR) and 413 bp (VtKF-  
185 VtKR)(Table 1).

186 On the other hand, when DNA from the 29 related *Vibrio* species were subjected to  
187 amplification, different results were obtained depending on the primer set and the  
188 thermocycler employed. The best specificity was obtained using Jvt1-Jvt2, since no  
189 positive amplifications were achieved in any of the PCR apparatus (Table 2; Fig. 2A).  
190 With the primer set VtF-VtR, amplicons of the expected size (816 bp) were observed  
191 for *V. proteolyticus* ATCC 15338<sup>T</sup>, *V. ezuriae* DSM 17533<sup>T</sup>, *V. nigripulchritudo* CECT

192 628<sup>T</sup>, *V. splendidus* CECT 528<sup>T</sup>, and *V. pomeroiyi* LMG 20537<sup>T</sup> when using the  
193 Biometra machine (Table 2; Fig. 2B). In addition, *V. mytili* CECT 632<sup>T</sup> also cross-  
194 reacted using these primers in the VWR apparatus (Table 2; Fig. 2B).

195 In the case of VtKF-VtKR primer set, positive reactions showing amplicons of the  
196 expected size (413 bp) were achieved for *V. pectenocida* A365<sup>T</sup> using the Biometra  
197 apparatus, *V. mytili* CECT 632<sup>T</sup> in the VWR machine, and *V. pomeroiyi* LMG 20537<sup>T</sup>  
198 regardless of the thermocycler utilized (Table 2; Fig. 2C).

199

### 200 3.3. Analysis of primers sequence

201 The location of the primers used in this study was analyzed in an alignment of the 16S  
202 rRNA gene sequences of all the *Vibrio* species showing positive amplification with any  
203 of the primer-sets studied (Fig. 3).

204 Primers Jvt1 and Jvt2 are both designed targeting hipervariable regions of the 16S  
205 rRNA gene. *V. ezurae* DSM 17533<sup>T</sup> shows 100% of similarity with *V. tapetis* CECT  
206 4600<sup>T</sup> in the region in which reverse primer (Jvt2) is designed, but it differs from the  
207 forward primer (Jvt1) in the last 5 nucleotides of the 3' end.

208 Sequences of all the *Vibrio* species, other than *V. tapetis*, amplified by primer pair VtF-  
209 VtR showed more than 70% similarity with the sequence of VtF, being in all cases at  
210 least 13 out 20 nucleotides of the 5' end identical to the sequence of this primer. In  
211 addition, VtR is targeting an area of the 16S rRNA gene in which all 6 amplified vibrios  
212 present a common sequence of 7 nucleotides with *V. tapetis* CECT 4600<sup>T</sup>  
213 (corresponding with the 5' end of the primer) and also show some ambiguous positions  
214 in the area complementary to the last five bases of the 3' end of the primer.

215 Regarding the primer pair VtKF-VtKR, *V. pectenocida* A365<sup>T</sup> showed 100% and 65%  
216 of similarity with the sequences complementary to VtKF and VtKR respectively.  
217 Sequences of *V. mytili* CECT 632<sup>T</sup> and *V. pomeroiyi* LMG 20537<sup>T</sup> also showed high  
218 complementarity to the reverse primer as well as in the area corresponding to the 5' end  
219 of the forward primer.

220

## 221 4. Discussion

222 Current methods for the detection of *Vibrio tapetis* involve the isolation of the  
223 microorganism from affected or healthy clams, and further biochemical identification as  
224 well as immunological detection. It has been often described that classical methods may  
225 fail in the detection of *V. tapetis* because the pathogen could not be isolated on culture

226 media, even after being detected by indirect procedures (i.e. immunofluorescence)(Castro  
227 et al., 1992, 1995). These facts can be explained by an antagonist relationship among  
228 representatives of the clam microbiota or by the overgrowth of other easy-growing  
229 bacteria (Castro et al., 2002).

