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# Genomic DNA cloning of rickettsia-like organisms (RLO) of Saint-Jacques scallop *Pecten maximus*: evaluation of prokaryote diagnosis by hybridization with a non-isotopically labelled probe and by polymerase chain reaction

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ABSTRACT: Genomic DNA fragments of a rickettsia-like organism (RLO) of Saint-Jacques scallop, *Pecten maximus*, were extracted from purified RLO and cloned. A DNA sequence of 1500 bp taken from one of the sequenced clones was coupled to horseradish peroxidase and the resulting probe was assayed for the diagnosis of RLO by nucleic acid hybridization. The specificity of the probe for RLO DNA was established from its lack of hybridization with scallop DNA. The sensitivity limit of the probe detected by enhanced chemiluminescence was determined to be around 500 ng of total RLO nucleic acids, equivalent to about $2.5 \times 10^6$ copies of RLO DNA. A pair of polymerase chain reaction (PCR) primers were synthesized that amplified a 1300 bp segment of the 1500 bp RLO fragment. After 30 cycles of amplification of total RLO nucleic acids, a product of the expected size was easily visible on agarose at all starting template concentrations between 100 ng and 10 pg of RLO total nucleic acids. The lowest level of sensitivity corresponds to about 100 fg of RLO DNA or about 50 genomic molecules. The specificity of the amplified DNA was confirmed by Southern blot analysis using the peroxidase-labelled probe and by the lack of amplified product with scallop DNA as the starting template.

## INTRODUCTION

Several molluscan species are associated with intracellular prokaryotes which are related to chlamydias or rickettsias according to their ultrastructural features (Weiss & Moulder 1984). Rickettsia-like organisms (RLO), with a typical non-complex developmental cycle, are frequently observed in the digestive gland of oysters and clams and in the gills of scallops (Gulka et al. 1983, Lauckner 1983, Elston 1986, Fries & Grant 1991, Fries et al. 1991). In most cases the effects of RLO on their hosts are unclear but some infections have been related to mortalities (Gulka et al. 1983, Gulka & Chang 1984). Recently, a gill RLO infection was described and reported in several European populations of Saint-Jacques scallops *Pecten maximus* (Le Gall et al. 1988a). On the basis of histological and epidemiological data, this RLO was assumed to be pathogenic (Le Gall et al. 1991). In order to better understand RLO biology and the nature of host-RLO interactions, especially the means of transmission, it is necessary to develop methods of descriptive and analytical epidemiol-

ogy. Taxonomic relationships have to be established between RLO's associated with other mollusc species and, in particular, to identify possible alternative hosts.

For all these purposes, a highly sensitive and specific diagnostic method is required for quantification of the infection. Typically, the detection of gill RLO relies on light microscopic examination of histological sections. While this technique is adequate for RLO diagnosis when relatively large and numerous colonies are present in the tissue samples, it is time-consuming and unreliable in the case of low-level infections. Light microscopy is inadequate for quantifying the infections and is not suitable for identifying and comparing RLO associated with different mollusc species.

In this context, DNA-based technologies are potentially useful for the identification of several kinds of pathogens, using either hybridization with cloned probes (Meinkoth & Wahl 1984, Pepin et al. 1990) or sequences amplified by polymerase chain reaction (PCR) (Kumar 1989, Linz et al. 1990).

For vertebrate pathogenic rickettsias, specific DNA probes have been developed and successfully used in sensitive and specific DNA/DNA hybridization analyses (Goff et al. 1988, Welburn & Gibson 1989). A chief advantage of this technology is the short time required for specific diagnosis compared to *in vitro* culture-based methods (Weiss 1981, Tamura 1988). This would be particularly interesting for the diagnosis of molluscan RLO that are non-culturable *in vivo* (Buchanan 1978). This difficulty is linked to the present lack of mollusc cell lines. However, direct DNA/DNA hybridization often lacks the ability to detect low levels of rickettsias present in some samples, such as in arthropod vectors. With the recent development of PCR, the detection of low numbers of rickettsias has proven possible by amplification of a specific fragment of target DNA (Tzianabos et al. 1989, Webb et al. 1990).

