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Molecular identification of Vibrio tapetis, the causative agent of the brown ring disease of *Ruditapes philippinarum*

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Abstract:

Vibrio tapetis is the marine bacterium responsible for the brown ring disease (BRD) affecting the manila clam, *Ruditapes philippinarum*. Identification of *V. tapetis* has been previously performed using biochemical criteria and serological procedures. All of these methods are time consuming and ill-adapted to individual screening. This study describes an oligonucleotidic probe (Vt446) and two PCR primers, deduced from the 16S rDNA sequence, allowing a fast and specific *V. tapetis* identification using dot blot hybridisation and species-specific primed PCR (SSP-PCR). The probe and primers have been tested on 60 strains, including referenced *Vibrio* sp., Gram negative and positive bacteria, marine bacteria samples and isolated clam bacteria. For all the 19 *V. tapetis* strains, the results of PCR assays consistently corroborated those of the agglutination tests. The detection limit was estimated to be 10^2 CFU ml^{-1}. The SSP-PCR method has resulted in *V. tapetis* detection in larvae, in diseased clams, and in asymptomatic broodstock clams that later developed BRD. In conclusion, the two SSP-PCR primers were useful for direct and fast identification of *V. tapetis* strains isolated in clams, and are well suited for the screening of individual *R. philippinarum* broodstock clams and larvae from the hatchery.

Keywords: Diagnostic; Detection; Bivalve; Vibriosis; SSP-PCR; DOT-BLOT hybridisation

Introduction

Bacteria (mainly vibrios) are often associated with larval bivalve mortalities (For reviews : McGladdery 1999, Sindermann 1990 and Paillard et al, 2004a). Few bacteria induce specific diseases in adults, therefore the etiology in adult bivalves has been demonstrated only for two major diseases: brown ring disease (BRD) in manila clams, Ruditapes philippinarum and R. decussatus (Paillard and Maes, 1990) and nocardiosis in the Pacific Oyster, Crassostrea gigas (Friedman et al., 1991; Friedman and Hedrick, 1991). BRD is a pathological condition affecting mainly adult R. philippinarum, but also juveniles, and has been associated with mass mortalities causing important economic losses in Europe (Paillard and Maes, 1989; Castro et al., 1992; Figueras et al., 1996). The etiological agent of BRD is a Vibrio named Vibrio P1 (Paillard and Maes, 1990; Paillard et al., 1994) or Vibrio tapetis (Borrego et al., 1996). The major sites of infection of V. tapetis in clams are the periostracal lamina and the extrapallial fluids (Paillard and Maes, 1995a; Paillard and Maes, 1995b; Allam et al., 1996). The colonisation of the shell matrix by V. tapetis induces a characteristic symptom: a brown deposit of melanised shell matrix on the inner surface of the valves (Paillard and Maes, 1994; Paillard and Maes, 1995b). The penetration of V. tapetis into haemolymph can induce mortality before the clam exhibits BRD symptoms (Allam et al., 2002; Paillard, 2004b). Identification of V. tapetis has been performed by using biochemical criteria (Borrego et al., 1996) and serological procedures (Paillard and Maes, 1995a; Paillard and Maes, 1995b; Allam et al., 1996; Allam et al., 2000; Castro et al., 1996; Nöel et al., 1996). Intraspecific characterisation of V. tapetis has been done by pulse-field gel electrophoresis, ribotyping and plasmid profiles (Castro et al., 1997; Romalde et al., 2002; Le Chevalier et al., 2003). However, these methods are time consuming and ill-adapted to individual screening of clams and to detect asymptomatic, but infected, clams, which is the required condition to enhance a hatchery strategy. In recent years, several methods allowing bacterial identification from

environmental samples have been developed on the basis of rRNA phylogenetic classification. These methods include in situ probing, dot blot hybridisation, sequencing of 16S rDNA clone libraries, single-strand conformation polymorphism (SSCP) analysis of a PCR-amplified variable region (Peters et al., 2000; Widjojoatmodjo et al., 1995) and denaturing gradient gel electrophoresis (DGGE) analysis (Gillan et al., 1998). Furthermore, methods have been developed for specific identification of bacteria. These methods include arbitrarily primed PCR (AP-PCR), randomly amplified polymorphic DNA (RAPD) and species-specific primed-PCR (SSP-PCR). In the majority of these examples, the targeted sequences are located outside the 16S or 23S rRNA genes (Hozbor et al., 1999; Lee et al., 1994; Pooler et al., 1996; Tilsala-Timisjarvi and Alatossava, 1998; Yamazaki and Nakamura, 1995). Variable regions of the 16S and 23S rRNA and tRNA genes have been used, among others, to specifically identify Coxialla burnetii (Ibrahim et al., 1997), Xanthomonas albilineans (Honeycutt et al., 1995) and Vibrio vulnificus (Aznar et al., 1994) using various methods.

In this study we describe a specific 16S rDNA probe and species-specific primers allowing a specific V. tapetis identification. For aquaculture purpose, we propose a SSP-PCR method applicable for fast and sensitive V. tapetis detection in larvae and broodstock from hatcheries.

Materials and methods

Bacterial strains. The 60 strains used in this study are listed in Table 2. This list includes the V. tapetis reference strain CECT 4600 and 18 other V. tapetis strains isolated from clams, cockles and fishes. Twelve of the latter strains (IS-1; IS-5; IS-7; IS-8; IS-9; 2.3; 8.17; 9.7; 11.2; RD0705; RP 1703; RP 6301) have been identified as V. tapetis on the basis of biochemical, serological and pathogenicity criteria (Maes, 1992; Maes and Paillard, 1992;