230 Due to the difficulty of the detection and isolation of this pathogen it was necessary to  
231 develop suitable, reliable, rapid and sensitive methods for fast detection of this  
232 pathogen. In the last years, PCR detection procedures have been developed for a high  
233 number of bacterial fish and shellfish pathogens with excellent advantages over the  
234 limitations implied by classical methodology (Beaz-Hidalgo et al., 2008; Brown et al.,  
235 1994; Del Cerro et al., 2002; Gonzalez et al., 2003; Lee et al., 1998; Nhung et al., 2007;  
236 Osorio et al., 1999; Romalde and Toranzo 2002; Romalde et al., 2004; Saulnier et al.,  
237 2000), as it was demonstrated in an experimental challenge with *V. tapetis* carried out  
238 by Drummond et al. (2007), which obtained 43 positive results by PCR *versus* only 3  
239 re-isolations from the inoculated clams.

240 For detection of *V. tapetis*, three PCR protocols were described including different  
241 primers pairs, namely Jvt1-Jvt2 (Romalde et al., 2007), VtF-VtR (Paillard et al., 2006),  
242 and VtKF-VtKR (Park et al., 2006), all of them targeting the 16S rRNA gene sequence.  
243 The present work includes a comparison of the three protocols on the basis of their  
244 sensitivity and specificity.

245 Differences in the sensitivity among the three PCR protocols used in this study were  
246 observed, being the most sensitive those using the VtF-VtR and the Jvt1-Jvt2 primer  
247 pairs which were able to amplify as low as 2-20 cells. This detection limit is similar to  
248 those obtained for other bacterial pathogens (Beaz-Hidalgo et al., 2008; Castro et al.,  
249 2010; Lee et al., 1998; Osorio et al., 1999; Romalde et al., 2004), and can be useful for  
250 detection of *V. tapetis* during the initial stages of infection. The primer set VtKF-VtKR  
251 showed poorer sensitivity although enough to detect *V. tapetis* acute infections. It has  
252 been described that an inverse relationship exists between sensitivity of PCR and the size  
253 of amplicons (Hossain et al., 2001; Otta et al., 1999). Contrarily, our results showed that  
254 the primer pair yielding the shorter amplicon also rendered the poorest detection limit.

255 Differences in specificity were also observed among the three PCR protocols analyzed.  
256 Thus, although all of them were able to correctly amplify all the *V. tapetis* strains, those  
257 employing primer sets VtF-VtR and VtKF-VtKR showed some cross-reactivity with  
258 other *Vibrio* species, including *V. proteolyticus*, *V. ezurae*, *V. nigripulchritudo*, *V.*

259 *splendidus*, *V. pomeroyi*, *V. mytili* and *V. pectenocida*, which yielded amplicons of the  
260 expected size in the correspondent PCR protocol.

261 The specificity of the protocols can be explained on the basis of the design of the  
262 primers. The 3' end has been described as a critical point for the correct priming and the  
263 posterior extension of the polymerase (Kwok et al., 1990; Sommer and Tautz, 1989).  
264 The GC content of the oligonucleotides is also important for the specificity and  
265 sensitivity of the PCR protocol. When a primer set is being designed, is strongly  
266 recommended the inclusion of C or G residues at 3' end in order to increase the priming  
267 efficiency and therefore, the specificity of the protocol. That so called GC clam  
268 provides enhanced specificity due to the stronger hydrogen bond (Apte and Daniel,  
269 2003; Hyndman & Mitsuhashi, 2003; Kidd and Ruano, 1995; Kwok et al., 1990). If we  
270 compare the number of G and C of these three PCR primer pairs, it can be observed that  
271 in the case of Jvt1-Jvt2 and VtKR-VtKR, at least in one of the primers, 4 out the last 5  
272 nucleotides at the 3' end are G or C, while the VtF-VtR primer set only presents two of  
273 these bases at the 3' end of their sequence. Furthermore, the presence of a T together  
274 with G or C within the 3' most triplet helps to prevent mismatch tolerance of primers  
275 with consecutive Gs or Cs (Apte and Daniel, 2003; Kidd and Ruano, 1995), as occurs in  
276 the most specific primers of this study, namely Jvt1-Jvt2. All these facts, specially the  
277 nucleotide composition at the 3' end of the primers and their complementary sequences  
278 in all the amplified vibrios, may explain the low specificity of the protocol described by  
279 Paillard et al (2006).