In the present study, a protocol has been developed for the extraction of DNA from purified *Pecten maximus* RLO. Several genomic fragments were cloned and one was assessed as a non-isotopic probe for the specific diagnosis of RLO. Subsequent to DNA sequencing of some DNA cloned fragments, specific oligonucleotides were synthesized and used as primers for PCR. The results show that PCR may provide a rapid, specific and highly sensitive test for RLO of *P. maximus*.

## MATERIALS AND METHODS

**Origin of animals.** Infected Saint-Jacques scallops *Pecten maximus* were collected by dredging in the St. Brieuc Bay (North Brittany, France) where the infection rate is generally near 100 %. For RLO purification, the most highly infected scallops were selected by light microscopic examination of gill tissue smears.

**RLO purification.** Rickettsia-like organisms were purified according to the protocol of Le Gall & Mialhe (1992). Briefly, 10 gills of parasitized *Pecten maximus* were dissected and homogeneized with an ultra-turrax. The homogenates were then sieved through nylon tissues to remove the larger cellular fragments. Several differential centrifugations on discontinuous sucrose gradients were performed to progressively concentrate the microorganisms. Purification was achieved with an isopycnic centrifugation on renographin (Scherring) discontinuous density gradient (14 %-21 %-28 %) (V/V). The purified RLO were collected and counted using a Malassez cell. At each purification operation, starting with 10 highly infected scallops, around $2 \times 10^8$ RLO were obtained.

**Total nucleic acid extraction from RLO.** A protocol was adapted from that described for the extraction of DNA from the vertebrate rickettsia *Coxiella burnetti* (Samuel et al. 1983, Vodkin et al. 1986). Purified RLO were resuspended in 1 ml of TE buffer pH 8.0 (Tris-HCl 10 mM, EDTA 1 mM) and incubated for 20 min at 37 °C with lysozyme at a final concentration of 1 mg $ml^{-1}$. After addition of sodium dodecyl sulfate (0.5 %) and proteinase K (100 µg $ml^{-1}$), a second incubation (2 h at 50 °C) resulted in complete lysis of the microorganisms. The sample was then extracted twice with phenol-chloroform (v/v) and once with chloroform. Total nucleic acids were recovered by absolute ethanol precipitation, washed with 70 % ethanol and then redissolved in TE buffer. The mean yield of this protocol was estimated to be about 14 µg of total nucleic acids from $2 \times 10^8$ purified RLO or 70 fg per RLO.

**DNA extraction from *Pecten maximus*.** As Saint-Jacques scallops *P. maximus* from Norway have been shown to be free from RLO infection (Le Gall et al. 1991), DNA was extracted from Norwegian scallop gill cells. The tissues were washed with sterile sea water to eliminate external contaminants, then homogenized and treated according to the protocol of Jeanpierre (1987).

**Cloning of RLO genomic DNA fragments.** Rickettsia-like organisms DNA was cut by digestion with *Eco*RI and *Hind*III endonucleases (Boehringer) and cloned into the pBluescript II plasmid vector (Stratagene) using the methods described by Maniatis et al. (1982). This vector carries (1) the gene for ampicillin resistance, (2) a polylinker, and (3) 2 promoter sequences present in the N-terminal portion of a lacZ gene fragment. Thus, vectors with the insert grow as white colonies in the XL1-Blue strain of *Escherichia coli* (Stratagen) which contains the lacZM15 gene on the F' episome.

**Probe preparation and blot hybridization conditions.** Inserts were excised from the vector by double

digestion with *Eco*RI and *Hind*III, separated through a 1 % agarose gel and purified from agarose using the Geneclean kit (Ozyme). Non-radioactive labelling and hybridizations were performed with the Amersham ECL kit (Amersham, France). Optimal hybridization buffer was supplied by the manufacturer and hybridization conditions were exactly those described in the kit (hybridization temperature 42 °C). This Amersham system allows direct probe labelling with horseradish peroxidase and detection of immobilized nucleic acids by the enhanced chemiluminescence emitted by the luminol and 4-iodophenol substrates. The resulting light is detected on autoradiographic films. Nucleic acid from test samples was denatured by alkali treatment and 100 µl aliquots were spotted onto a nylon membrane (Hybond N+, Amersham). The membrane was then dried at room temperature and baked for 2 h at 80 °C under vacuum before hybridization with the probes.

