Quantitative Detection of Viable *Flavobacterium psychrophilum* in Chum Salmon *Oncorhynchus keta* by Colony Blotting and Immunostaining

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(Received March 4, 2008)

**ABSTRACT**—Quantitative detection of viable *Flavobacterium psychrophilum*, the etiological agent of bacterial coldwater disease, was evaluated by colony blotting and immunostaining. Bacterial colonies isolated from chum salmon *Oncorhynchus keta* ovarian fluids on a modified *Cytophaga* agar plate were blotted onto a nitrocellulose membrane and immunostained with antiserum against *F. psychrophilum*. Although the blotted colonies were strongly or weakly stained with the antiserum, blots from colonies of *F. psychrophilum* were distinguishable from those of other yellowish colonies by digital processing of the colony-blotted membrane photograph with an image-analyzing software. It was also confirmed that 12 strains of *F. psychrophilum* were all positive by the present method, while the subjected six isolates, which formed yellowish colonies but were not identified as *F. psychrophilum* by PCR targeting *gyrB* gene, and other reference six strains, *F. branchiophilum*, *F. limicola*, *F. granuli*, *Pseudomonas flavescens*, *P. fluorescens*, *Chryseobacterium daecheongense*, were all negative. From these results, the present procedure using colony blotting and immunostaining is useful for quantitative detection of viable *F. psychrophilum* from ovarian fluids and kidneys of chum salmon.

**Key words:** *Flavobacterium psychrophilum*, bacterial coldwater disease, BCWD, colony blotting, immunostaining, digital processing

*Flavobacterium psychrophilum* (Bernardet et al., 1996), gram-negative and yellow-pigmented bacteria, is the etiological agent of bacterial coldwater disease (BCWD), which causes severe losses in aquaculture and resource enhancement of salmonid fish (Holt et al., 1993; Nematollahi et al., 2003). *F. psychrophilum* is generally detected in clinically infected coho salmon *Oncorhynchus kisutch* (Wood and Yasutake, 1956), sockeye salmon *O. nerka*, chinook salmon *O. tshawytscha* (Rucker et al., 1953), Atlantic salmon *Salmo salar* (Cipriano, 2005) and rainbow trout *O. mykiss* (Bernardet and Kerouault, 1989; Lorenzen et al., 1997) mainly in European and North American countries. Outbreaks of BCWD have been reported in coho salmon (Wakabayashi et al., 1991), ayu *Plecoglossus altivelis* (Iida and Mizokami, 1996) and masu salmon *O. masou* (Amita et al., 2000) since the 1980s in Japan, especially in Hokkaido areas, a northern part of Japan, industrial damages due to BCWD have increased in salmonid fish farm since around 2000.

Chum salmon is one of the most important species in Hokkaido coastal fisheries, because approximately one billion chum salmon fry has been released every year since 1980, and over 30 million of adult fish have returned to the coastal areas of Hokkaido since middle of the 1980s (Watanabe, 1999). Although no mass mortalities due to acute BCWD has been reported in chum salmon, it is considered that *F. psychrophilum* could be an opportunistic pathogen to chum salmon because it is isolated or detected from chronically dead juvenile of chum salmon (Holt et al., 1993). In coho salmon and rainbow trout, *F. psychrophilum* is highly detectable from ovarian fluids (Baliarda et al., 2002), and it was reported that fish mortalities increased with a rising of inoculated doses of viable *F. psychrophilum* in experimental infection (Madsen and Dalsgaard, 1999; Garcia et al., 2000). Moreover *F. psychrophilum* isolates from adult...
of chum salmon also showed pathogenicity to salmonid fishes including chum salmon (Misaka and Suzuki, 2007).

Several detection methods for *F. psychrophilum* have been developed, such as culture isolation with modified *Cytophaga* agar (Wakabayashi and Egusa, 1974; Wakabayashi et al., 1991), PCR (Toyama et al., 1994; Izumi and Wakabayashi, 2000) and immunofluorescence antibody technique (IFAT) (Madetoja and Wiklund, 2002). Unfortunately, it is very difficult to evaluate the influence of *F. psychrophilum* to chum salmon resource in the coastal areas of Hokkaido, because many yellowish bacteria forming similar colonies to *F. psychrophilum* were always isolated together. No technique has been established for rapid, quantitative and specific detection of viable *F. psychrophilum*. Thus, in the present study, we evaluated the quantitative detection of viable *F. psychrophilum* in ovarian fluids and kidneys of chum salmon by colony blot and immunostaining with antiserum against *F. psychrophilum*.