280

281 In summary, the PCR protocol including the primer set Jvt1-Jvt2 has proved to be the  
282 highest specific and sensitive procedure for the detection of *V. tapetis*. Therefore, we  
283 propose this PCR protocol as the most adequate for an accurate detection of *V. tapetis* in  
284 diagnostic pathology as well as in epidemiological studies of this clam pathogen.

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### 287 **Acknowledgements**

288 This work was supported in part by grants AGL2006-13208-C02-01 and AGL2010-  
289 18438 from the Ministerio de Ciencia e Innovación (Spain). S.B. and A.D. acknowledge  
290 the Ministerio de Ciencia e Innovación and the Xunta de Galicia (Spain) for research  
291 fellowships.

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414 Table 1.- Strains of *Vibrio tapetis* included in this study and results obtained with the three PCR detection protocols employed. Results for each  
 415 primer set in two thermocyclers are shown.

Strain	Host	Country/date of isolation	Jvt1-Jvt2		VtF-VtR		VtKF-VtKR	
			VWR	Biometra	VWR	Biometra	VWR	Biometra
CECT 4600 <sup>1</sup>	<i>Ruditapes philippinarum</i>	France, 1990	+	+	+	+	+	+
GR0202RD	<i>R. decussatus</i>	Spain, 1994	+	+	+	+	+	+
HH6087	<i>Hippoglossus hippoglossus</i>	United Kingdom. 2001	+	+	+	+	+	+
GR0705RD	<i>R. decussatus</i>	Spain, 1994	+	+	+	+	+	+
CMJ 10.7	<i>R. philippinarum</i>	Spain, 2005	+	+	+	+	+	+
C 11.25	<i>R. philippinarum</i>	Spain, 2005	+	+	+	+	+	+
102	<i>R. philippinarum</i>	Ireland, 2005	+	+	+	+	+	+
127	<i>R. philippinarum</i>	Ireland, 2005	+	+	+	+	+	+
IS 1	<i>R. philippinarum</i>	France, 1988	+	+	+	+	+	+
IS 8	<i>Venerupis aurea</i>	France, 1990	+	+	+	+	+	+
IS 9	<i>Cerastoderma edulis</i>	France, 1990	+	+	+	+	+	+
B 2.3	<i>R. philippinarum</i>	France, 1991	+	+	+	+	+	+
B 8.3	<i>R. philippinarum</i>	France, 1991	+	+	+	+	+	+
B 9.3	<i>R. philippinarum</i>	France, 1991	+	+	+	+	+	+
GR1703RP	<i>R. philippinarum</i>	Spain, 1994	+	+	+	+	+	+
LP2	<i>Symphodus melops</i>	Norway, 1999	+	+	+	+	+	+
C0620701B	<i>Umbrina cirrosa</i>	Spain, 2007	+	+	+	+	+	+
C0620701H	<i>U. cirrosa</i>	Spain, 2007	+	+	+	+	+	+
C0620701R	<i>U. cirrosa</i>	Spain, 2007	+	+	+	+	+	+
a200	<i>Dicologlossa cuneata</i>	Spain, 2005	+	+	+	+	+	+
a201	<i>D. cuneata</i>	Spain, 2005	+	+	+	+	+	+
a204	<i>D. cuneata</i>	Spain, 2005	+	+	+	+	+	+
a255	<i>D. cuneata</i>	Spain, 2005	+	+	+	+	+	+

416 +, specific amplification for the primer set; -, no amplification detected. CECT: Spanish Collection of Type Cultures, Valencia, Spain.

417 Table 2.- Strains<sup>a</sup> of related *Vibrio* species included in this study to test the specificity of  
 418 PCR protocols. Results for each primer set in two thermocyclers are shown<sup>b</sup>.