**Sequencing and synthesis of oligonucleotide primers.** The nucleotide sequences of 4 cloned fragments were determined using the classical Sanger chain termination method (Sanger et al. 1977). Two oligonucleotide primers that flank the DNA segment to be amplified by PCR (Fig. 1) were synthesized with a Milligene Cyclone DNA apparatus, using ß-cyanoethyl phosphoramidite chemistry. They were designated R151 and R152. Each primer contained 22 nucleotides and was designed to have a Tm of 66 °C. Both primers had limited internal complementarity and little identity with other DNA sequences, as revealed by a search in GenBank. R151 and R152 had the sequence 5'-TGTGATGCTCTAAGCCTGACTG-3' and 5'-TGTACTTTGCCTGACCTTGCCT-3', respectively. The oligonucleotides were cleaved from the columns with concentrated $NH_4OH$, deprotected at 55 °C overnight, filtered on Ultrafree (Millipore), desalted and redissolved in deionised $H_2O$.

**PCR amplifications.** Polymerase chain reaction amplifications were performed according to standard procedures (Guatelli et al. 1989). Briefly, 50 µl of reaction mixture contained RLO DNA, 100 µM of the 4 dNTPs, 250 ng of each oligonucleotide primer, 3 units of Taq DNA polymerase (Cetus), 5 µl of 10-fold concentrated reaction buffer (Cetus), $MgCl_2$ at different final concentrations and distilled water to give a final volume of 50 µl. The reaction mixtures were overlaid with paraffin oil to prevent evaporation and subjected to 30 cycles of amplification in a programmable heating block (Techne). During each cycle, the samples were incubated at 92 °C for 2 min (denaturation of the template), cooled to 55 °C for 2 min (annealing of the primers) and heated for 2 min at 70 °C (polymerisation of nucleotides). Finally, the samples were incubated for an additional 5 min at 70 °C and cooled to room temperature. Subsequently, 10 µl (20 % of each sample) was separated by electrophoresis in 2 % agarose gel and visualized under UV light to analyse the purity and size of the amplified products. The specificity of amplification was confirmed by Southern blot analysis using the peroxidase-labelled DNA fragment. The sensitivity of PCR was determined using different levels of starting template (10-fold dilutions) of purified RLO total nucleic acids from 100 ng to 1 pg.

## RESULTS

### Extraction and cloning of RLO nucleic acid

Nucleic acids were extracted from purified RLO. Because the mean yield of total nucleic acids per RLO was around 70 fg, several hundred million RLO were needed. To obtain the quantity of DNA necessary for our experiments several operations of RLO purification from infected scallop gills had to be performed. Attempts at RLO DNA purification by isopycnic centrifugation through CsCl gradient or by enzymatic digestion with RNase failed because of the low content of DNA in the total nucleic acid population, estimated to be about 1 %. Following these failed attempts, all the subsequent experiments were undertaken with RLO total nucleic acids, with the assumption of a 1 % DNA content. After digestion of RLO genomic DNA with *Eco*RI and *Hind*III and transformation of the XL1-Blue strain of *Escherichia coli*, several dozen recombinant colonies were isolated. Among these colonies, 23 were randomly selected and the inserts were characterized on the basis of their size (ranging between 300 and 2000 bp) and restriction enzyme pattern. A 1500 bp insert from one of these colonies containing the plasmid pKS15 was selected for hybridization studies.

### Specificity and sensitivity of a peroxidase-labelled probe from pKS15

The results of hybridization experiments performed with the peroxidase-labelled 1500 bp insert from pKS15 are summarized in Table 1. First, using the plasmid pKS15 as target DNA, the sensitivity limit of the hybridization assay was estimated to be around 20 pg. Considering that the size of the plasmid target is 4500 bp (MW: $3 \times 10^6$), the sensitivity limit can be estimated to be around $4.5 \times 10^6$ target copies.

The specificity of the probe was investigated using DNA from *Pecten maximus* scallops originating from Norway, a RLO-free area (Le Gall et al. 1991). No hybridization signal was detected whatever the blotted quantity of scallop DNA. These results clearly indi-

