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# Detection of Flavobacterium psychrophilum in fry of rainbow trout by RT-PCR

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Summary

**The paper describes the occurence of Flavobacterium psychrophilum as a concurrent pathogen with infectious hematopoietic necrosis virus in rainbow trout fry in Croatia. A molecular technique was applied for F. psychrophilum detection due to the failure of standard bacterial identification on agar plates. As the tissue material had already been used up for RNA extraction and detection of RNA virus, classical, well-described PCR could not be performed. Consequently, one step RT-PCR was adapted for F. psychrophilum identification. This method proved to be a valuable diagnostic tool for F. psychrophilum detection in mixed co-infections with RNA viruses.**

**Keywords: Flavobacterium psychrophilum, RT-PCR, rainbow trout**

*Flavobacterium psychrophilum* is the aetiological agent of „rainbow trout fry syndrome" (RTFS) and „bacterial cold-water diseases" (BCWD), primarily in rainbow trout (*Oncorhynchus mykiss*) and coho salmon (*Oncorhynchus kisutch*) (9). RTFS can cause up to 90% fish mortality with high economic looses (10). Characteristic clinical signs of disease are exophtalmia, skin ulcers, necrosis of fins („fin rot") and anemia of gills and internal organs (3), although bacteria can be isolated from fish without any signs of disease (10).

Infection with *F. psychrophilum* can increase fish susceptibility to other diseases (11). Incidence of *F. psychrophilum* in mixed infection with: infectious hematopoietic necrosis virus (IHNV) (6), infectious pancreatic necrosis virus (IPNV) (7) and virus causing erythrocytic inclusion body syndrome (EIBS) (4) were reported previously. Dual infections with other bacteria and some parasites were also found (4).

Traditional detection of *F. psychrophilum* is based on a specific agar medium cultivation at optimal temperature 18°C (2) and has been rather difficult because of the slow growth of the bacteria cells and overgrowth or inhibition of rapidly multiplying bacteria from water (11). Hence, another methods, including serological and molecular techniques, for rapid and more sensitive detection of *F. psychrophilum* were developed. Polymerase chain reaction (PCR) based on the amplification of specific parts of 16S rRNA (4) and gyrB gene (8) are described.

Although *Cytophaga*-like bacteria were reported as the pathogenic organisms isolated in trout farming in Croatia (12), there were no reports of recently detected *F. psychrophilum*. In the 2005, first occurence of infectious hematopoietic necrosis virus (IHNV) in rainbow trout fry, imported to one Croatian farm, was detected (unpublished data). However, some external signs (gill's anemia, necrosis of fins) suggested possibility of mixed infection with *F. psychrophilum*.

The aim of this study was to identify *Flavobacterium psychrophilum* in mixed infection with RNA viruses in fry of rainbow trout by RT-PCR. The comparison between nucleotide sequences of *F. psychrophilum* from Crotian aquaculture and known sequences of CSF 259-93 and ATCC 49418 strains from GenBank, was also shown in the present study.

## Material and methods

**Sampling.** Rainbow trout fry (n = 12, average weight = 36.44 g, average length = 15.18 cm) from a freshwater farm in Croatia were sampled just upon their import from the neighbouring country. Detailed external and internal observation prior to virusological and bacteriological examination were accomplished. Virusological examination by RT-PCR and cell culture isolation showed the presence of infectious hematopoietic necrosis virus (IHNV) in all analysed fish (unpublished data). Isolated RNA used for the virus detection was a starting material for the concomitant bacterial pathogen detection. Therefore one step reverse transcription-polymerase chain reaction (RT-PCR) method was applied for the *F. psychrophilum* identification.

**Bacteriological examination.** For routine bacterial analysis, individual fish samples were taken from liver, spleen and kidney, placed onto Tryptic soy agar (TSA, BD-BBL) and incubated at 22°C. Two samples of pooled tissue homogenates were placed onto Tryptone yeast extract salts plates (TYES, 0.4% tryptone, 0.05% yeast extract, 0.0074% anhydrous calcium chloride, 0.05% magnesium sulphate heptahydrate, pH 7.2) for specific *F. psychrophilum* determination.

**RT-PCR.** 12 pools of tissue samples (spleen, heart, kidney, brain) from every fry were stored at –80°C. After homogenization, tissue was proceeded for direct RNA extraction with TRI reagent (MRC, USA), following the manufacturer's instructions. One step RT-PCR (Access RT-PCR System, Promega) was carried out for the amplification of the gene fragment encoding 16S rRNA. Reaction was performed in 50-µl reaction mixtures containing 4 µl of RNA template, AMV/Tfl 1x reaction buffer, 1 mM $MgSO_4$, 200 µM of each dNTP, 1 µM of each primer (FP1 5'-CTTAGTTGGCATCAACAC-3' and FP3 5'-ACACTGG-

CAGTCTTGCTA-3') (5) and 5 U of AMV reverse transcriptase and Tfl DNA polimerase (Access RT-PCR system, Promega). Reaction conditions were: 45 min. of cDNA synthesis at 45°C, initial denaturation at 94°C for 2 min. followed by 40 cycles of amplification (denaturation at 94°C for 40 s, annealing at 55°C for 1 min., extension at 68°C for 2 min.), and a final 7-min. elongation period at 68°C. Amplified products were analysed by 1.7% agarose gel electrophoresis.

**Sequence analysis.** RT-PCR products were purified by a QIAquick gel extraction kit (Qiagen) and sequenced by an „ABI PRISM® 3100-Avant Genetic Analyzer" DNA sequencer (Applied Biosystems). Sequencing was repeated twice for the result authenticity. The National Center for Biotechnology Informations BLASTx server was used to find out degree of homology with similar sequences in GenBank (1).

