An improved enzyme-linked immunosorbent assay (ELISA) for detection of *Flavobacterium psychrophilum* isolated from salmon and rainbow trout

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**Abstract**

A double antibody sandwich enzyme-linked immunosorbent assay (ELISA) was developed in order to improve the diagnostic of the diseases caused by *Flavobacterium psychrophilum* in salmon and trout. With incubation steps of 1 hour at 37°C for coating antibodies and 30 minutes at 37°C for antigens and conjugates, the detection limit was in the range of 1 x 10⁴ cells ml⁻¹. Strains isolated from salmon and rainbow trout were successfully identified in an individual ELISA, which confirms the validity of the method for detection of *F. psychrophilum* serotypes. This biotin-avidin short protocol ELISA could be the technique of choice for seroepidemiological studies because it is a specific, sensitive, rapid and inexpensive procedure.

**Introduction**

*Flavobacterium psychrophilum* (syn. *Cytophaga psychrophila*, *Flexibacter psychrophilus*) remains a major cause of rainbow trout fry syndrome (RTFS) and bacterial cold water disease (BCWD) in wild and hatchery-reared salmonids, particularly in Europe, America and Japan (Austin & Austin, 1999). Additionally, in the past decade, the host range of this bacterium appears to have broadened, with several more non-salmonid fish species being affected (Amita *et al.*, 2000).

Currently, a laboratory definitive diagnosis of this infectious disease requires the classic isolation of the bacterium on agar media followed by the taxonomical analysis which may take several days to yield results (Lorenzen *et al.*, 1997). Since there is a need for more rapid diagnosis, methods for detection of antigens could help to obviate the shortcomings of conventional methods. However, the specificity and sensitivity of these serological techniques largely described in the literature is determined by the methodology of the immunoassays and varied depending on the antigen and antiserum used (Crump *et al.*, 2001).

Recently, a microtiter agglutination method and an ELISA technique have been developed for detection of *F. psychrophilum* serotypes (Lorenzen & Olesen, 1997; Izumi & Wakabayashi, 1999). However, these researchers reported the inability of the methods to differentiate between *F. psychrophilum* serotypes unless cross-absorbed antisera and “O” antigens were used.
Thus, here we report the development of a double antibody sandwich short protocol ELISA based on whole-cell antigens and unabsorbed polyclonal rabbit antisera. The method was improved by the use of a biotin-avidin system and optimised as simple, routine and inexpensive diagnostic tool for the rapid (four hours or less), specific and sensitive detection of *F. psychrophilum* serotypes.

**Materials and Methods**

**Bacterial strains and growth conditions**

*F. psychrophilum* strains included in the study were isolated from salmon (*Oncorhynchus kisutch*), rainbow trout (*O. mykiss*), and other reference bacterial strains from the National Collection of Industrial and Marine Bacteria (NCIMB, Aberdeen, Scotland) and belonging to different species of genus *Flavobacterium* (*F. columnare*, *F. succinicans*) as well as *Flexibacter maritimus* and *Aeromonas salmonicida* were used to test the specificity of the assay. Bacteria were routinely cultured in modified Anacker and Ordal agar medium (MAOA) (*Flavobacterium* strains) (Toranzo & Barja, 1993), tryptone soy agar (TSA, Oxoid) (*Aeromonas salmonicida* isolate) and *Flexibacter maritimus* medium (FMM) (Pazos et al., 1996). The taxonomical position of the bacterial strains was confirmed using morphological, physiological and biochemical test and API systems as previously described (Pazos et al., 1993; Santos et al., 1992; Santos et al., 1993).

**Antigens**

Bacterial cells harvested from a 48-72 hours culture at 18°C on agar plates were pelleted and formalized in a 0.3% (v/v) saline buffer (phosphate buffered saline pH 7.4).

