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DETECTION OF *RENIBACTERIUM SALMONINARUM* IN TISSUE OF BROOK TROUT (*SALVELINUS FONTINALIS*) BY NESTED RT–PCR

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Summary

Pathogenic bacterium *Renibacterium salmoninarum* causes kidney disease with high mortality rate and considerable economic losses in salmonid farming. Thus, application of fast and sensitive method for *R. salmoninarum* diagnosis is of great importance. This paper describes detection of *R. salmoninarum* in brook trout tissue with gross clinical signs of disease by nested RT–PCR. Determination of partial sequence of bacterial *msa* gene was done prior to comparison with similar sequences from different *R. salmoninarum* isolates. Nested RT–PCR proved to be a rapid and valuable diagnostic tool for *R. salmoninarum* detection, and sequence analysis confirmed previously reported genetic uniformity of this bacteria.

Key words: *Renibacterium salmoninarum*, nested RT–PCR, *msa* gene sequence, brook trout

INTRODUCTION

Renibacterium salmoninarum is a small, rod–shaped, Gram–positive bacterium that causes bacterial kidney disease (BKD) reported from many different countries worldwide. This disease is a systemic, chronic and sometimes fatal to salmonid fish, with primary hosts of the genera *Oncorhynchus*, *Salmo* and *Salvelinus* (Evenden et al., 1993; Wiens and Kaattari, 2003). Clinical signs of BKD include loss of appetite, lethargy, dark colouration, exophthalmia, bloody ascites and bleedings around cloaca and at base of fins (Fryer and

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Lannan, 1993). The most obvious internal signs are the grey–white focal granulomatous lesions in the kidney and other internal organs. *R. salmoninarum* spreads horizontally and vertically, but gamete cells are one of the most common infection route, despite their disinfection (Bruno and Munro, 1986). BKD in Croatia is regulated by »Decree on the measures of animal health protection against infectious and parasite diseases« issued yearly by Ministry of Agriculture, Forestry and Water Management. *R. salmoninarum* is amongst the most prevalent pathogenic bacteria in trout farming in Croatia (Križanac and Teskeredžić, 1980; Kapetanović et al., 2005; Oraić and Zrnčić, 2005), as well as in Slovenia (Jenčič, 2005). Different virulence of *R. salmoninarum* strains is associated with a 57 kDa immunodominant antigen (p57) also known as the major soluble antigen (MSA). p57 agglutinates salmonid leukocytes and rabbit erythrocytes, but paradoxically suppresses the host immune response (Fredriksen et al., 1997; Hamel, 2005). Because of its abundance on the bacterial cell surface and in secreted form through the host tissue, p57 is an appropriate detection marker in a number of immunological and molecular assays for *R. salmoninarum* identification (O'Farrell and Strom, 1999). These techniques are preferred more then culturing of this fastidious bacterium due to the cysteine requirement for growth and incubation length (Wiens and Kaattari, 2003). For instance, minimum of 12 weeks are recommended for bacterium isol ation in primary cultures from tissues of carrier fish without clinical signs of BKD (Benediktsdóttir et al., 1991; Hirvelä–Koski et al., 2006). Enzyme–linked immunosorbent assay (ELISA) and fluorescent–antibody assay (FAT) are commonly used in *R. salmoninarum* diagnostics, but although they can be quantitative, poor quality control of antibody and lack of required sensitivity in subclinical cases appeared to be limitations (Griffiths et al., 1996; Powell et al., 2005). In order to increase sensitivity and rapidity of BKD diagnosis, several PCR and RT (reverse transcription) — PCR assays were developed (Miriam et al., 1997; OIE, 2006). A nested reverse transcription–PCR based on *msa* (p57) gene sequence amplification proved to be highly sensitive in viable bacteria detection (Cook and Lynch, 1999). Königsson et al. (2005) have developed fluorescent PCR system for detection of the 16S rRNA gene including a mimic molecule to reduce reaction inhibitors and thus false negative results. A quantitative PCR assay was used for *R. salmoninarum* detection in Chinook salmon (Powell et al., 2005) and proved to be approximately 10–fold more sensitive than quantitative FAT (Rhodes et al., 2006). Application of reliable, fast and sensitive technique for *R. salmoninarum* detection is necessary to prevent bacterium spreading, especially in subclinical cases.