## Results and discussion

External observation indicated pale gills with haemorrhagic areas and necrosis of fin tissue (fig. 1). There were no evidence of macroscopic changes of visceral organs. However, haemorrhagic arreas of gills could be a result of both *F. psychrophilum* and IHNV infection found previously (unpublished data). For determination of the pathogen prevalence, immunohistochemistry (7) and real-time PCR should be performed.

All attempts to isolate *F. psychrophilum* on TSA and TYES agar failed, probably due to the high inoculation temperature of 22°C (2), that is used in our laboratory for routine bacterial screening. Therefore, RNA which had been used for IHNV detection and characterisation was applied for *F. psychrophilum* identification. One step RT-PCR was successfully adapted and amplification products of expected size (971 bp) were obtained (fig. 2).

Sequence analysis of RT-PCR products confirmed the presence of *F. psychrophilum* 16S rRNA. Comparison of Croatian *F. psychrophilum* 16S rRNA with known sequences from GenBank showed 100% identity with strain CSF 259-93 (fig. 3). Studies on differences between CSF 259-93 strain (virulent in trout challenge model) and ATCC 49418 strain (avirulent in trout challenge model) demonstrated the polymorphism of 16S rRNA gene with two sequence variants (6 base difference) (13). Soule at al. (14) indicated presence of both sequence variants in ATCC 49418, and just one in CSF 259-93 strain. Finding of CSF 259-93 strain specific 16S rRNA sequence in Croatian *F. psychrophilum* does not, however, abolish the presence of the other sequence variant due to the possibility of more abundant template to exceed the signal from the other sequence during the determination process (14).

In conclusion, one step RT-PCR is a valuable method for the rapid and sensitive *F. psychrophilum* detection in mixed infections with RNA viruses. Fast bacteria determination could help preventing their dissemination in water environment. For the further genotypization and quantification of this pathogen, additional methods should be performed.

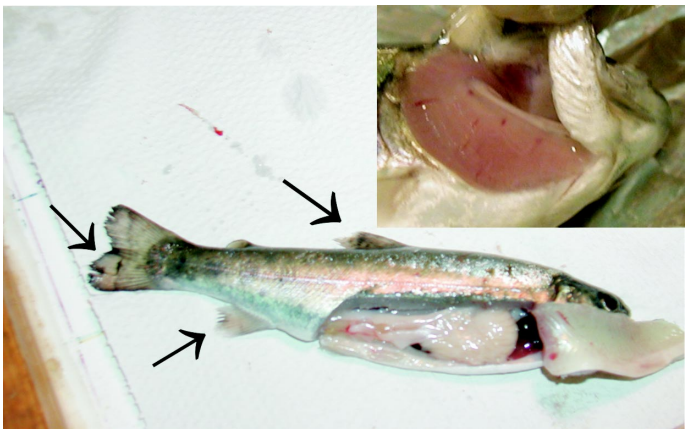

**Fig. 1. Necrosis of fins (black arrows) and small haemorrhagic areas on the gills (right upper corn) of rainbow trout fry, as the external signs of disease**

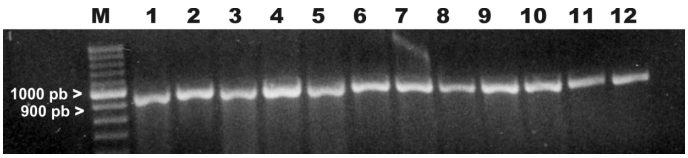


**Fig. 2. Agarose gel electrophoresis patterns of amplificated parts of *F. psychrophilum* 16S rRNA (971 bp) from 12 pooled tissue samples (1-12) of infected rainbow trout fry. M-molecular marker (100 bp)**

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Croatian        ATGCCGCGTGCAGGATGACGGTCCTATGGATTGTAAACTGCTTTTGCACAGGAAGAAACA 224
CSF 259-93      ATGCCGCGTGCAGGATGACGGTCCTATGGATTGTAAACTGCTTTTGCACAGGAAGAAACA 420
ATCC 49418      ATGCCGCGTGCAGGATGACGGTCCTATGGATTGTAAACTGCTTTTGCACAGGAAGAAACA 420
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Croatian        CTCGGTCGTGACCGAGCTTGACGGTACTGTGAGAATAAGGATCGGCTAACTCCGTGCCAG 284
CSF 259-93      CTCGGTCGTGACCGAGCTTGACGGTACTGTGAGAATAAGGATCGGCTAACTCCGTGCCAG 480
ATCC 49418      CTACCTCGTGAGGTAGCTTGACGGTACTGTGAGAATAAGGATCGGCTAACTCCGTGCCAG 480
                **   ******   **************************************************

Croatian        CAGCCGCGGTAATACGGAGGATCCAAGCGTTATCCGGAATCATTGGGTTTAAAGGGTCCG 344
CSF 259-93      CAGCCGCGGTAATACGGAGGATCCAAGCGTTATCCGGAATCATTGGGTTTAAAGGGTCCG 540
ATCC 49418      CAGCCGCGGTAATACGGAGGATCCAAGCGTTATCCGGAATCATTGGGTTTAAAGGGTCCG 540
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**Fig. 3. Comparison of 16S rRNA short nucleotide region of the amplified fragment of *Flavobacterium psychrophilum* cDNA from Croatian isolate against GenBank available CSF 259-93 (accession number AY662494) and ATCC 49418 (accession number AY662493) sequences. Croatian and CSF 259-93 isolates showed 100% identity, while they differed from ATCC 49418 in 6 bp**

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