Paillard et al., 1994; Novoa et al., 1998; Choquet, 2004) and genetic tests. Genetic tests included DNA/DNA Hybridisation for strains IS-,; IS-5, IS-7, 2.3, 8.17, 9.7, and 11.2; molecular typing using Ribotyping; Randomly Amplified Polymorphic DNA (RAPD) and Pulse-Field Gel Electrophoresis (PFGE) for these 12 strains (Borrego et al., 1996; Castro et al., 1997; Romalde et al., 2002). Four of the strains (P16b; UK6; P21; 29B) have been more recently identified as V. tapetis on the basis of biochemical, serological and pathogenicity criteria (Allam et al., 1996; 2000; 2002; Paillard et al., 1997; Paillard et al., 2004b). DNA Gyrase B phylogenetic studies were performed on four strains, RD0705, IS-5, IS-7 and UK6 and P16 and have shown that these strains are grouped in the same clade as the reference V. tapetis CECT 46000 (Choquet, 2004). Two Vibrio sp., LP2 and HH6087, recently isolated from fishes, Symphodus melops and Hyppoglossus hyppoglossus, respectively, suffering mortalities have been identified as V. tapetis on the basis of serological test, phenotypic and genetic characterisation (16S rRNA gene for HH6087 and DNA-DNA hybridisation for LP2, and the Gyrase B gene for both strains) (Jensen et al., 2003; Reid et al., 2003; Choquet, 2004). Previous and recent studies have shown that the V. tapetis isolates (strains IS1, IS5, IS7, IS8, IS9, P21, P16b, UK6, LP2 and HH6087) are able to induce the BRD symptoms (Maes, 1992; Allam et al., 2000; Choquet, 2004). The strain RD0202, a Vibrio *sp* biochemically similar to V. tapetis except for the lack of lactose fermentation, do not show positive agglutination with V. tapetis anti-serum, but is able to reproduce BRD symptoms and mortalities in R. decussatus and R. philippinarum (Novoa et al., 1998; Choquet, 2004). The two strains, P9 and GM4, are not V. tapetis related species, moreover they do not provoke mortality in clams and only GM4 can induce BRD symptoms in very low prevalence (15%) compared to V. tapetis strains (60 to 90%) (Choquet, 2004).

For the present study, Vibrios and unrelated Gram positive and negative bacteria, and marine bacteria. V. tapetis (CECT 4600) were grown at 20°C on Trypticase soy agar (TSA, Difco)

adjusted to 1.5% NaCl. Other marine bacteria were grown at 20°C in Marine Agar (Difco). The reference strains were grown under the conditions recommended by the ACTT.

Clams

Larvae of Ruditapes philippinarum (18 days, length = 120µm) were purchased from the SATMAR hatchery (Gatteville, France). Adults (30-40 mm) were sampled from clam beds cultured by the SATMAR society in the Chausey Islands.

Larval culture and challenge experiment

Larvae were placed in a glass beaker (2 L of sterile sea water, SSW), at 5 larvae/ml (on average 10,000 larvae per beaker). Challenge experiments with V. tapetis CECT 4600 (10^4 CFU ml^{-1}) were performed 2 days after reception and over a period of 15 days using three different conditions (control larvae, challenged larvae, and no larvae but with V. tapetis alone in seawater), all done in triplicate. V. tapetis concentration was determined using a linear regression equation describing a relationship between Optical Density (OD at 492 nm) and Total Heterotrophe Bacteria Counts (THBC) as first described in Paillard and Maes, 1990.

The larvae were fed with a mix of algae (Isochrisis galbana, Pavlova lutheri, and Chaetoceros pumilum). Larval culture temperatures were performed at 18 and 23°C, in order to satisfy the host (23°C is generally used in the hatchery) and the pathogen (V. tapetis optimal growth temperature is 18°C) (Paillard et al., 1997; Paillard et al., 2004b). Sea water and algal food were renewed 3 times a week. V. tapetis detection was performed in larvae after 2 (T2) and 15 days (T15) of culture. For this, the larvae in each beaker were collected on a gridded filter (around 100 larvae were sampled), rinsed three times with SSW and then blended for 30s in 100 µl of SSW using a Polytron™ blender. Larval homogenates and aliquots of 100 µl sea

water sampled from beakers containing no larvae were stored below -20°C until used for DNA extraction.

BRD diagnostic in broodstock adults

The BRD symptom is characterized by a brown organic conchiolin deposit at the inner face of the valves. This conchiolin deposit can be partially or completely covered by shell layers as a function of the shell repair process (Paillard and Maes, 1994). The development of and recovery from BRD is assessed by scoring macroscopic symptoms according to the classification system established by Paillard and Maes (1994) and recently modified by (Paillard 2004a, b). In this portion of the study, three main groups of clams have been considered: the asymptomatic ones which present no BRD symptoms, the diseased ones, which present a conchiolin deposit partially covered by shell layers and the recovered clams, which have completely covered the conchiolin deposit by calcified shell layers (Table 3). In fact, the clams which developed the disease during the experiment, independently of the issue, correspond to group 2 and 3.

Diagnosis of BRD and detection of *V. tapetis* by PCR in broodstock clams

Three hundred and twenty adult clams were marked individually with a permanent marker pen and then maintained in an aquarium (250 L) at 14°C with a continuous renewal of seawater (cf. results Table 3). Once a day, they were fed with a mix of algae (Pavlova lutheri, Isochrisis galbana and Chaetoceros pumilum). At each sampling time (T0 and T30, 30 days after the first sampling T0), the clams were placed in an anaesthetised solution (5% $MgCl_2$ in a 70/30 solution of distilled water/ seawater) until they opened their valves. After elimination of the pallial fluid, the extrapallial fluid was collected through a needle inserted in the sinusal, peripheral and central compartment as described by Paillard (2004b). Extrapallial fluids were

frozen immediately at -20°C to be used later for V. tapetis detection. BRD diagnosis was then individually performed on clams through binocular microscope observation as described above. The clams were then quickly returned to the aquarium to limit mortality due to the sampling.