	Jvt1-Jvt2		VtF-VtR		VtkF-VtkR	
	VWR	Biometra	VWR	Biometra	VWR	Biometra
<i>V. aestuarianus</i> ATCC 35048 <sup>T</sup>	–	–	–	–	–	–
<i>V. alginolyticus</i> CCM2575	–	–	–	–	–	–
<i>V. anguillarum</i> ATCC 43306 <sup>T</sup>	–	–	–	–	–	–
<i>V. campbellii</i> ATCC25920 <sup>T</sup>	–	–	–	–	–	–
<i>V. cyclitrophicus</i> LMG 21359 <sup>T</sup>	–	–	–	–	–	–
<i>V. diazotrophicus</i> CECT 627 <sup>T</sup>	–	–	–	–	–	–
<i>V. ezurae</i> DSM 17533 <sup>T</sup>	–	–	+	+	–	–
<i>V. fluvialis</i> CECT 4217 <sup>T</sup>	–	–	–	–	–	–
<i>V. furnisii</i> CECT 4203 <sup>T</sup>	–	–	–	–	–	–
<i>V. haliotocoli</i> JCM21271 <sup>T</sup>	–	–	–	–	–	–
<i>V. harveyi</i> RA 58.2	–	–	–	–	–	–
<i>V. lentus</i> CECT 5110 <sup>T</sup>	–	–	–	–	–	–
<i>V. logei</i> NCIMB1443	–	–	–	–	–	–
<i>V. mediterranei</i> CECT 621 <sup>T</sup>	–	–	–	–	–	–
<i>V. mimicus</i> CECT 4218 <sup>T</sup>	–	–	–	–	–	–
<i>V. mytili</i> CECT 632 <sup>T</sup>	–	–	+	–	–	+
<i>V. nereis</i> ATCC 25917 <sup>T</sup>	–	–	–	–	–	–
<i>V. nigripulchritudo</i> CECT 628 <sup>T</sup>	–	–	+	+	–	–
<i>V. ordalii</i> NCMIB 2167 <sup>T</sup>	–	–	–	–	–	–
<i>V. orientalis</i> CECT 629 <sup>T</sup>	–	–	–	–	–	–
<i>V. parahaemolyticus</i> ATCC 27969	–	–	–	–	–	–
<i>V. pectenecida</i> A365 <sup>T</sup>	–	–	–	–	+	–
<i>V. penaeicida</i> AM101	–	–	–	–	–	–
<i>V. pomeroiy</i> LMG 20537 <sup>T</sup>	–	–	+	+	+	+
<i>V. ponticus</i> CECT 5869 <sup>T</sup>	–	–	–	–	–	–
<i>V. proteolyticus</i> ATCC 15338 <sup>T</sup>	–	–	+	+	–	–
<i>V. splendidus</i> CECT 528 <sup>T</sup>	–	–	+	+	–	–
<i>V. tasmaniensis</i> LMG 21574 <sup>T</sup>	–	–	–	–	–	–
<i>V. vulnificus</i> ATCC 27562 <sup>T</sup>	–	–	–	–	–	–

419 <sup>a</sup> ATCC, American Type Culture Collection, Rockville, MD, USA; CCM, Czech  
 420 Collection of Microorganisms, Czech Republic; LMG, BCCM/LMG Bacteria  
 421 Collection, Gent, Belgium; CECT, Spanish Collection of Type Cultures, Valencia,  
 422 Spain; DSM, German Collection of Microorganisms and Cell Cultures; JCM, Japan  
 423 Collection of Microorganisms; NCIMB: National of Industrial and Marine Bacterial  
 424 Ltd, Aberdeen, UK; *V. harveyi* RA 58.2 belongs to the Laboratory collection and strains  
 425 of *V. pectenecida* and *V. penaeicida* were kindly donated by Drs. Lambert and Goarant  
 426 from the IFREMER in Plouzane (France) and New Caledonia respectively.  
 427 <sup>b</sup> +, specific amplification for the primer set; –, no amplification detected.

428 **Figure legends**

429

430 **Figure 1.-** Sensitivity of the PCR protocols for detection of *V. tapetis* using Jvt1-Jvt2  
431 (A), VtF-VtR (B), and VtKF-VtKR (C) primer sets. Lanes: M, PCR Marker (50-2000  
432 bp ladder, Sigma); 1-11 and 13-23, Serial dilutions of DNA extracted from the type  
433 strain CECT 4600<sup>T</sup>, ranging from 1000 ng/μl to 1 ag/μl.; 12 and 24, negative control  
434 (water). 1 to 12, amplifications performed in a T-Professional basic (Biometra)  
435 thermocycler; 13 to 24, amplifications performed in an Uno Cyler (VWR)  
436 thermocycler. Numbers on the left indicate the position of molecular size marker in bp.  
437 Numbers on the right indicate the size of the amplified products and the restriction  
438 fragment in bp.