Materials and Methods

Strain and plate cultivation

*F. psychrophilum* strains and other bacterial isolates were listed in Table 1. A total of 12 strains of *F. psychrophilum* from salmonid and ayu, i.e. NCMB1947T (type strain), Fp-B, FPC814, Fp-D, FPC840, Fp-A, Fp-C, Fp-W, Fp-K, Fp-O, Fp-T and Fp-Z, and reference 6 strains, i.e. *F. branchiophilum* ATCC 35035, *F. limicola* NBRC 103156T, *F. granuli* NBRC102009T, *Pseudomonas flavescens* NBRC 103044T, *P. fluorescens* NBRC 101042, *Chryseobacterium daecheongense* NBRC 102008T, were used in this study. Moreover, unidentified yellowish bacterial isolates from the ovarian fluids and the kidneys of chum salmon, Fp-Q, Fp-R, Y-01, Y-02, Y-03, Y-04, were used. These bacteria were cultured using modified *Cytophaga* agar (CAM; 0.2% tryptone, 0.05% yeast extract, 0.02% beef extract, 0.02% sodium acetate, 0.02% calcium chloride, 1.5% agar, pH 7.2; Wakabayashi and Egusa, 1974) at 15°C for 5–7 days. CAM was also used for bacterial isolation from fish samples, and numbers of viable bacteria were expressed as colony forming unit (CFU).

**Bacterial isolation from fish ovarian fluids and kidneys**

Ovarian fluid and kidney samples were taken from matured females of chum salmon at Shizunai River, southeastern Hokkaido, on October 27, 2006. The samples were taken aseptically and stored at −80°C until use. A hundred microliter of each ovarian fluid was spread onto CAM and incubated at 15°C for 5–7 days for isolation of *F. psychrophilum*.

**Colony blotting and immunostaining**

NCMB1947T was cultured in CBm (CAM without agar) with shaking for 24 h and the stationary phase (about 10^5 CFU/ml) of the cultured bacteria was used as positive control. A ten microliter of positive control and

<table>
<thead>
<tr>
<th>Species</th>
<th>Strains/Isolates</th>
<th>Host fish/Source of isolation</th>
<th>Isolation year</th>
<th>Location</th>
</tr>
</thead>
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<tr>
<td>Flavobacterium psychrophilum</td>
<td>NCMB1947T</td>
<td>coho salmon</td>
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colonized bacteria on CAm were blotted onto nitrocellulose (NC) membrane (Advantec). The blotted membranes were incubated in the buffer-1 (0.1 M maleic acid, 0.15 M NaCl, pH 7.5) contained 5% (w/v) of skim milk at room temperature for 30 min with shaking. After three times washing with the buffer-1 for 20 min, the membrane was immunostained with rabbit antiserum against NCMB1947T at room temperature for 2 h. Followed by a washing with the buffer-1 for 20 min, the membrane was treated with alkaline phosphatase conjugated swine antiserum against rabbit immunoglobulins (Dako) at room temperature for 1 h. All antisera were diluted at 1:500 with the buffer-1 before use. The immunostained membrane was immersed in the buffer-2 (100 mM Tris-HCl, 100 mM NaCl, 50 mM MgCl₂, pH 9.5) containing substrate (0.034% nitro blue tetrazolium chloride and 0.018% 5-bromo-4-chloro-3-indolylphosphate disodium salt) at room temperature for 5 min. The color reaction was stopped by washing the membrane in TE buffer (10 mM Tris-HCl, 1 mM EDTA, pH 8.0).

Digital processing

Digital photographs of the colony blotted membranes were taken using digital camera (Fine Pix V10, Fujifilm, Japan) in 2,592 × 1,944 pixel, and digitally processed with software Image J (Version 1.36). The red part out of RGB (red, green and blue) colors was selected from the digital photographs. The threshold of the depth in color was adjusted using the signal of NCMB1947T on the membrane as the positive control, i.e., upper limit and lower limit of the threshold were adjusted to the both ends of modal group including the depth in color of positive control.