At T0, fluids of 212 clams were individually sampled. Aliquots of 100 µl from each clam was frozen for further analysis. The rest of fluids were distributed in 23 pools of 8 to 10 individuals (14 pools of 10 individuals and 9 pools of 8) and used for *V. tapetis* detection by PCR. Microscopic examinations for signs of BRD were performed in all clams sampled for fluids, but only 112 individuals opened their valves sufficiently in the anaesthetised solution to be successfully diagnosed. At T30, BRD diagnosis was carried out on all survivors (n = 229) including at least 51 clams also sampled at T0. For these clams, V. tapetis detection was performed using the individual samples collected at T0 and conserved at -20°C. Therefore, three categories of clams could be distinguished on the basis of V. tapetis detection at T0 and BRD diagnosis at T0 and T30: Category I, no V. tapetis at T0 and no BRD at T30; Category II, V. tapetis detection at T0 and BRD symptoms at T30; Category III, V. tapetis detection at T0 and BRD recovery at T30. At T30, fluids from clams in categories II and III were grouped into two pools of 9-10 individuals each, which were used for V. tapetis detection (Table 3).. The 8 individuals in category I were grouped into a single pool. Another pool of individuals sampled only at T30 and showing no BRD symptoms (n=12) was also included. In total, *V. tapetis* detection was performed using the individual samples of 58 clams (Table 3).

DNA extraction and 16S rDNA amplification

DNA was extracted from 100 µl of larval homogenate, sea water or the extrapallial fluid of adults according to the chelex method (Saulnier et al., 2000). For bacterial suspensions, total

DNA was prepared from 1.5 ml of saturated liquid culture according to Ausubel and collaborators (1990).

The 16S rDNA were PCR-amplified using the universal primers 616V and 1492R (Table 1) according to Kalmbach et al., (1997). PCR products were purified from agarose gels using the Ultrafree-DA kit (Millipore).

Probe design and DOT BLOT hybridisation

The Vt446 probe was deduced by the authors from the 16S rRNA gene sequence of the V. tapetis CECT 4600 strain (Accession n°: Y08430) by comparison with the sequences of 45 referenced Vibrio strains (sequence alignments not shown). Only the sequences of at least 1.4 kb were selected. The accession numbers of these strains are AB000278, AB000393, AB013297, AF007115, AF022409, AF064556, AF064557, AF064559, AF064637, AJ007045, AJ132227, M98446, U46579, U57919, X16895, X70640, X70641, X70643, X74689, X74691, X74692, X74693, X74698, X74701, X74704 to X74708, X74710 to X74717, X74719, X74721, X74723, X74725, X76333, X76335, X99761, X99762, and Z31657. The definition of this probe in the region of the 450th nucleotide of the 16S rRNA gene (Escherichia coli numbering) is in agreement with the target sites proposed for the molecular identification of the genus Vibrio (Dorsch et al., 1992).

Vt446 dot blot hybridisation were performed on 2 µl of alkali-denatured PCR-amplified 16S rDNA, or 200 ng of genomic DNA, onto a Hybond N+ membrane according to the manufacturer's recommendations (Amersham). V. tapetis CECT 4600 DNA and Herring sperm DNA (Sigma) were used as positive and negative control, respectively. The Vt446 and the EUB338 oligonucleotidic probes (Table 1) were digoxigenine-labeled using a DIG Oligonucleotide 3'-End Labelling Kit (Boehringer). The EUB338 universal probe was designed by Aman et al. (1990). The DIG oligonucleotide labelling EUB338 probe was used

to control the efficiency of the probe hybridisation and of the revelation process. Overnight hybridisation was performed at 54°C in Tris-Cl pH 8 50 mM containing 3 M tetramethylammonium chloride (Sigma), 2 mM EDTA, 5X Denhardt's and 0.1% SDS. The membranes were washed twice in 2X SSC, 0.1% SDS at room temperature for 10 min and once for 30 min at 59°C in the hybridisation solution without Denhardt's. Positive signals were detected using the DIG Nucleic Acid Detection Kit (Boehringer).

Agglutination test

The agglutination assay was carried out as follows: 20 µl of liquid culture bacteria were centrifuged at 13,000 g. The bacteria were resuspended in 5 µl of phosphate buffered saline (PBS) and 5 µl of an anti V. tapetis (CECT 4600) polyclonal antibody solution (Pasteur, France) were added. Anti V. cholerae (Pasteur, France) antibody was used as a negative control. The agglutination test was performed only for *Vibrio* species.

PCR primers design

The reverse primer, VtR, partially overlaps the Vt446 probe (Fig. 1) and this sequence is only present in the 16S rRNA gene of 8 bacteria (Accession n°: AF007115, AF055811, AF055817, AF055833, D89929, M64339, U64006, and X74710). The alignment of these sequences with the 16S rDNA of V. tapetis allowed the definition of the forward primer, VtF. In addition to V. tapetis, the sequence of this primer is 100% identical to sequences of only V. pectenicida (Accession n°: Y13830) and V. rumoence (Accession n°: AB013297). Moreover, at least 3 nucleotides at the terminal 3'end of the VtF primer are absent from the VtF-complementary sequences of all the other 16S rDNA. Therefore, we expected that this pair of primers would allow the amplification of a 416 bp fragment specifically from the V. tapetis 16S rDNA. Sequences of the VtF and VtR primers are presented in Table 1. PCR reactions were

performed in 50 µl of 10 mM Tris-Cl, 50 mM KCl containing 200 µM of each dNTP, 1.5 mM $MgCl_2$, 400 µM of each primer, 0.2 U of Taq DNA polymerase (Sigma), and 1 to 5 µl of bacterial suspension or 200 ng of genomic DNA. The thermal profile consisted of 35 cycles of 1 min at 94°C, 1 min at 63°C, and 45 s at 72°C in a GeneAmp^R PCR system 9700 (Applied Biosystem). The positive control consisted of V. tapetis CECT 4600 DNA while the negative control was deionized water. To evaluate the sensitivity of the method, serial dilutions from 10 to 10^6 CFU/ml of a culture of V. tapetis were performed in SSW. The CFU/ml was determined by plating onto marine agar. PCR-amplified DNA fragments were visualised under U.V. illumination after agarose gel electrophoresis (1% W/V) and ethydium bromide staining. Ladders (100 pb DNA Ladder Promega (Figs. 3-5) and smart ladder Eurogentec (Fig. 6)) were used as a molecular weight marker.

Probes and primers synthesis

Probes and primers were synthesized by, and purchased from, PROLIGO (France) and EUROBIO (France).

