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Rapid and low-level toxic PCR-based method for routine identification of *Flavobacterium psychrophilum*

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Summary We describe a rapid, low-toxicity and simple method for the detection of the bacterial fish pathogen *Flavobacterium psychrophilum*. The method, based on the polymerase chain reaction (PCR), combined the electrophoresis of PCR products in a vertical agarose gel and a modified methylene blue stain. DNA was amplified directly either from bacterial suspensions or from tissues experimentally infected with *F. psychrophilum*, using different non-toxic commercial DNA extraction kits. The protocol allowed to detect 15 to 150 cells of the pathogen in bacterial suspension, without prior DNA extraction, and 7500 to 75,000 cells in seeded spleen tissue and ovarian fluid using Dynabeads DNA DIRECT™ extraction system. This method, which has the advantage of not using hazardous products, is proposed as a fast tool for routine identification of *F. psychrophilum*.

Key words *Flavobacterium psychrophilum* · Low toxicity · Methylene blue · Vertical electrophoresis · Fish pathogenic bacteria

Introduction

Flavobacterium psychrophilum has been known as the causative agent of coldwater disease (CWD) in fish since 1948 [3]. The same bacterium has been shown to be the pathogen involved in the rainbow trout fry syndrome (RTFS) in farmed fish [2, 13, 18], occasionally also being isolated from non-salmonid fish [7, 10]. Identification of this fish pathogen is generally based on the study of its phenotypical and serological characteristics [1, 8, 11, 12, 22]. However, identification is usually delayed or impeded by rather fastidious growth and weak reactivity in most biochemical tests. Serological methods such as immunofluorescence (IF), enzyme-linked immunosorbent assay (ELISA) and slide agglutination test are expensive and time consuming, and require the preparation of specific antisera against the main pathogenic serotypes [8, 11, 22]. Attempts have been made to change these systems for more effective DNA-based methods. Recently, some authors have described primer pairs for specific PCR amplification of small subunit 16S rRNA gene of *F. psychrophilum* [20, 21]. This technique provides sensitive and fast diagnosis of the diseases caused by this pathogen.

In this safety-conscious age we live in, it is desirable to develop procedures that do not use products that are toxic to the worker nor produce negative environmental side effects. Presently, most of the described PCR-based detection methods use hazardous products. Many DNA extraction protocols still include steps using organic solvents, such as phenol/chloroform, that are considered highly toxic. Moreover, detection of PCR products in agarose gels typically involves the use of fluorescent intercalating dye ethidium bromide and UV light. Although this system is rapid, simple to perform and very sensitive, it has important problems: ethidium bromide has carcinogenic effects that complicate handling and disposal of the chemical, and ultraviolet radiation is dangerous, particularly to eyes. Several studies have been made to replace these methods of extraction and visualization of DNA by others without these disadvantages [6, 16, 17].

The aim of this study was to develop a fast, sensitive, economic and low-toxicity PCR-based method for routine diagnosis of disease caused by *F. psychrophilum*. The usefulness and sensitivity of this method were assessed. The method avoids the exposure of the operator to hazardous products and the utilization of sophisticated equipment to treat the dangerous residues.

Materials and methods

Bacterial strain and growth conditions *Flavobacterium psychrophilum* NCIMB 1947 was the bacterial strain used for the study. The bacterium was stored at -80°C in modified Anacker and Ordal broth [19] with 15% glycerol. The strain was routinely grown on modified Anacker and Ordal agar plates and cultured at 15°C for 3-4 days.

Preparation of samples prior to PCR Assays were carried out with pure bacterial culture and experimentally infected fish homogenate. The NCIMB 1947 strain was grown as described above, scrubbed off the agar plates and subsequently diluted in sterile phosphate buffered saline to obtain 3×10^8 to 3×10^1 cells per ml (McFarlan scale no 1). Spleen and ovarian fluid were removed from gravid female rainbow trouts free of *F. psychrophilum*, homogenized in phosphate buffered saline 50% (w/v) for 30 s, and seeded with known amounts of bacterial suspensions giving final concentrations of 1.5×10^8 to 1.5×10^1 CFU per ml in the extracts.

Bacterial numbers were verified by plating dilutions onto modified Anacker and Ordal agar, incubating at 15°C for 3-4 days and counting the colonies produced.

DNA extraction DNA was extracted using three low-toxicity commercial systems: Chelex 100 (Sigma, Madrid, Spain), InstaGene Matrix (BioRad, Hercules, USA) and Dynabeads DNA DIRECT™ (DynaL, Oslo, Norway). DNA purification was performed according to the manufacturer's protocol. Bacterial suspensions and inoculated fish homogenates, without prior DNA extraction, were also used as templates for PCR amplification.