**Rabbit antibodies**

Polyclonal antisera for diagnostic purposes were produced against *F. psychrophilum* strains NCIMB 1947 and PT 4.1 and named anti-NCIMB 1947 and anti-PT4.1, respectively. Two kilogram New Zealand white rabbits were immunised intravenously at three days intervals with 0.2, 0.4, 0.8 and 1.0 ml of the appropriate formol-fixed bacteria adjusted to 1 x 10^9 cells ml^-1 (McFarland standard nº 3). Two weeks later a booster inoculum of four injections (1.0 ml/dose) administered as before were given. Anaesthetised rabbits were bled by cardiac puncture eight days after the last injection. Sera recovered were aliquoted undiluted and frozen at -20°C until use. Preimmune sera were previously collected from the rabbits. Antibody levels were determined in 96-well round-bottomed microtiter plates (Corning, New York) by agglutination as described by Lorenzen & Olesen (1997).

The immunoglobulin G fraction (IgG) from antisera was purified through a pre-packed protein A sepharose affinity chromatography column according to the manufacture’s protocol (HiTrap™ Protein A, Amersham Pharmacia Biotech AB, Sweden). Protein concentration of purified antibodies was determined by the method of Bradford (1976) using the commercial kit available from Bio-Rad (Bio-Rad Laboratories). Purified antibodies were kept at -20°C.

**Conjugates**

The IgG was biotinylated with a hydroxysuccinimide ester of biotin (Sigma Chemical Co., St. Louis, USA) as described by
Tijssen (1987). Stock biotin labelled conjugates were stored at -20°C.

Enzyme-linked immunosorbent assay
The rapid biotin-avidin assay was performed according to the double antibody sandwich method (Rangdale & Way, 1995). Dilutions of the reagents (unlabelled coating antibodies, biotin conjugates and extravidin peroxidase) were determined in previous checkerboard titration with appropriate positive and negative controls. Different volumes, temperatures and incubation times were also evaluated. Working conditions were chosen that showed maximum reactivity of the positive and minimum reactivity of the negative specimen.

The wells of flat-bottomed microtiter plates (Nunc-Immuno™ Plate MaxiSorp™ Surface, Denmark) were sensitised with 100µl of each purified anti-\textit{F. psychrophilum} serum diluted in 0.05M sodium carbonate-bicarbonate buffer pH 9.6. Plates were incubated for 1 hour at 37°C, washed three times with 0.01M phosphate buffered saline (PBS) pH 7.4 supplemented with 0.05% Tween 20 (PBS-T) and subsequently blocked with 100µl of PBS-T containing 1% bovine serum albumin (PBS-T-BSA) for 30 min at 37°C to minimise unspecific adsorption.

For all the following steps, PBS-T-BSA was used as diluent, each well receiving 100µl and incubations were performed at 37°C for 30 min unless otherwise indicated.

Once blocked, plates were washed three times with PBS-T before addition of antigen (formalin killed bacterial cells) and positive and negative controls. After sample incubation, plates were washed as above and diluted biotinylated conjugate of each antiserum (anti-NCIMB 1947 and anti-PT 4.1) were added.

Following another incubation and washing step, the wells received ExtrAvidin-Horseradish Peroxidase (Sigma Co.) dilution. Unbound material was removed and plates developed with the substrate 0-Phenylenediamine Dihydrochloride (OPD, Sigma Co.) diluted in 0.05M phosphate-citrate buffer pH 5.0 containing 0.03% sodium perborate (Sigma Co.) and incubated for 15 min in the dark at room temperature. The reaction was stopped by the addition of 1M H\textsubscript{2}SO\textsubscript{4}. Absorbance values were immediately read with an ELISA microplate reader (Bio-Rad Model 550) at 492 nm. Results were considered positive if absorbance exceeded at least two standard deviations above the mean of negative controls.