Results

Probe specificity

The V. tapetis probe, Vt446, was tested for specificity by comparison of dot blot hybridisation onto the PCR-amplified 16S rDNA from 50 different bacteria strains (Table 2). The Vt446 probe hybridised specifically to the PCR-amplified 16S rDNA, or genomic DNA (data not shown), of the V. tapetis (Vt) reference strain and all other V. tapetis strains (IS-1, IS-8, IS-5, IS-9, IS-7, P21, 29b, P16b, UK6) isolated from different clams (Fig 2; Table 2). All of these strains also reacted by agglutination with the anti V. tapetis polyclonal antibodies (Table 2).

PCR primers specificity and detection limit

The VtF and VtR primers were designed to amplify a 416 bp fragment from the V. tapetis CECT4600 16S rDNA (Fig.1). The specificity of the PCR was tested with the purified DNA of the 60 different bacterial strains (Table 2). As suspected, a 416 bp PCR product was obtained only from V. tapetis CECT 4600 and from other V. tapetis isolates (Fig. 3, lanes 1, 3-9). Whatever the method, Vt446 dot blot hybridisation, PCR amplification, or the agglutination test, all the 19 V. tapetis strains (CECT 4600 and clam isolates) reacted positively (Table 2). The strains non pathogenic for clams, GM4 and P9, were negative in the PCR assay and presented only weak positive results with the agglutination test and dot blot hybridisation (Table 2). The strain Vibrio sp., RD 0202, showed a positive reaction by PCR, but a negative result with the agglutination test (Table 2).

To evaluate the sensitivity of this method, serial dilutions from 10 to 10^6 CFU/ml of a V. tapetis liquid culture were performed and was verified by the plate count method. The detection limit was around 10^2 CFU ml^{-1} (Fig. 4).

Detection of V. tapetis by PCR in clams

Larvae

Two days (T2) after reception from the hatchery, no V. tapetis were detected by PCR in larvae. After 15 days of culture (T15) at 18°C (Fig. 5) and 23°C (data not shown), a 416 bp PCR product was obtained in one of the six control beakers (at 23°C). For all batches challenged with V. tapetis at both temperatures, the presence of a single band of 416 bp was obtained (lanes I1, I2, I3, I1, I2, I3) (Fig. 5). In the seawater without larvae but containing V. tapetis CECT 4600 as control positive, as expected a 416 bp PCR product was obtained at T2 and also at T15.

Adult broodstock

At T0, the prevalence of BRD was very low (0.89%; 1/112) (Table 3), but 7 out of 23 (30.4%) batches generated a band of appropriate size when examined by PCR. At T30, the proportion of total clams that had developed BRD (Table 3) reached 51 % (n= 229), but 27% of them had recovered, as indicated by shell deposition over the organic deposit. For the clams which were diagnosed at both T0 and T30 (n = 51), V. tapetis presence was determined in each clam at T0 (Fig. 6). At T0, a positive PCR reaction was observed in some asymptomatic clams, which had developed BRD by T30 (Fig. 6, lanes 4-6, 10, 11) or recovered from BRD at T30 (lanes 7-9). In the recovered clams, V. tapetis was still present, but at concentrations near the detection limit. Negative reactions at T0 were observed in clams that were asymptomatic at both T0 and T30 (Fig. 6, lanes 12, 13). At T30, V. tapetis PCR detection was performed on clams in categories I, II, and III (see in Material and Methods). Positive reactions were found only in clams from categories II and III. In the clams from category III (recovered clams), V. tapetis was nevertheless present but at concentrations near the detection limit. In clams from category I (asymptomatic), V. tapetis was not detected.

During the experiment, cumulative mortality reached 28% at T30 (Table 3). The dead clams belonged to all groups and the mortality could have been due to the stress associated with anaesthesia, fluid sampling, BRD examination as well as BRD development during the experiment.

Discussion

Identification of pathogenic bacteria isolated from diseased organisms is a very important challenge for pathologists. Phenotypic characterisation of bacteria (serotyping, physiology, biochemical properties, and metabolism) shows low reproducibility and instability depending on the culture conditions. Moreover, characterisation systems for marine bacteria are not very

reliable and are time consuming. Molecular diagnostics, based on specific DNA fragment detection, compared to phenotypic characterisation, constitutes a reliable and rapid identification for numerous isolates. Many molecular identification methods, such as species specific probe hybridisation, sequencing of 16S rDNA, SSCP and DGGE have been developed to identify pathogenic bacteria, especially those inducing infections in humans (Yamamoto, 2002; Peters et al., 2000). However, to date, few authors have reported molecular identification of the bacteria involved in diseases of marine bivalves. The present results have demonstrated that Dot blot hybridisation method using the Vt446 probe allowed the identification of V. tapetis strains. The Vt446 probe could be used for molecular identification of bacteria from environmental samples (seawater, marine mud) by bacterial colony hybridisation of or in situ probing. Nevertheless, this method is time consuming (2-3 days), requires several steps and moreover some weak false positive reactions were suspected for GM4 and P9 strains. Therefore, we have developed a one step procedure on the basis of the PCR assay for rapid detection (one day) in clam aquaculture.

The specificity of PCR has been demonstrated to detect only V. tapetis reference strain, CECT 4600, and the nineteen other V. tapetis isolates. Moreover, for all the 19 V. tapetis strains, the results of PCR and the agglutination tests systematically corroborated each other. Identification of V. tapetis using PCR seems to be more efficient than hybridisation or agglutination. This was illustrated by the strains P9 and GM4, both isolated from a clam, which were weakly positive for agglutination and hybridisation, and negative for PCR, suggesting a false positive hybridisation. Recent studies have demonstrated that both GM4 and P9 are actually not R. philippinarum pathogens and do not correspond to V. tapetis species (Choquet, 2004).