PCR amplification All PCR amplifications were performed with Ready-to-go PCR beads (Amersham Pharmacia Biotech, Piscataway, USA) by adding one pmol of primers and 0.5 μl of template (extracted DNA, bacterial suspension or fish homogenate) to each PCR bead.

Primers PSY1 (5'-GTTGGCATCAACACACT-3') and PSY2 (5'-CGATCCTACTTGCCTAG-3') described by Toyama et al. [20] were used. Samples were subjected to 40 cycles of amplification in a DNA thermal cycler (Mastercycler personal Eppendorf [Eppendorf, Hamburg, Germany]). A preheating step at 94°C for 5 min was included. The amplification cycles used for denaturation, primer annealing to the template and primer extension were: 94°C for 30 s, 51°C for 90 s and 72°C for 120 s. A final extension step of 5 min at 72°C was also carried out. PCR tubes were then held at 4°C . Negative control (no template) was included in each batch of PCR reaction. Total PCR amplification time was 4 h.

Analysis of PCR products PCR products were simultaneously analyzed by (i) conventional horizontal 1% agarose gel electro-

phoresis (100 V, 1 h) followed by 5-min ethidium bromide staining (HAGE/EB method), and (ii) our proposed alternative low-toxicity method based on the use of vertical agarose gel electrophoresis and methylene blue staining (VAGE/MeB method). Briefly, PCR products were electrophoresed vertically for 30 min at 100 V on a 1% (w/v) agarose gel (1 mm thick) and visualized using a modification of the methylene blue stain previously described by Johnson et al. [9]. After electrophoresis, gels were submerged for 20 min in 10 μg per ml MeB in deionized water and then rinsed with tap water. Subsequently gels were scanned (Macintosh ColorOne Scanner 1200/30), air-dried on a Whatman paper and stored between two plastic sheets.

In order to evaluate the potential usefulness of our method for the analysis of PCR products, serial dilutions of pure culture and seeded tissue samples, with or without prior DNA extraction, were analyzed in parallel by VAGE/MeB and HAGE/EB procedures.

Results and Discussion

The usefulness of our VAGE/MeB method for the analysis of PCR products, obtained from bacterial suspensions and experimentally infected spleen and ovarian fluid samples, was evaluated. The PCR amplification of *F. psychrophilum* DNA using PSY1 and PSY2 primers generated a single product with the expected 1089 bp length. No amplification products were obtained from negative control reactions. The detection level of both HAGE/EB and VAGE/MeB methods was about 15 to 150 cells for pure bacterial cultures (Fig. 1) and 7500 to 75,000 cells for seeded spleen tissue and ovarian fluid (Fig. 2). The VAGE/MeB method provides a good visualization of PCR products, similar to that obtained with the conventional method, in only 50–55 min. The 1 mm thick gel allows easy staining and destaining of the gel, so the washing step (1 hour) of the original staining method was replaced by a simple rinse in tap water. Besides, vertical electrophoresis is faster than the horizontal one so the time of the entire process was also reduced. We also found that the methylene blue solution can be reused 10–15 times.

To develop a complete low-toxicity protocol, we also evaluated different non-toxic methods to obtain DNA for the PCR amplification. There were no differences at the detection level for PCR products obtained directly from the bacterial suspension or after DNA extraction with Chelex 100, Instagene and Dynabeads. The detection level (15 to 150 cells) was similar to that obtained in other recent studies in which phenol/chloroform was the system used for DNA extraction [15, 21]. In contrast, the detection level for spleen tissue and ovarian fluid seeded with *F. psychrophilum* (7500 to 75,000 cells) was lower than that obtained with pure cultures. This reduction of the sensitivity in comparison with pure culture can be explained by the presence of host DNA and of various

undefined amplification inhibitors [14, 21]. Dynabeads DNA DIRECT™ proved to be the only extraction system which allows the detection of the pathogen in fish fluid and tissues (Fig. 2). The seeded fish homogenates used directly for the PCR, without previous extraction, were negative.

In conclusion, the proposed VAGE/MeB was found to be a rapid, inexpensive and low-toxicity protocol for the detection of *F. psychrophilum*. The technique is simple enough to be used in small laboratories where the facilities and budgets are limited. We suggest that DNA can be routinely amplified from whole *F. psychrophilum* cells without prior extraction, as has been reported for other fish pathogens [4, 5]. The entire process of amplification, gel electrophoresis and visualization of PCR products can be performed in about 5 h compared to 7–8 hours for HAGE/EB plus phenol chloroform extraction. The detection is possible in infected tissues using the Dynabeads extraction method. In this case the total time of the process is only increased by 15–20 minutes.