Results and Discussion
The agglutinating titer of the diagnostic antisera obtained in this study ranged from 1/128 to 1/256 against the homologous antigen, similar to that described by other authors (Lorenzen & Olesen, 1997). The protein concentration of the IgG preparations ranged from 6.2 mg ml\textsuperscript{-1} (anti-NCIMB 1947) to 3.5 mg ml\textsuperscript{-1} (anti-PT 4.1). In the ELISA, the most consistent results (A\textsubscript{492nm} value about 2.0 for the homologous antigen) were obtained using coating antibodies at 2.5 µg ml\textsuperscript{-1}, biotinylated IgG at 1/1000 and extravidin peroxidase at 1/5000 dilution. These findings were slightly higher than in another study (Rangdale & Way, 1995), in which an ELISA was designed for detection of \textit{F. psychrophilum} directly from fish spleen tissue.
The antisera produced in the present work were specific in ELISA for their homologous bacteria; thus, both salmon strains reacted strongly with anti-NCIMB 1947 but not with anti-PT 4.1, while both rainbow trout strains reacted strongly with anti-PT 4.1 but not with anti-NCIMB 1947. According to these results, the strains were divided into two groups: serotypes 1 and 2. The serotype 1 comprised NCIMB 1947 and NCIMB 2282. The serotype 2 comprised PT 4.1 and NCIMB 13384.

In previous works performed with a microtiter agglutination method (Wakabayashi et al., 1994; Izumi & Wakabayashi, 1999), it was indicated that *F. psychrophilum* isolates from coho salmon and rainbow trout shared common antigen(s) and serotypes were only detected when absorbed antisera and the thermostable “O” antigen were used. Thus, the ELISA results described here compare favourably with the microtiter test, particularly because using whole-cell preparations distinguish 4 isolates into 2 serotypes without the need for absorption. This appears to be a new observation as all other workers (Wakabayashi et al., 1994; Lorenzen & Olesen, 1997; Rangdale & Way, 1995) have found that antibodies to *F. psychrophilum* have to be absorbed out to yield serotype-specific antibodies in both agglutination and ELISA methods. Differences in the immunization schedules used to raise rabbit antisera as well as in the concentrations of reagents used in the ELISA methodology could probably explain the results obtained in the present study.

Specificity of the ELISA was also investigated by introducing other bacterial antigens into the assay. Thus, cross-reactions were not observed with other closely related *Flavobacterium* spp. bacteria, neither with *Flexibacter maritimus* and *Aeromonas salmonicida*.

Sensitivity of this rapid ELISA was analysed with serial dilutions of *F. psychrophilum* whole-cells. The standard curves obtained were very similar for anti-NCIMB 1947 and anti-PT 4.1. The ELISA was able to detect $1 \times 10^4$ cells of *F. psychrophilum* per ml.

Although two sandwich ELISA were reported in earlier papers with an overnight incubation step for coating antibodies and 1 or 2 hours for the conjugates (Lorenzen & Olesen, 1997; Rangdale & Way, 1995), the present ELISA consists of one incubation step of 1 hour and three of 30 minutes each with the antigens, biotinylated IgGs and avidin-peroxidase. Moreover, the ELISA can still be adapted if precoated plates are used, in which case the assay takes about three hours.

In short, several conditions make this ELISA procedure very attractive as a diagnostic option for *F. psychrophilum* diseases: 1) can be performed in most laboratories, 2) requires no specialised equipment, 3) visual interpretation of a positive or negative reaction is possible with a naked eye, 4) large numbers of samples can be examined in microtiter plates at the same time, 5) can provide results in a few hours, and 6) allows serotyping and epidemiological studies.

In this sense, because the knowledge about serotype distribution of *F. psychrophilum* is still rare, types should be evaluated in every country in order to ensure an optimal formulation of future vaccines. The introduction of an ELISA system based on a pool of anti-*F. psychrophilum* sera reacting with serotypes
most commonly isolated from salmon and rainbow trout might be soon investigated for practical and economical reasons.

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References