The species-specific primed-PCR (SSP-PCR) method, developed in this study, could be also applied routinely to identify V. tapetis from among all the strains isolated from shellfish and

fishes suffering mortality in the field or hatchery. The SSP-PCR identification could be confirmed in a second step, at a physiological level. Their pathogenicity could be evaluated in vivo by BRD reproduction (Paillard and Maes, 1990) and by clam mortality tests (Allam et al., 2002) and in vitro by cytotoxicity assays (Choquet et al., 2004). These biological confirmations have been recently carried out for all the V. tapetis strains and for some Vibrio sp. which have shown positive results by SSP-PCR (Paillard, 2004a; Choquet, 2004). This is the case for the Vibrio sp, RD0202, which showed a positive reaction by SSP-PCR in the present study and which showed high pathogenicity against R. philippinarum after experimental challenge and in in vitro tests (Novoa et al., 1998; Choquet, 2004). DNA-DNA hybridisation and MLST (MultiLocus Sequence Technique) are in progress to confirm the relationship of RD0202 to V. tapetis.

Shellfish concentrate microorganisms in their tissues and fluids from surrounding waters during filtration and feeding processes and are recognised as reservoirs for numerous human pathogens. Outbreaks of shellfish-associated infection have increased considerably since the early 1970's (Potasman et al., 2002). Therefore, numerous applications of PCR-derived methods have been developed to detect human bacterial pathogens, such as Salmonella, Aeromonas hydrophila, E. coli and vibrios, in shellfish (Toze, 1999; Hervio-Heath et al., 2002). Molecular methods have been recently developed to identify bacterial pathogens for marine fishes and crustaceans, such as Yersinia ruckeri, A. salmonicida, Renibacterium salmoninarum in salmon (Hiney et al., 1992); Altinok et al., 2001; Nilsson and Strom, 2002), Photobacterium damsela in seabass, striped bass and seabream (Osorio et al., 1999) and V. penaeicida in shrimp (Saulnier et al., 2000). Bacterial diseases have been often reported in bivalve larvae, but few have been described in adults (Paillard et al., 2004b). For instance, few molecular assays have been developed to detect bivalve bacterial pathogens. V. tapetis

detection is very important for BRD diagnosis because the clinical signs cannot be observed in larvae and juveniles (under 2 mm), during the incubation period of 5 to 10 days before symptoms are observed in juveniles and adults, or in cases when the pathogen enters directly into tissues inducing death without provoking symptoms. The SSP-PCR method described in this study was developed primarily to detect this pathogen in larvae and adult broodstock in the hatchery. The utility of method for following infection and disease progression using haemolymph or extrapallial fluid sampling of individual adult clams was subsequently demonstrated.

For the first time, V. tapetis was detected in larvae by the SSP-PCR assay. Until now, V. tapetis had never been isolated or detected in clams smaller than 2 mm in length when using ELISA methods. This could be due to the lower sensitivity of this latter method, since V. tapetis detection limit using ELISA technique is about $5 \cdot 10^4$ CFU ml^{-1} in SSW or in fluids (Allam et al., 2002; Paillard, 2004a; Noël et al, 1996). Larvae are generally very sensitive to pathogenic vibrios and cannot harbour concentrations higher than 10^5 CFU ml^{-1} without suffering mortalities (Tubiash, 1973). In the present study, the larval challenge experiment with V. tapetis was performed at 10^4 CFU ml^{-1} without causing mortalities. In previous experiments (Paillard et al., unpublished data) V. tapetis at this concentration did not induce severe mortalities in clam larvae; however, a concentration of 10^6 CFU ml^{-1} was able to induce mortalities of 70% and 50% in D larvae and in metamorphic larvae after nine days of culture, respectively. In the present study, a positive reaction by PCR was obtained in one of the three control beakers after 15 days of larval culture at 23°C; this suggest that some batches of larvae were previously contaminated at low level in the hatchery. The SSP-PCR detection assay seems to be effective for detecting lower concentrations than 5 10^4 CFU ml^{-1} of V. tapetis (detection limit for ELISA) in larvae and could be applied routinely in hatcheries to prevent larval and juvenile mortality episodes and to limit dissemination of BRD.

Another study in which BRD progression was followed in individual clams used ELISA to detect V. tapetis also showed that, during recovery, V. tapetis burdens decreased and were at very low levels, approaching the detection limit for ELISA, when clam had completely recovered (Paillard, 2004a; Paillard and Ford, unpublished data). Nevertheless, ELISA detection did not permit distinguishing infected, but asymptomatic, clams from uninfected ones, because V. tapetis burdens in both cases were generally too close to the ELISA detection limit (Paillard, 2004a). In the present study, the SSP-PCR method allows the detection of V. tapetis in infected, but asymptomatic clams: asymptomatic clams that were scored positive for V. tapetis at T0 developed BRD symptoms at T30. This result suggests that SSP-PCR reaction could be used to detect early infections. The fact that some clams remained uninfected during the whole experiment (no V. tapetis detected and no BRD at T30), and that the pathogen can also be eliminated with the recovery process, even if they are maintained in contact with V. tapetis infected clams suggests that "V. tapetis resistant" broodstock could be identified using the SSP-PCR method. In addition, the SSP-PCR method could also be applied to confirm that seed clams are free of V. tapetis. For these purposes, development of a quantitative PCR method is in progress using these specific primers. In conclusion, the SSP-PR method is sensitive, specific and rapid for identifying V. tapetis among isolates from diseased and moribund clams. The two SSP-PCR primers are useful for direct and fast identification of V. tapetis in individual R. philippinarum. Moreover, the rapidity and specificity of SSP-PCR suggest that this latter method is well adapted to detect V. tapetis in all stages of clams from larvae to adults in hatcheries, as well as individuals from clam beds. This molecular diagnostic method should be used to confirm V. tapetis free clams, in order to enhance venerid culture along the north European Atlantic coast. Nevertheless, the possibility that a wild bacteria type, unreferenced in the databases, will positively hybridise with the Vt446 probe and/or result in a positive SSP-PCR amplification cannot be excluded.

Further improvements of molecular diagnostic tests are currently in progress in order to develop real-time quantification methods. The methods developed in the present study for clams will be applied, after modification, for the screening of V. tapetis in bivalve species other than clams, including fishes. These methods will be of great value for monitoring V. tapetis in the marine environment in order to establish its host species among invertebrates and vertebrates and its geographical distribution around the world.