**pKSRE 15: extremity A**

```
          10         20         30         40         50         60
AAGCTTCACG CTTCCTCAGG CTGTCGATAT TCAAAAAATT AAAGCAAAAT ACACTGATGG
          70         80         90        100        110        120
TGTACTTTGC CTGACCTTGC CTAAACAAAT CAACGTAGAC GTTCAATAAT AAGAGAACAG
         130        140        150        160        170        180
AGCTGGTTTA TTAAACCGGC TCGACTTTGC ATTCGTTACT ACTACAATAT GGCTGGTTCT
         190        200        210        220        230        240
ATAACTGTTG TAATAGCGCT TTAGGGATAT CTTCTCTTTG AATGCCTTTA TCATCTTATC
         250        260        270
TGCAAACCGC TTGCTATCTT ATAGAGGGAG ATCCTTAAG
```

**pKSRE 15: extremity B**

```
          10         20         30         40         50         60
GAATTCCGTA ACTGTGTTTT ATTCTGAATG ATCTAAAAAA TAAACAAGCG TTTTAAACTG
          70         80         90        100        110        120
GTGTTTTNGT ATTGGTTTGA ATGCGTATTA AACAGAATTT TCTGAAAAAA AGATGCTGAG
         130        140        150        160        170        180
TTGACTATAT TGTTAAGACT TCATTGTGAT GCTCTAAGCC TGACTGGGGC TCTTATNGCT
         190        200        210        220        230        240
GATCAGATAT CTGTTAGTGT TATTGAAAGT GGCAGCGTTA CTGGTCAGAG TTATTGACGG
         250
GCATGATATA T
```

**pKSRE 9: extremity A**

```
          10         20         30         40         50         60
AAGCTTTTNG GGCTNATGCA NGCTNATCNA ATATGNCATG TATGAAAGCA ATGAGTATGT
          70         80         90        100        110        120
TTAAATACGA TCGNCTTTGG TTGTGCANCT AAGCCTTGAC GGGACGNNGC TGGACGAAGN
         130        140        150        160        170
TTTCNATGGC GAAGCCNAGA GCCTAACGAT GAACGGATCA GACTTTTTGT TCA
```

**pKSRE 7: extremity A**

```
          10         20         30         40         50         60
AAGCTTCTAG TGATTCACAA AATGAGGCGG CAGGGCAGAA ATACCAAAGA CAAAAATTCG
          70         80         90        100        110        120
TTCAGGCAAG TCAAAGGAGG TATTTTTCTG TTGCATGGCT TCATTGAATG TTTGGTGCAT
         130        140        150        160        170        180
ATGGCCCGAT GCCAATGAGA TTGCCCCTGC TGTTTTGTTT TTGCACCAGT CGCGCCATAA
         190        200        210        220        230        240
AACAGGTTGC CAGTGGGTGN AGTCTGGGTA ATATCAGGTA ATCACCGCCT TTTNCCAGCT

T
```

**pKSRE 18: extremity A**

```
          10         20         30         40         50         60
AAGCTTGTTC CATTCACAAC TGACCACAAT GCAATTGAAG ATAGCCTATT ATGACACGGC
          70         80         90        100        110        120
TTCGGTGTAT CGCTGACATT TTTTTATCAA TCTGCAGGTA TAGACCCGAC AGATCATCCC
         130        140        150        160        170        180
ATCTTCAGTC ATTCAGATAT AAAGTCCCAG CCCTTTACTT TTATACCGAG GCTCCTGAAG
         190        200        210        220        230        240
AATACCGATA ATCAGACATT CTGAATAACT CATTTTTATA GACTATATAG TCTTTCCCAA
         250        260        270        280        290
CGGATAACCC TTACNAACNA TCGTATTCCA ACCCGATAGG AATACCTCAT CACTTNA
```

Fig. 1. Sequence of insert extremities determined by Sanger dideoxynucleotide method (Sanger et al. 1977). For pKSRE 15, both extremities were sequenced (A: *Hind*III extremity; B: *Eco*RI extremity). For pKSRE 9, pKSRE 7 and pKSRE 18, only *Hind*III extremity was sequenced

Table 1. Hybridizations with peroxidase-labelled 1500 bp RLO probe. –: negative signal; +: positive signal; nd: not determined

| Target DNA | Quantity of nucleic acid deposited | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| | 10 µg | 5 µg | 1 µg | 500 ng | 100 ng | 10 ng | 1 ng | 100 pg | 20 pg | 10 pg |
| pKS15 | + | + | + | + | + | + | + | + | + | – |
| *Pecten maximus* | – | – | – | nd | nd | nd | nd | nd | nd | nd |
| RLO | nd | + | nd | + | – | – | – | – | – | nd |