439

440 **Figure 2.-** Non-specific PCR products obtained for *Vibrio* sp. strains using Jvt1-Jvt2  
441 (A), VtF-VtR (B), and VtKF-VtKR (C) primer sets. Lanes: M, PCR Marker (50-2000  
442 bp ladder, Sigma); 1 and 18, *V. parahaemolyticus* ATCC 27969; 2 and 19, *V.*  
443 *pectenicida* A365<sup>T</sup>; 3 and 20, *V. mediterranei* CECT 621<sup>T</sup>; 4 and 21, *V. proteolyticus*  
444 ATCC 15338<sup>T</sup>; 5 and 22, *V. nereis* ATCC 25917<sup>T</sup>; 6 and 23, *V. cyclitrophicus* LMG  
445 21359<sup>T</sup>; 7 and 24, *V. ezurae* DSM 17533<sup>T</sup>; 8 and 25, *V. nigripulchritudo* CECT 628<sup>T</sup>; 9  
446 and 26, *V. splendidus* CECT 528<sup>T</sup>; 10 and 27, *V. lentus* CECT 5110<sup>T</sup>; 11 and 28, *V.*  
447 *orientalis* CECT 629<sup>T</sup>; 12 and 29, *V. campbellii* ATCC25920<sup>T</sup>; 13 and 30, *V. pomeroyi*  
448 LMG 20537<sup>T</sup>; 14 and 31, *V. mytili* CECT 632<sup>T</sup>; 15 and 32, *V. furnisii* CECT 4203<sup>T</sup>; 16  
449 and 33, positive control (*V. tapetis* CECT4600<sup>T</sup>); 17 and 34, negative control (water). 1  
450 to 17, amplifications performed in a T-Professional basic (Biometra) thermocycler; 18  
451 to 34, amplifications performed in an Uno Cyler (VWR) thermocycler. Numbers on  
452 the left indicate the position of molecular size marker in bp. Numbers on the right  
453 indicate the size of the amplified products and the restriction fragment in bp.

454

455 **Figure 3.-** Alignment of the 16S rRNA gene sequences of the three strains of *V. tapetis*,  
456 CECT4600<sup>T</sup> (NR026361) GR0202RD (FR797810) and HH6087 (AY800101),  
457 representatives of the different genetic groups described within this bacterial species,  
458 and the sequences of the *Vibrio* species with cross-reactivity in the PCR protocols using  
459 primer pairs VtF-VtR and VtKF-VtKR, including *V. nigripulchritudo* ATCC27043<sup>T</sup>  
460 (X74717), *V. splendidus* ATCC33125<sup>T</sup> (X74724) *V. penaeicida* DSM14398<sup>T</sup>

461 (AJ421444), *V. pectenica* A365<sup>T</sup> (Y13830), *V. ezurae* HDS1-1<sup>T</sup> (AY426980), *V.*  
462 *pomeroyi* LMG20537<sup>T</sup> (AJ491290), *V. mytili* CECT632<sup>T</sup> (X99761) and *V. proteolyticus*  
463 ATCC15338<sup>T</sup> (X74723). The location of the each primer-set used in this study is  
464 indicated. Sequence annotations based on 16S rRNA gene sequence of *V. tapetis* CECT  
465 4600<sup>T</sup>.  
466