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Figure captions

Figure 1. Localisation of the Vibrio tapetis 16S rRNA gene oligonucleotidic probe Vt446 (bold letters) and species-specific primers VtF and VtR (underlined). Positions are referenced to the 16S rRNA genes of E. coli (accession V00348) and V. tapetis (accession Y08430), respectively. * : identical nucleotides.

Figure 2. Dot Blot hybridisation with the Vt446 (A) or the EUB338 (B) probe on PCR-amplified 16S rDNA from strains of V. tapetis (Vt), other Vibrio species (V, Vf, Val, Vp, Vs, Van) their strain names are bracketed as defined in Table 2. Hs : negative control (herring sperm DNA), C : positive control of the revelation procedure.

Figure 3. PCR from V. tapetis reference strain (Vt), V. tapetis isolates (2.3; 8.17; 9.7; 11.2; RD 0202; RP 1703) and two Vibrio sp. (GM4 and P9). ∅ : negative control.

Figure 4. PCR from V. tapetis reference strain (Vt). Serial dilutions from 10 to 10^6 UFC/ml of liquid culture. ∅ : negative control.

Figure 5. PCR detection of V. tapetis in larvae incubated at 18°C, after two days (not underlined) and after 15 days (underlined). Two different conditions in replicates: C, Control unchallenged larvae; I, V. tapetis challenged larvae. Vt, V. tapetis in sea water. ∅ : negative control.

Figure 6. SSP-PCR detection of V. tapetis in extrapallial fluid derived from individual clam genitor after reception (T0). Genomic of DNA of V. tapetis CECT 4600 (lane 1), V. fischeri and V. splendidus (lanes 2 and 3) were used as positive and negative controls. Asymptomatic

clams at T0 which have developed BRD at T30 (lanes 4-6, 10 and 11), those which have recovered from BRD at T30 (lanes 7-9) and which were healthy at T30 (lanes 12 and 13). ∅ : negative control.

Table 1. Nucleotidic sequences of the probes and primers used.

Probe	Specificity	Sequence (5' - 3')	Target site	Reference
EUB338	Bacteria	GCTGCCTCCCGTAGGAGT	16S (337-354) [a]	Aman et al., 1990
616V	Universal	AGAGTTTGATYMTGGCTCAG	16S (8-27) [a]	Kalmbach et al., 1997
1492R	Universal	CGGYTACCTTGTTACGAC	16S (1493-1510) [a]	Kalmbach et al., 1997
Vt446	*V. tapetis*	AGCGTGCATCCTTGACGT	16S (446-463) [b]	this study
VtF	*V. tapetis*	CGAGCGGAAACGAGAAGTAG	16S (55-75) [b]	this study
VtR	*V. tapetis*	GGATGCACGCTATTAACGTACA	16S (450-472) [b]	this study

[a] E. coli (Accession V00348) numbering, [b] V. tapetis (Accession Y08430) numbering

Table 2. Bacterial strains used to confirm the V. tapetis specificity of the Vt446 probe and the PCR reaction. -, negative reaction. +, weak positive reaction, +++, strong positive reaction. nd, not determined. ATCC, American Type Culture Collection; CECT, Colleccīōn Espagnola de Cultivos Tipos ; CIP, Collection de l'Institut Pasteur.

Bacteria	Strain designation	Source	Origin	Hybridisation Vt446	PCR	Agglutination
Vibrio tapetis	Vt	Collection	CECT 4600	+++	+++	+++
Vibrio fischeri	Vf	Collection	ACTT 7744	-	-	-
Vibrio anguillarum	Van	Collection	ACTT 19105	-	-	-
Vibrio alginolyticus	Val	Collection	ACTT 17749	-	-	-
Vibrio splendidus	Vs.	Collection	ACTT 25914	-	-	-
Vibrio pectenicida	Vp	Collection	CIP 105190	-	-	-
Vibrio natriegens	Vn	Collection	ACTT 14048	-	-	-
Vibrio haloplanktis	Vh	Collection	ACTT 14393	-	-	-
Vibrio costicola	Vco	Collection	ACTT 33508	-	-	-
Vibrio damsela	Vd	Collection	ACTT 33536	-	-	-
Vibrio carchariae	Vca	Collection	ACTT 35084	-	-	-
Vibrio mytili	Vm	Collection	CECT 632	-	-	-
Vibrio sp. 56	T9a-3β	*R. philippinarum*	Sligo, Ireland	-	-	-
Vibrio sp. UK	UKC	*R. philippinarum*	United kingdom	-	-	-
Vibrio sp. IRL	BSI	*R. philippinarum*	Sligo, Ireland	-	-	-
Vibrio sp.	GM3	*R. philippinarum*	Morbihan Gulf, France	-	-	-
Vibrio sp.	GM2	*R. philippinarum*	Morbihan Gulf, France	-	-	-
Vibrio sp.	GM4	*R. philippinarum*	Morbihan Gulf, France	+	-	+
Vibrio sp.	2a-4	*R. philippinarum*	Sligo, Ireland	-	-	-
Vibrio sp.	T9a-3α	*R. philippinarum*	Sligo, Ireland	-	-	-
Vibrio sp.	GL1	*R. philippinarum*	Glénan, France	-	-	
Vibrio sp.	GL2	*R. philippinarum*	Glénan, France	-	-	-
Vibrio sp.	GL4	*R. philippinarum*	Glénan, France	-	-	-
Vibrio sp.	GL5	*R. philippinarum*	Glénan, France	-	-	-
Vibrio sp.	GL6	*R. philippinarum*	Glénan, France	-	-	-
Vibrio sp. Rd S	RD0202	*R. decussatus*	Galicia, Spain	nd	+++	-
V. tapetis Rp 29-a	IS-1	*R. philippinarum*	Landeda, France	+++	+++	+++
V. tapetis Rp 29	IS-5	*R. philippinarum*	Landeda, France	+++	+++	+++
V. tapetis Rp 56	IS-7	*R. philippinarum*	Quiberon, France	+++	+++	+++
V. tapetis Va 56	IS-8	*V. aurea*	Quiberon, France	+++	+++	+++
V. tapetis Ce 56	IS-9	*C. edule*	Quiberon, France	+++	+++	+++
V. tapetis Rp 29-b	29-b	*R. philippinarum*	Landeda, France	+++	+++	+++
V. tapetis Rpn 56	P16b	*R. philippinarum*	Morbihan Gulf, France	+++	+++	+++
V. tapetis Rp UK	UK6	*R. philippinarum*	United kingdom	+++	+++	+++
V. tapetis Rp 29	P21	*R. philippinarum*	Bay of Brest, France	+++	+++	+++
V. tapetis Rd S	RD0705	*R. decussatus*	Galicia, Spain	nd	+++	+++
V. tapetis Rp S	RP1703	*R. philippinarum*	Galicia, Spain	nd	+++	+++
V. tapetis Rp S	RP6301	*R. philippinarum*	Galicia, Spain	nd	+++	+++
V. tapetis Rp 29	2.3	*R. philippinarum*	Landeda, France	nd	+++	+++
V. tapetis Rp 29	8.17	*R. philippinarum*	Landeda, France	nd	+++	+++
V. tapetis Rp 29	9.7	*R. philippinarum*	Landeda, France	nd	+++	+++
V. tapetis Rp 29	11.2	*R. philippinarum*	Landeda, France	nd	+++	+++
V. tapetis Hh UK	HH6087	*H. hippoglossus*	Scotland, UK	nd	+++	+++
V. tapetis Sm NO	LP2	*S. melops*	Bergen, Norway	nd	+++	+++
Halomonas sp. 33	P9	*R. philippinarum*	Marennes, France	+	-	+
Pseudomonas aeruginosa	PAO1		Our collection	-	-	nd
Escherichia coli DH5			Our collection	-	-	nd
Bacillus subtillis			Our collection	-	-	nd
Lactococcus lactis sp *lactis*			Our collection	-	-	nd
Enterococcus. faecalis			Our collection	-	-	nd
Roseobacter sp.	X129	Sea water	Portzic, France	-	-	nd