Biological characteristics of unidentified yellowish bacteria from chum salmon

NCMB1947T and unidentified yellowish bacterial strains, Fp-Q, Fp-R, Y-01, Y-02, Y-03 and Y-04, were subjected to biological tests for motility, catalase, oxidase, acid production from glucose, oxidation/fermentation, degradation of casein, gelatin, starch and tyrosine, growth at 37°C according to standard procedures. Morphological observation of bacteria was carried out by microscopy with magnification of 1,000 after Gram stain. Those bacteria were also subjected to PCR amplification targeting gyrB gene of F. psychrophilum as described below.

Identification of F. psychrophilum by PCR

Identification as F. psychrophilum was performed by PCR with the primers targeting gyrB gene of F. psychrophilum according to the method of Izumi and Wakabayashi (2000). Bacterial colonies were boiled in 20 μL of distilled water for 5 min, and centrifuged at 8,000 × g for 5 min, and a portion of the resultant supernatant was used as a template for PCR amplification.

Influence of ovarian fluids and kidneys in the quantitative detection of F. psychrophilum

Each two samples of 50 mg kidney and 50 μL ovarian fluid were mixed with 100 μL of NCMB1947T cultured in CBm and inoculated on CAm to examine the influence of these tissues in the quantitative detection of F. psychrophilum.

Results and Discussion

As a result of bacterial isolation from an ovarian fluid of chum salmon, many bacteria including yellowish colonies were colonized on a CAm plate (Fig. 1A). All of the bacterial colonies on the CAm and three positive control of F. psychrophilum (indicated by arrows in Fig. 1B) were blotted onto a NC membrane for immunostaining with antiserum against F. psychrophilum (Fig. 1B). The blots of the yellowish colonies were strongly or weakly stained with the antiserum, and the threshold of the depth in color of the red part out of RGB colors exhibited bimodal with Image J. The upper limit and lower limit of

![Fig. 1. Colony blot for quantitative detection of Flavobacterium psychrophilum from an ovarian fluid of chum salmon. (A), Colonies on modified Cytophaga agar. A 100 μL of an ovarian fluid of chum salmon was cultured on the agar. (B), A nitrocellulose membrane after colony blotting. (C), Digital picture after processing by Image J. Arrows on B and C indicate NCMB1947T as a positive control. Number 1–18 on the figures indicate the strongly immunostained colonies. Bar = 2 cm.](image-url)
the threshold were adjusted to the both ends of modal group including the depth in color of positive control (Fig. 2), then the strongly stained eighteen blots (number 1–18 colonies in Fig. 1B) appeared by this digital processing (Fig. 1C). All of the colonies on the CAM were subcultured for identification with PCR targeting gyrB gene of F. psychrophilum. PCR products with 1,017 bp corresponding to the target region were amplified only from the colonies strongly immunostained with the antiserum except number 3 colony in Fig. 1B and also exhibited positive signals in Fig. 1C, but no PCR product was observed from the colony exhibiting weakly immunostained in Fig. 1B. It is known that the present PCR targeting gyrB gene is useful for detection and/or identification of F. psychrophilum (Izumi and Wakabayashi, 2000; Izumi et al., 2005), thus it was suggested that the colonies except number 3 colony with positive reaction in both immunostaining and digital processing were all identified as F. psychrophilum, moreover that F. psychrophilum was distinguishable from other yellowish bacteria cultured on CAM by the present method. Although only number 3 colony was strongly immunostained but was not identified as F. psychrophilum in this study, it is easily detectable not as F. psychrophilum by whitish color of this colony. Anyway, it is a noteworthy fact that an unknown whitish bacterial colony was strongly immunostained in the present method. It is necessary to make a couple of blot onto each NC membrane with cultured NCMB1947T as positive control and the standard of the threshold of the depth in color of F. psychrophilum. We must investigate the appropriate number of bacteria using as positive control for improvement of this method in future because the number of bacteria blotting to NC membrane per unit area might affect the depth in color of immunostaining.