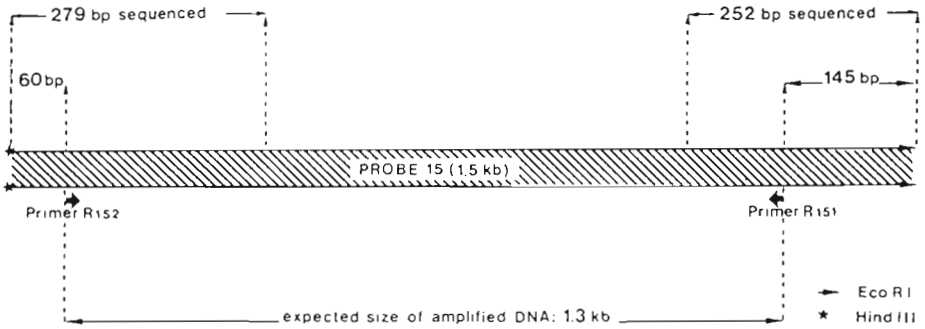


Fig. 2. Localization of primers R151 and R152 on probe 15 (1.5 kb), defining a 1.3 kb region to be amplified by polymerase chain reaction

cated that the probe failed to hybridize with genomic sequences of the RLO host species and confirmed its specificity for RLO DNA. Positive hybridization signals were observed for RLO total nucleic acid blots equivalent to 5 µg and 500 ng, respectively. Since we estimate the DNA content to be about 1%, the sensitivity limit for RLO DNA would be around 5 ng. Taking into account the size of Rickettsiales genomes, estimated to be $1.8 \times 10^6$ bp (MW $1.2 \times 10^9$) (Myers & Wisserman 1981), the sensitivity limit of hybridization would be about $2.5 \times 10^6$ RLO genomes. This estimation is in good agreement with the sensitivity limits deduced from the model experiments with pKS15.

### Sensitivity and specificity of PCR amplification

Some knowledge of RLO DNA sequences was a prequisite for PCR experiments since specific primers are needed. Several sequences corresponding to the extremities of different cloned restriction fragments were obtained and are listed in Fig. 1. A comparison of these RLO sequences with those contained in the nucleic acid data banks (EMBL, GenBank) did not reveal any significant homologies. The PCR primers R151 and R152 were selected from the extremities of the pKS15 insert. This primer pair should direct the synthesis of a 1300 bp fragment (Fig. 2).

In order to confirm the specificity of the amplification and to select optimal $MgCl_2$ concentrations, preliminary experiments were performed with 100 ng of RLO total nucleic acids and 10 µg of *Pecten maximus* DNA. After electrophoretic separation of the amplification products, the expected 1300 bp band was observed for the RLO DNA samples (Fig. 3A), but additional minor bands were also obtained. After Southern-blotting of the amplification products and hybridization with the DNA probe of RLO, the expected 1300 bp band only reveals positive but not the additional bands (Fig. 3B). On the other hand, using the DNA of *P. maximus* as substrate, the electrophoretic pattern of amplified products did not reveal any clearly distinguishable band, thereby confirming the specificity of amplification. The observed electrophoretic patterns of the amplified products were independent of the $MgCl_2$ concentration.

Using 3.5 mM $MgCl_2$, the sensitivity of the PCR was determined by using different levels of starting templates, prepared by 10-fold serial dilutions of purified RLO total nucleic acids. The electrophoretic patterns of amplified products obtained after 30 cycles of amplification from 100 ng to 10 fg of starting templates are shown in Fig. 4. Down to 10 pg of starting template, the expected size band was observed in the electrophoretic pattern, this band being unique for 100 pg and for 10 pg. With less template no bands were visible and on the basis of our estimation of a 1 % DNA content, it may be assumed that the sensitivity limit for PCR detection would be about 100 fg or 50 RLO genomes.