Roseobacter galleaciensis	X34	Collection	CIP 105210	-	-	nd
Roseobacter sp.	X20	Sea water	Portzic, France	-	-	nd
Roseobacter sp.	X6	Sea water	Portzic, France	-	-	nd
Vibrio sp.	DO1	Sea water	Portzic, France	-	-	-
Bacillus sp.	D02	Sea water	Portzic, France	-	-	-
Bacillus sp.	D22	Sea water	Portzic, France	-	-	-
Pseudoalteromonas sp.	D41a	Sea water	Portzic, France	-	-	-
Pseudoalteromonas sp.	D41b	Sea water	Portzic, France	-	-	-
Vibrio sp.	D66	Sea water	Portzic, France	-	-	-

Table 3 : Genitor sampling at T0 and T30 and BRD diagnosis. n, number; % , pourcentage of BRD and of clam survivors (in bold character) ; sam. , sampled.

		T0				**T30**									
		sampled		**no sam.**	**Total**	**sam. T0-T30**		**sam. only T30**		**Total sam. T30**		**no sam.T30**		**Total Clam**	
		n	**%**	**n**	**n**	**n**	**%**	**n**	**%**	**n**	**%**	**n**	**%**	**n**	**%**
Fluid sampling	Clam used	212		108	320	51		13		64		166		230	
BRD DIAGNOSTIC	Asymptomatic	111	99,11			8	15,69	12	92,31	20	31,25	92	55,76	112	48,91
	Diseased	1	0,89			18	35,29			18	28,13	38	23,03	56	24,45
	Recovered	0	0			25	49,02	1	7,69	26	19,70	35	21,21	61	26,64
	Total D+R	0	0			43	84,31			44	68,75	73	44,24	117	51,09
	total	112			112	51		13		64	100,00	165		229	
Fluid +BRD	total	112			112			13		64		165		229	
	Total clam	212		108	**320**	51		13		64		166		**230**	**71,875**

```
E. coli     60 AGTCGAACGGTAACAGGAAGAAGCTTGCT-357-GAGGAAGGGAGTAAAGTTAATACCTTTGCTCATTGACGTTA
V. tapetis  37 AGTCGAGCGGAAACGAGAAGTAGCTTGCT-358-GAGGAAGGGGTGTACGTTAATAGCGTGCATCCTTGACGTTA
               ****** *** ***  **** ********     *********    * ******* * *   ** *********
```