The purpose of this work was to confirm *R. salmoninarum* presence in brook trouts with clinical signs of BKD by nested RT–PCR assay. Additionally, sequence analysis of short nested RT–PCR product was performed for bacterium characterization and comparison with other isolates.

MATERIALS AND METHODS

Sample collection

In the January 2007, on the request of the holder of one Slovenian fish farm, inspection of health status was conducted on brook trouts, *Salvelinus fontinalis* (Mitchill, 1814), with increased mortality rate. BKD was diagnosed at the farm by the Veterinary Inspection previously. Moribund brook trouts (n=10, age 14 months) were caught from the pool by netting. Detailed clinical observation was done prior to sample collection and bacteriological examination. Two pools of different organs (spleen, heart, kidney and brain) were collected from the fish and stored in Opti MEM I with GlutaMAX medium (Gibco). Samples were delivered to the laboratory in portable electrical refrigerator at 4ºC and immediately homogenized by Ultra Turrax T8 homogenizer (IKA Works).

RNA isolation and nested RT–PCR

Total RNA was extracted from tissue homogenates by TRI reagent (MRC, USA), following the manufacturer's instructions. One step RT–PCR (Access RT–PCR System, Promega) was carried out for the first amplification of the *msa* gene fragment of *R. salmoninarum* as described previously (Vardić et al., 2006). Additional nested reaction was performed in 50 l reaction mixtures containing: 10 µl of cDNA template, 1x reaction buffer, 1,5 mM $MgCl_2$, 200 µM of each dNTP and AmpliTaq DNA polymerase (Roche). Specific primers used in both reactions (1µM) are reported previously (Cook and Lynch, 1999; Miriam et al., 1997), and reaction conditions for the second amplification were: initial denaturation at 94ºC for 5 min followed by 35 cycles of 94ºC for 40 s, 60ºC for 45 s and 72ºC for 30 s, with final elongation period at 72ºC for 5 min. Amplified products were analysed by 1,7% agarose gel electrophoresis. To avoid false positive results, control reactions, in which reverse transcriptase was omitted from the first RT–PCR amplification were performed (Cook and Lynch, 1999).

Sequence analysis

Nested PCR products were purified by QIAquick gel extraction kit (Qiagen) and sequenced in both directions commercially using »ABI PRISM® 3100–Avant Genetic Analyzer« DNA sequencer (DNA service, IRB). The European Bioinformatics Institute (EMBL–EBI) WU–Blast2 web server was used to identify similar sequences. Thereafter, sequences were compared by ClustalW (Thompson et al., 1997) to find out degree of homology.

RESULTS AND DISCUSSION

Clinical observation

Dark colouration of skin, formation of cavities in the musculature and haemorrhagic areas around fins and cloaca were the predominant clinical signs in examined brook trouts (Fig. 1, a and b). Skin lesions with superficial blisters and cavitations formation filled with white, yellowish or haemorrhagic fluid are reported previously in atypical BKD together with ocular lesions (Hoffmann et al., 1984; Jansson, 2002; Wiens and Kaattari, 2003). Internally, grey–white nodules were detected through the kidney tissue (Fig. 1, c), and these focal granulomas where also seen in the heart, spleen and liver, which were enlarged (Fig. 1, d).