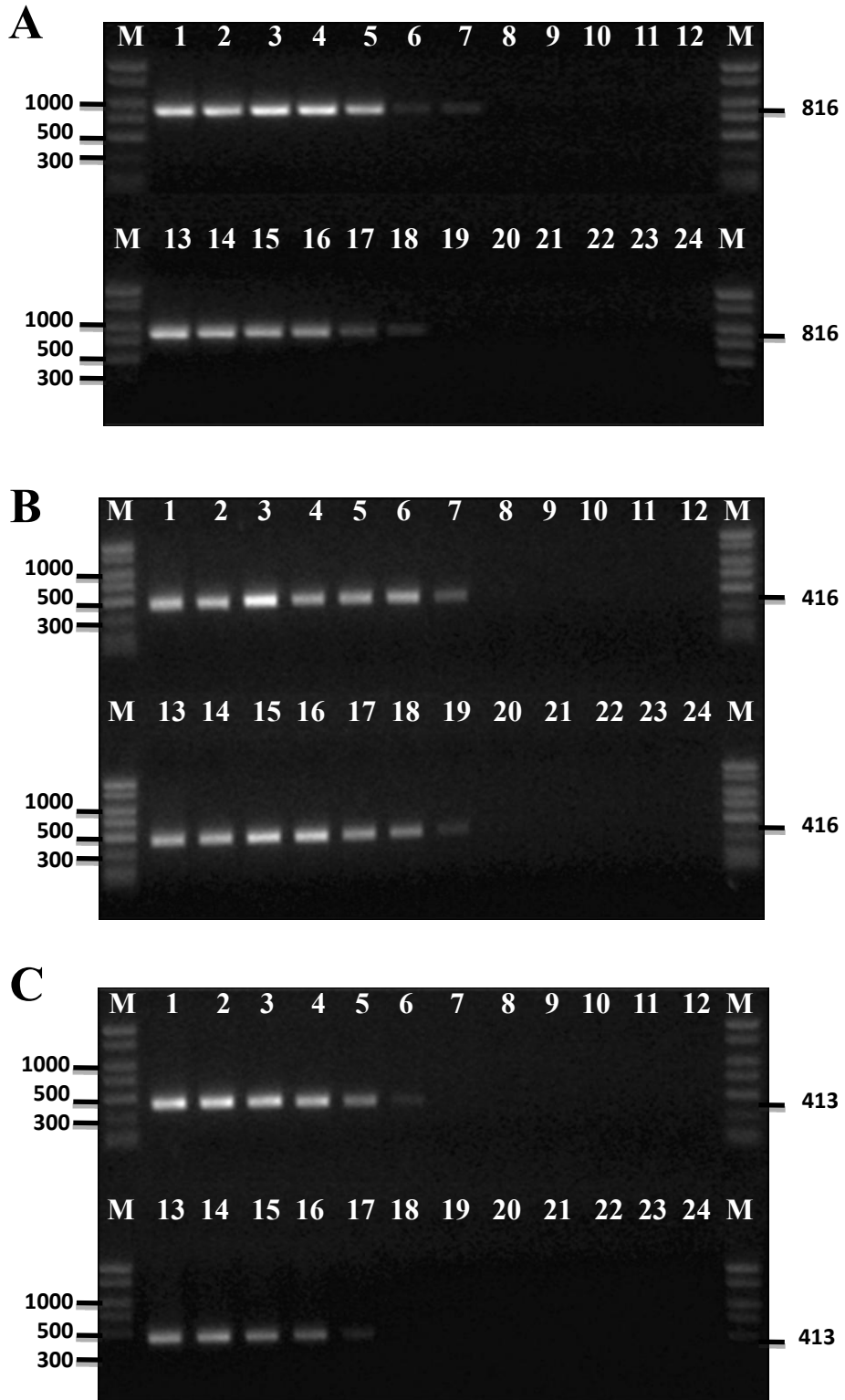


Fig. 1.- Balboa et al.