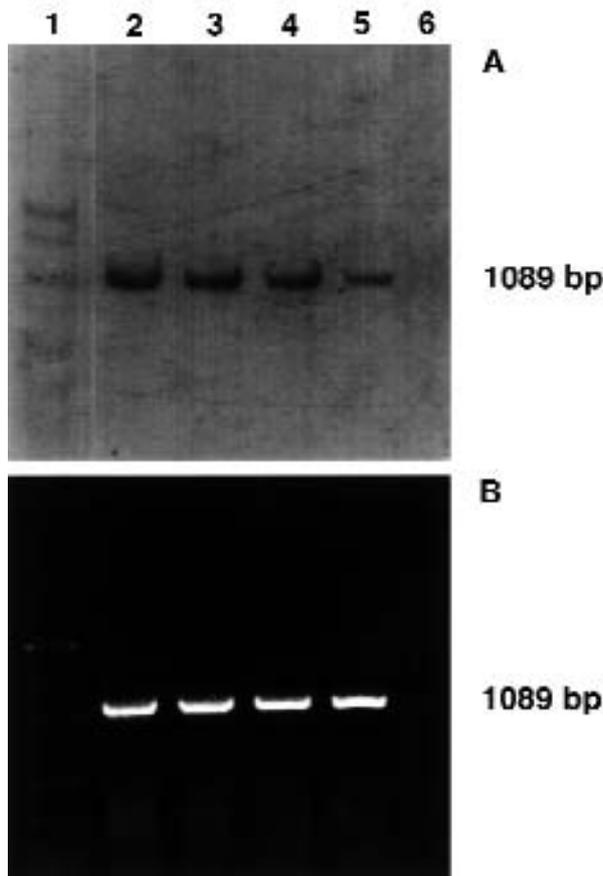


Fig. 1 Visualization of PCR products from *Flavobacterium psychrophilum* analyzed by VAGE/MeB (A) and HAGE/EB (B) procedures. Duplicate sets of PCR products from suspensions of 1.5×10^5 (lane 2), 1.5×10^4 (lane 3), 1.5×10^3 (lane 4), 1.5×10^2 (lane 5), 1.5×10^1 (lane 6) *F. psychrophilum* cells were electrophoresed into agarose gels. Lane 1, PCR marker. Detection level is between 1.5×10^1 and 1.5×10^2 cells

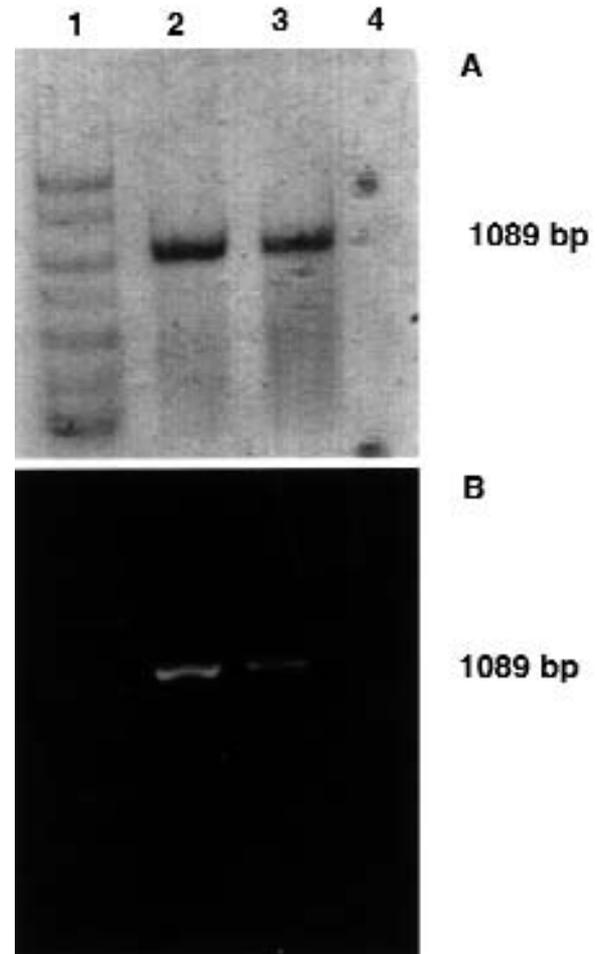


Fig. 2 Analysis by VAGE/MeB (A) and HAGE/EB (B) procedures of PCR products from DNA extracted with Dynabeads from trout spleen seeded with *Flavobacterium psychrophilum*. Duplicate sets of samples spiked with 7.5×10^5 (2), 7.5×10^4 (3), 7.5×10^3 (4) *F. psychrophilum* cells were electrophoresed into agarose gels. Lane 1, PCR marker. Detection level is between 7.5×10^3 and 7.5×10^4 cells

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