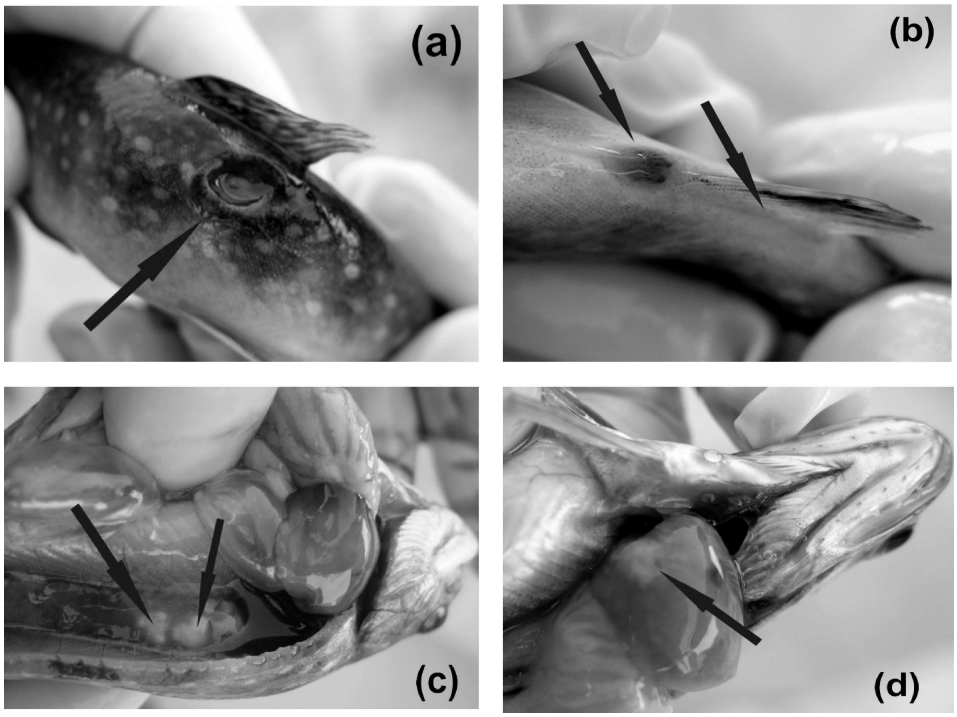

Figure 1. External and internal gross signs of BKD in infected brook trout: a) cavitations in the skeletal muscle; b) bleedings around cloaca and at base of fins; c) granulomatous foci through the kidney; d) enlarged liver with granulomas
Slika 1. Vanjski i unutrašnji simptomi bakterijske bolesti bubrega u zaraženim potočnim pastrvama: a) kavitacije u skeletnim mišićima; b) krvarenja oko kloake i na bazi peraja; c) granulomatozne tvorevine na bubregu; d) povećana jetra s granulomima

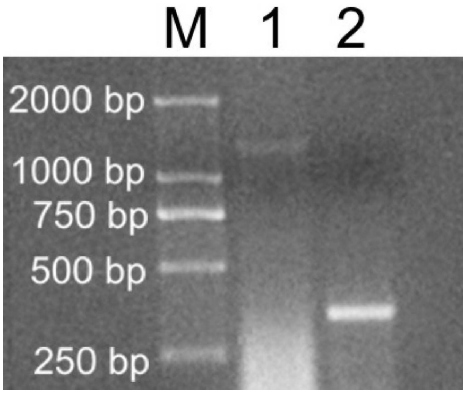

Figure 2. Agarose gel electrophoresis of RT–PCR products (line 1, 1356 bp) and nested PCR products (line 2, 346 bp) amplified from RNA extracts from pooled tissues of brook trouts infected with R. salmoninarum. *M — DNA molecular marker*
Slika 2. Elektroforeza u gelu agaroze produkata reakcije RT–PCR (stupac 1, 1356 pb) i produkata reakcije »nested« PCR (stupac 2, 346 pb) umnoženih iz RNA skupnih uzoraka tkiva potočnih pastrva zaraženih bakterijom R. salmoninarum. *M — DNA molekularni biljeg*

Nested RT–PCR detection of R. salmoninarum and sequence analysis

For the fast and sensitive *R. salmoninarum* diagnosis nested RT–PCR method was applied directly on the fish tissue. Characteristic RT–PCR products for the first and the second (nested) reactions were obtained (1356 bp and 346 bp) from brook trout samples (Fig. 2). Kidney tissue, ovarian fluid, eggs and the whole blood are the usual starting material for the PCR–based detection of *R. salmoninarum* (OIE, 2006; Cook and Lynch, 1999).