Figure 1

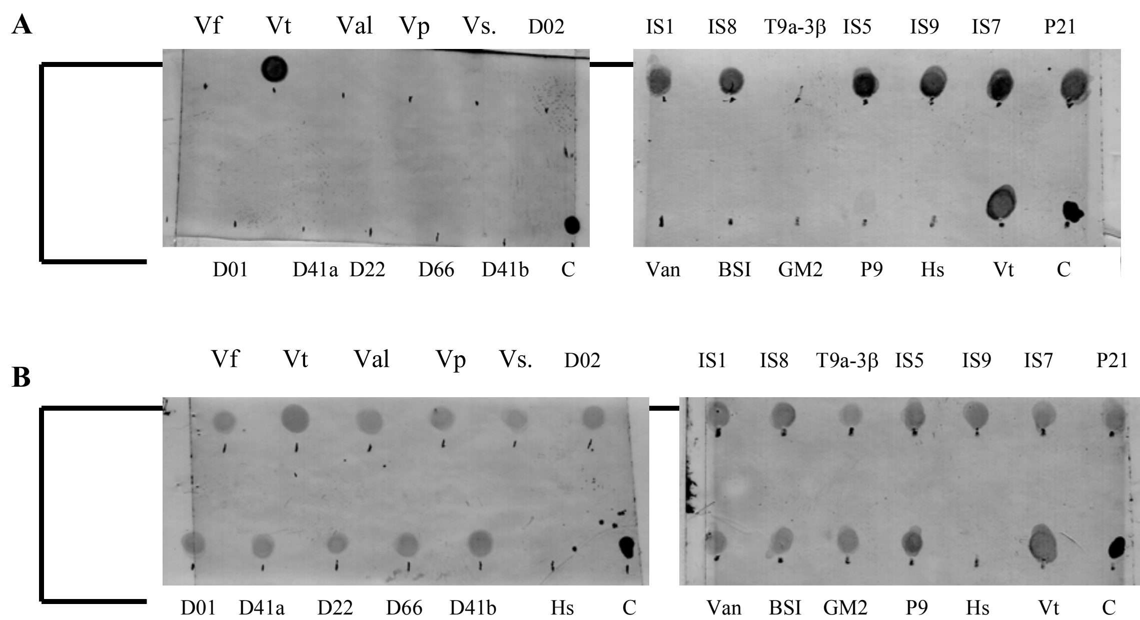

Figure 2

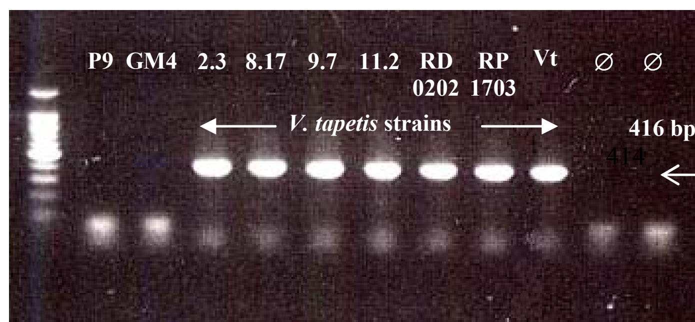

Figure 3

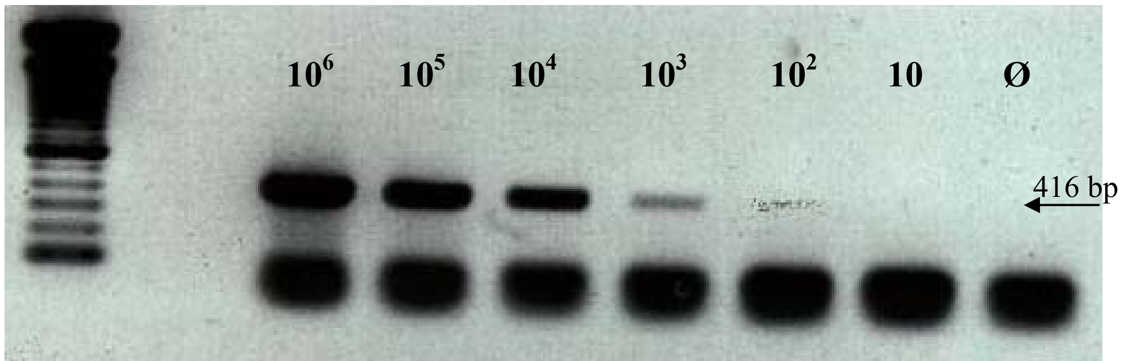

Figure 4

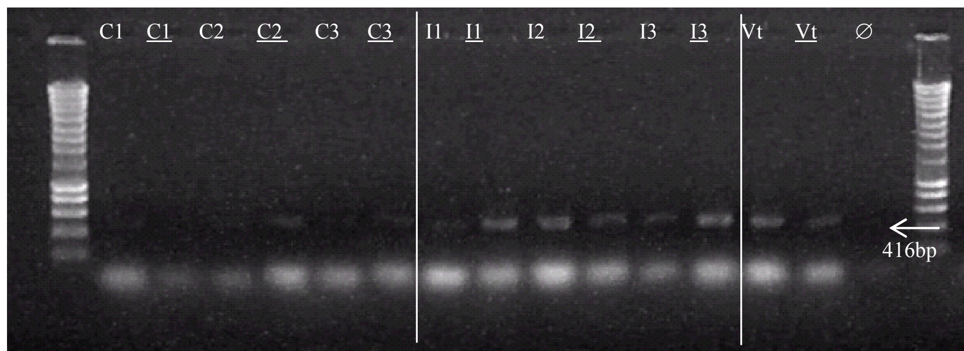

Figure 5

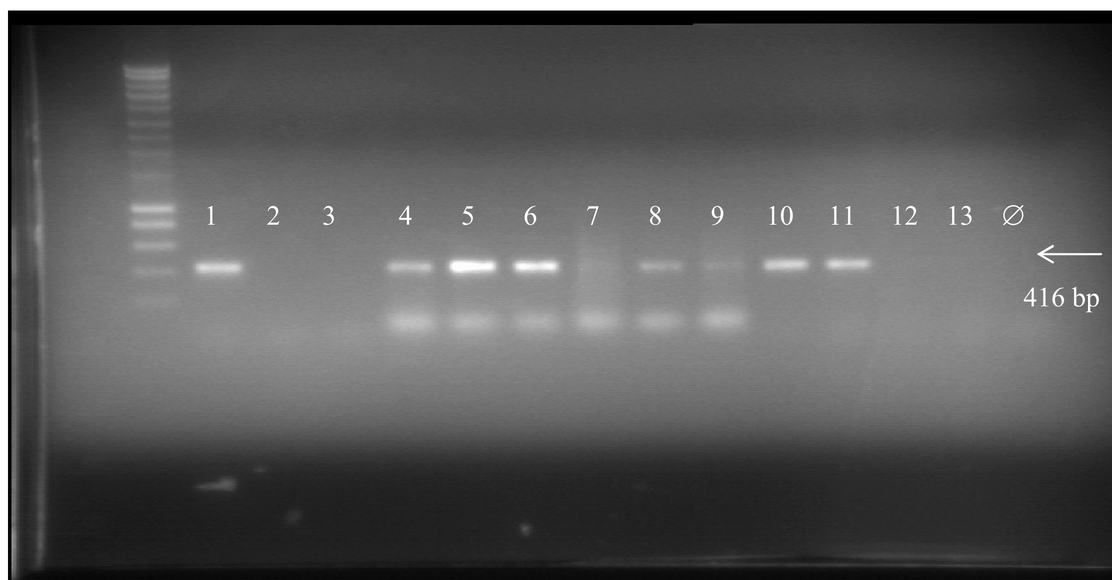

Figure 6