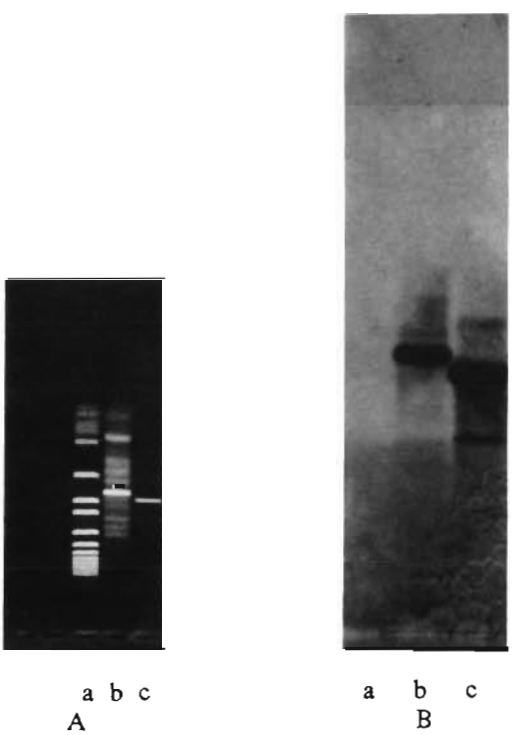


Fig. 3. Electrophoresis in 2% agarose gel (A) and Southern blot analysis (B) of the PCR amplified products. Lane a: molecular weight marker: 1 Kb DNA leader (Gibco BRL); lane b: PCR amplification of RLO total nucleic acids (100 ng); lane c: probe 15 (1.5 Kb) as positive control

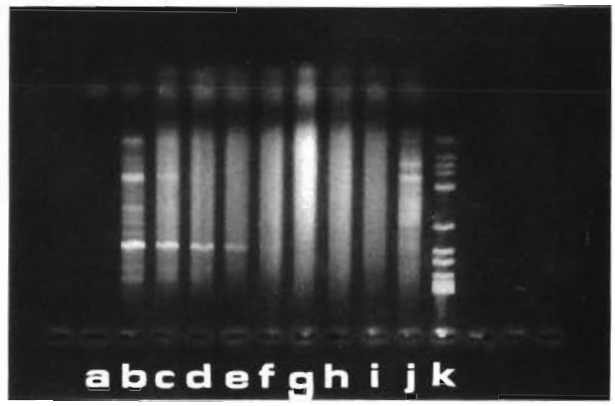


Fig. 4. PCR amplification of RLO total nucleic acids (lanes b to i: 10fold serial dilution from 100 ng to 10 fg) and *Pecten maximus* DNA (lane j). Lane a: negative control without DNA; lane k: molecular weight marker: 1 Kb DNA leader (Gibco BRL)

## DISCUSSION

The development of DNA-based technologies for molluscan RLO was undertaken to open the way for epidemiological and taxonomic studies. The lack of suitable *in vitro* systems for cultivation of molluscan RLO (Buchanan 1978) compelled us to purify them directly from infected scallop gills (Le Gall & Mialhe 1992). A nucleic acid extraction protocol adapted from that previously established for *Coxiella burnetti* (Samuel et al. 1983, Vodkin et al. 1986) proved to be successful for the extraction of RLO total nucleic acids. Purification of RLO DNA from total nucleic acids was also attempted but failed because of the very low yields, both of RLO themselves and of RLO DNA. Thus, all experiments were subsequently performed with total nucleic acids, with an RLO DNA content estimated to be about 1 %.

The cloning of RLO DNA fragments was easily performed by using Bluescript vector and the XLI Blue strain of *Escherichia coli*. The ease of cloning, leading to numerous specific DNA fragments, must be compared to the hybridoma technology necessary for the preparation of specific antibodies. The latter is much more time-consuming and is inferior in terms of reliability, yield and specificity of the reagents (Mialhe et al. 1988, 1992). The chief interest of this set of cloned RLO DNA fragments consisted of their potential application for the development and evaluation of DNA-based diagnostic methods, which could provide an alternative to light microscopy. The most direct application was to use the fragments directly for DNA hybridizations. These were undertaken only with non-isotopic labels because such diagnostic techniques will have to be performed in marine laboratories, which are generally not authorized to handle radioactive substances. The hybridization and detection system we used was based on the direct labelling of DNA with horseradish peroxidase using a commercially available kit, but other similar methods would be expected to give comparable results.