However, in this work we reported pooled spleen, heart, kidney and brain from five fish per sample as an adequate material for successful detection of *R. salmoninarum.* This is important because total RNA isolated from such pooled samples could be also used as a starting material for virological and other bacteria analysis. As the starting material for the initial RT–PCR reaction was not simply bacterial RNA, small amounts of specific product were detected in the first reaction (Fig. 2, Line 1). Second amplification yields much larger quantities of expected product (Fig. 2, Line 2) and is thus necessary for the accurate *R. salmoninarum* diagnosis. Furthermore, this method detects viable cells, because bacterial mRNA has a short half–life unlike DNA or rRNA, and its detection corresponds to the bacterial viability (Cook and Lynch, 1999; Malorny et al., 2003; Sheridan et al., 1998).

A cross–contamination between samples and thus false positive results is reported as a main limitation of the nested RT–PCR method (Belák and Thorén, 2001). Nevertheless, horizontal cross–contamination was not de-

tected here, because control reactions without reverse transcriptase failed to produce detectable products of amplification (results not shown). Moreover, brown trout's samples without clinical signs of BKD from the same farm were analyzed together with infected samples and they proved to be negative. T a o et al. (2004) reported a one tube nested RT–PCR which is together with a »real–time« PCR more advanced assay without risk of cross–contamination. Another problem in PCR–based methods is inhibition of the amplification reaction that brings false negative results. Using mimic molecules as internal controls, amounts of inhibitors in kidney and other complex biological material could be reduced (K ö n i g s s o n et al., 2005).

Sequence analysis

Protein p57 is encoded by the three genes. While *msa 1* and *msa 2* are both necessary for the full pathogenicity (C o a d y et al., 2006), *msa 3*, a duplication of *msa 1*, is not present in all isolates of *R. salmoninarum* (R h o d e s et al., 2004). Coding sequence, that is identical for all of the mentioned *msa* genes, was analyzed in this work. Nucleotide sequence determination of amplified nested RT–PCR product confirmed the presence of *msa* gene fragment. Comparison of determined sequence with known *msa* sequences from GenBank (Fig. 3) showed 100% identity with *R. salmoninarum* strain ATCC33209 (accession number AF123888 for msa1 and AF123889 for msa2) and strain

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Subject         AGGAAGCTCCTTTGTTTATAACAAAGATGGTCCTGCCAAGGAACTGAAAGTCTGGGGGAC 180
ATCC33209       AGGAAGCTCCTTTGTTTATAACAAAGATGGTCCTGCCAAGGAACTGAAAGTCTGGGGGAC 180
684             AGGAAGCTCCTTTGTTTATAACAAAGATGGTCCTGCCAAGGAACTGAAAGTCTGGGGGAC 180
                ************************************************************

Subject         GACTCTAAACGTAGGTTTCGGGTGTAACGATAATGCGCCATCCCCAATGGGATGTGGCCC 240
ATCC33209       GACTCTAAACGTAGGTTTCGGGTGTAACGATAATGCGCCATCCCCAATGGGATGTGGCCC 240
684             GACTCTAAACGTAGGTTTCGGGTGTAACGATAATGAGCCATCCCCAATGGGATGTGGCCC 240
                ********************************** ************************