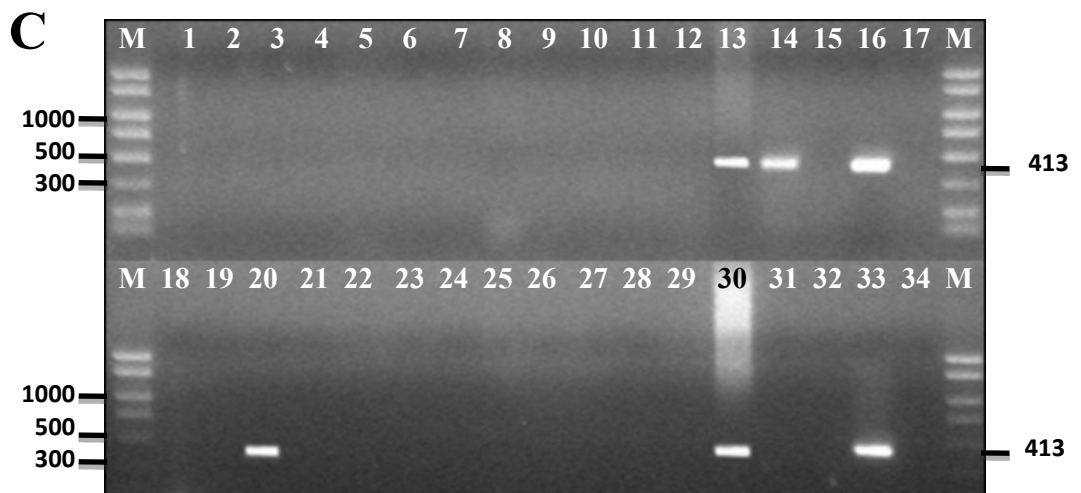
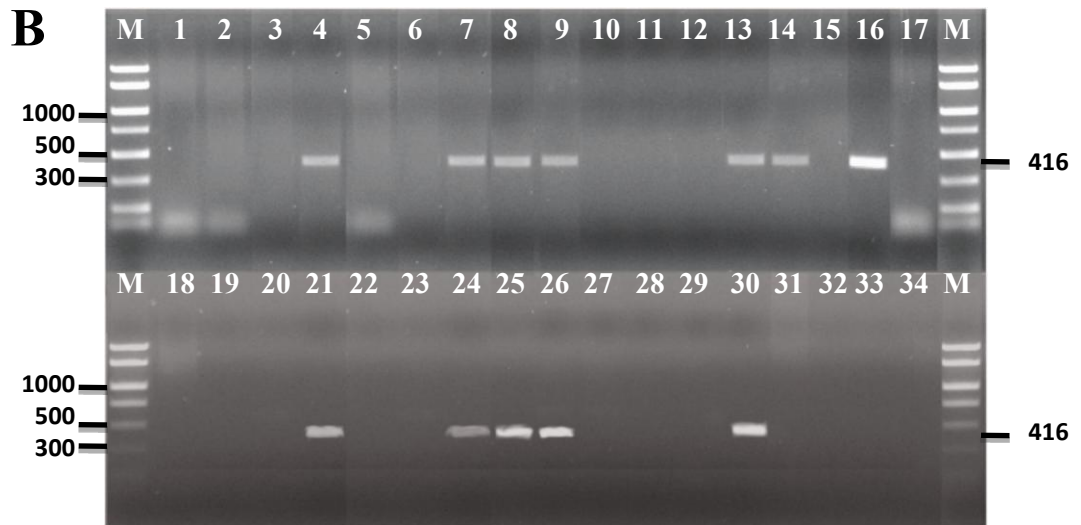
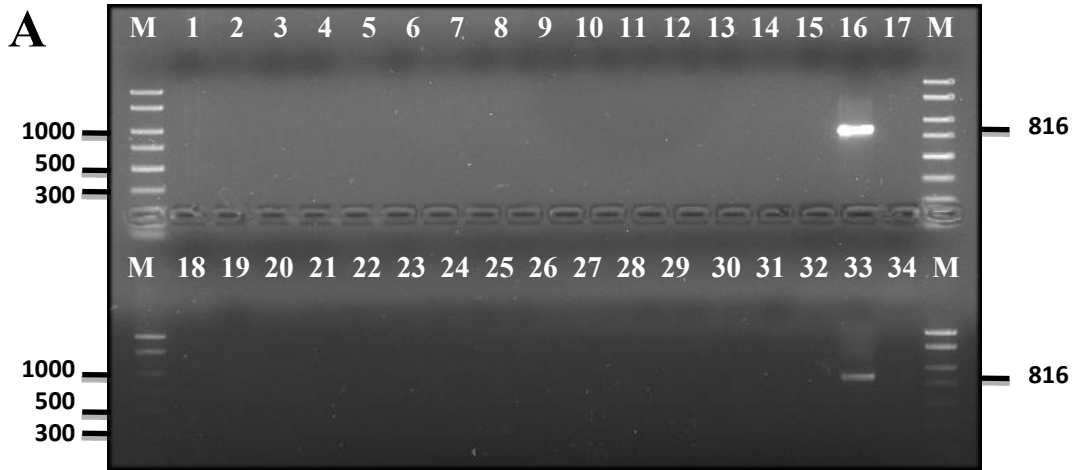


Fig. 2.- Balboa et al.

Figure 3