Specificity appeared to be very good since no signal was observed with a host DNA equivalent to 20 times the sensitivity limit determined for RLO total nucleic acids. The sensitivity of detection, estimated at about $2.5 \times 10^6$ copies, is low but in relative agreement with data published for other non-isotopic probes specific for different intracellular prokaryotes, like mollicutes (Razin et al. 1987), chlamydias (Rasmussen & Timms 1991) or rickettsias (Goff et al. 1988). The sensitivity limits are generally between 0.01 and 1 ng of genomic DNA, which is equivalent to about $10^3$ to $10^6$ organisms. The present results for *Pecten maximus* RLO may be explained by the large quantity of RNA present in the samples. However, the sensitivity limit determined with the recombinant plasmid pKS15 was similar ($4.5 \times 10^6$ copies), which may indicate that the probe labelling system or hybridization/detection method remain to be optimized. Some improvements of sensitivity might be achieved by using a combination of several cloned DNA fragments, constituting a few thousand base pairs (Razin et al. 1987). However, because the sensitivity is rather low, a straightforward hybridization method appears unsuitable for RLO diagnosis.

On the other hand, these probes constitute a unique set of tools for undertaking taxonomic comparisons between RLO associated with different molluscan species. Further investigations for determination of probe specificity would be essential before probes can be used as a diagnostic or epidemiological tool and would be especially useful for ascertaining whether or not sympatric mollusc species may be alternate hosts for RLO. In this respect, it is worth noting that in Brittany, RLO-infected *Pecten maximus* populations are mixed with other scallop species belonging to the *Chlamys* genus, in which gill RLO colonies have also been observed (Le Gall et al. 1988b).

Inside the order of Rickettsiales, it will become possible to determine the taxonomic relationships between this molluscan RLO and microorganisms, such as *Coxiella* sp. or *Wolbachia postica*, that show similar morphological features and that also exhibit intravacuolar growth (Weiss & Moulder 1984, Leu et al. 1989). Another way of carrying out these large scale taxonomic studies would be to compare ribosomal DNA (rDNA) sequences (Woese 1987), since rDNA sequences have been obtained for several rickettsias associated with vertebrates and/or arthropods. Because of the homologies which exist between the rDNA sequences from several rickettsias, the characterization

of *Pecten maximus* RLO rDNA would be based on PCR amplification by using primers corresponding to highly conserved sequences, as recently performed for *Vibrio* spp. bacteria (Rehnstam et al. 1989).

The relative inadequency of DNA probe sensitivities for RLO diagnostics led us to investigate PCR methodologies (Innis et al. 1990). This powerful nucleic acid amplification technique facilitates the synthesis of easily detectable amounts of DNA from only a few copies of the target nucleic acid sequences. However, because PCR amplifies the target DNA between 2 specific primers, it was first necessary to undertake sequencing of the cloned *Pecten maximus* RLO DNA fragments due to the complete absence of molluscan RLO DNA sequence information. The resulting sequences were assumed to be specific by their absence from data banks, and particularly when compared with all known rickettsial sequences. Our results seem to justify this assumption. A primer pair was arbitrarily selected from the DNA fragment cloned in pKS15, for which both extremities were sequenced. These 22-mer primers flank a target sequence equivalent to 1300 bp, a fragment size easily identifiable after electrophoresis on agarose gel.

Under optimal experimental conditions, the PCR appeared highly specific since no amplification was obtained with *Pecten maximus* DNA. It was also highly sensitive on the basis of serial dilution assays with RLO nucleic acids. The sensitivity limit, estimated to be 10 pg of RLO total nucleic acids corresponding to a few dozen RLO, agreed well with results obtained for purified DNA from vertebrate rickettsias (Tzianabos et al. 1989). Recently, papers describing the PCR amplification of clinical samples infected with *Rickettsia rickettsii* (Tzianabos et al. 1989) and *Rickettsia typhii* (Webb et al. 1990) have been published; similar experiments will now have to be performed for the detection by PCR of RLO in *P. maximus* samples. The essential aims of this research will be to obtain a very sensitive RLO detection method suitable for epidemiological studies. Firstly, it should be possible to identify early stages of infection in gill samples. Secondly, by applying PCR to ovocyte samples, it should allow us to ascertain whether or not transovarian transmission in Saint-Jacques scallop occurs (Le Gall et al. 1991); such a transmission has been demonstrated in several arthropod vectors (Burgdorfer & Brinton 1975) and, based on histological examinations, has been suspected for molluscan RLO (Fries et al. 1991). Taking into account the large geographical distribution of scallop rickettsiosis and the increasing development in the production of scallop spat in hatcheries, a better understanding of RLO transmission is urgent and would be served well by a rapid progress in the development of new diagnostic methods.

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