Subject         GGCAAATAAACCTGTTGCATCCGGACTTCAGCCTCCGGCGGGTCCTCTTGGCGGCGGTAC 300
ATCC33209       GGCAAATAAACCTGTTGCATCCGGACTTCAGCCTCCGGCGGGTCCTCTTGGCGGCGGTAC 300
684             GGCAAATAAACCTGTTGCATCCGGACTTCAGCCTCCGGCGGGTCCTCTTGGCGGCGGTAC 300
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Figure 3. Comparison of a partial msa gene sequences between detected R. salmoninarum *isolate from brook trout (subject), virulent strain from Chinook salmon (ATCC33209) and strain from brown trout with enhanced virulence (684). The only found difference was C to A substitution (grey colour).*
Slika 3. Usporedba nukleotidnih sljedova odsječka gena msa iz bakterije R. salmoninarum *pronađene u potočnim pastrvama (subject), virulentnog soja iz kraljevskog lososa (ATCC33209) i soja s pojačanom virulencijom iz potočnih pastrva (684). Jedina zabilježena razlika je supstitucija C u A (istaknuto sivom bojom)*

MT239 (access. no. AF123890 for msa1 and AF123891 for msa2), but one nucleotide difference by comparison with strain 684 (access no. AF458101 for msa1 and AF458102 for msa2).

This is in agreement with the fact that *R. salmoninarum* is the only species within *Renibacterium* genus with considerable degree of genetic uniformity among isolates (Grayson et al., 2000; Starliper, 1996). Wiens et al. (2002) reported that the only difference between several isolates in *msa* coding sequence is a single C–to–A substitution (Ala^{139}–to–Glu). It is associated with enhanced biological activity of p57 from *R. salmoninarum* strain 684. Such mutational event is not characteristic for specific host or geographical area, because it was found in *R. salmoninarum* from brown trout (*Salmo trutta*) in Norway (strain 684) and Atlantic salmon (*Salmo salar*) in Nova Scotia (strain K2A2) (Cook and Lynch, 1999; Wiens et al., 2002).

Partial nucleotide sequence of *R. salmoninarum* p57 detected in brook trouts here proved to be identical to both virulent ATCC33209 and attenuated strain MT239, showing that this short *msa* sequence is not sufficient proof of virulence. Isolation of bacterium and other methods for characterisation, including agglutination test, analysis of p57 expression and tRNA spacer regions should be performed (Alexander et al., 2001). Finding of a third *msa* gene in *R. salmoninarum* would also be interesting.

In summary, we confirmed the presence of *R. salmoninarum*, particularly *msa* gene, in the brook trouts with clinical signs of BKD by nested RT–PCR. Pooled samples of spleen, heart, kidney and brain proved to be an adequate material for *R. salmoninarum* detection by this molecular method. Partial coding sequence of the *msa* gene was identical to previously described ATCC33209 and MT239 strains, and additional analyses are required for more detailed characterisation of the infectious agent from diseased brook trouts.

Sažetak

DETEKCIJA *RENIBACTERIUM SALMONINARUM* U TKIVU POTOČNE ZLATOVČICE (*SALVELINUS FONTINALIS*) METODOM »NESTED« RT–PCR

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Bakterija *Renibacterium salmoninarum* uzrokuje bakterijsku bolest bubrega karakteriziranu visokom stopom smrtnosti i značajnim ekonomskim gubitcima u uzgoju salmonidnih riba. Zato je primjena brze i osjetljive metode za detekciju *R. salmoninarum* veoma važna. U radu je opisan nalaz *R. salmoninarum* u tkivu potočne zlatovčice s karakterističnim kliničkim znakovima bolesti metodom »nested« RT–PCR. Određen je nukleotidni slijed dijela bakterijskog gena *msa*, koji je zatim uspoređen sa sličnim sljedovima iz različitih izolata *R. salmoninarum*. »Nested« RT–PCR pokazao se brzom i korisnom dijagnostičkom metodom u detekciji *R. salmoninarum*, a analiza nukleotidnoga slijeda *msa* potvrdila je prije uočenu genetičku jednolikost ovih bakterija.

Ključne riječi: *Renibacterium salmoninarum*, »nested« RT–PCR, nukleotidni slijed gena *msa*, potočna zlatovčica

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