To evaluate specificity of the present method, a total of 24 bacterial strains listed in Table 1 were cultured on CAM to subject to the colony blot with immunostaining and digital processing (Fig. 3). It was confirmed that all bacterial isolates were grown on CAM (Fig. 3A), and that the blotted colonies were strongly or weakly stained with rabbit antiserum against NCMB1947T (Fig. 3B). All blotted colonies of F. psychrophilum strains on NC membrane strongly stained and appeared by digital processing with Image J in the same way as in Fig. 1, while blots of the other bacterial colonies including unknown yellowish bacteria on the membrane disappeared (Fig. 3C). In Table 2, biological characteristics of F. psychrophilum NCMB1947T and the unknown yellowish bacteria, Fp-Q, Fp-R, Y-01, Y-02, Y-03, Y-04. NCMB1947T was weakly refractile, and oxidase and catalase positive, no acid produced from glucose, no change in

![Image](image-url)
viable ods have still some problems in quantitative detection for and PCR (Toyama et al., 1994; Madetoja and Wiklund, 2002). In these points, the present detection method of F. psychrophilum with CAm plate cultivation and immunostaining was improved.

Next, influence of ovarian fluids and kidney homogenates to quantitative detection of F. psychrophilum by the present method with CAm was evaluated (Table 3). In two independent experiments, 326 and 201 colonies of F. psychrophilum appeared on the CAm by spreading of approximately 200 CFU of NCMB1947T mixed with ovarian fluids although less than ten colonies were observed by spreading of the ovarian fluids only. The same tendency was observed in kidney homogenates, meaning that 219 and 369 colonies were observed on CAm by spreading of approximately 200 CFU of NCMB1947T mixed with kidney homogenates but almost few colonies were observed by spreading of the kidney homogenates only. It was therefore confirmed that there could be little influence by ovarian fluids and kidney homogenates to colonization of F. psychrophilum on CAm. Growth of F. psychrophilum is sometimes inhibited by growth of other bacteria such as Pseudomonas spp. (Tirola et al., 2002), and which makes a problem for quantitative detection of F. psychrophilum.
Moreover, detection of *F. psychrophilum* by plate cultivation needs relatively long time rather than other method such as PCR. Kumagai et al., (2004) described that supplementation of tobramycin to medium might be effective for the selective cultivation of *F. psychrophilum*. It was suggested the supplementation of glucose to medium improve the growth speed of *F. psychrophilum* (Cepeda et al., 2004). Although those problems were not observed in the present study, if being observed, supplementation of tobramycin and glucose to CAM might be useful for the improvement of cultivation of *F. psychrophilum*.

Some colonies were not immunostained uniformly in the depth of color such as the number 12 colony in Fig. 1 and the number 8 colony in Fig. 3. The reason of this phenomenon is unclear, however, it might be due to the form of the colony. That is to say bacteria of these colony might be not blotted uniformly onto a NC membrane because of the unevenness form of the colony.

In conclusion, viable *F. psychrophilum* in ovarian fluids and kidneys of chum salmon was distinguishable from other kind of yellowish bacteria by colony blot with immunostaining. Moreover, ovarian fluids and kidney tissue homogenates had little effects in colonization of *F. psychrophilum*. Thus, the present method was considered to be useful for the quantitative detection of viable *F. psychrophilum*. It was recently suggested that *F. psychrophilum* could be transmitted vertically in salmonid (Brown et al. 1997; Kumagai et al. 2000). In a case of *Renibacterium salmoninarum*, the etiological agent of bacterial kidney disease (BKD), vertical transmission occurred due to intraovular infection by rising concentration of *R. salmoninarum* in ovarian fluid (Lee and Evelyn, 1989). Thus, elucidation and quantitative detection of viable *F. psychrophilum* in ovarian fluids in chum salmon is important to assess a possibility of its vertical transmission. Moreover, viable *F. psychrophilum* was detectable from BCWD-affected rainbow trout kidneys in experimental infection, and kidneys are one of the affected organs in BCWD (Madsen and Dalsgaard, 1999). Thus, in our future study, we would elucidate relationship between mortality of chum salmon and viable numbers of *F. psychrophilum* in those kidneys by the present method.

**Acknowledgements**

We greatly thank Dr. S. Izumi, Gunma Prefectural Fisheries Experiment Station for providing of rabbit serum against NCMB1947. This study was supported by a grant from the Hokkaido government.

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