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Utilization of molecular methods to relate Red Mark Syndrome affecting *Oncorhynchus mykiss* to an unculturable *Rickettsiales* organism

Francesca Cecchini, Marisa Manzano*, Lucilla Iacumin, Giuseppe Comi, Donatella Volpatti, Elena Galletti, Marco Galeotti

Dipartimento di Scienze AgroAlimentari, Ambientali e Animali, University of Udine, Italy.

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ABSTRACT

Red Mark Syndrome (RMS) is an inflammatory skin condition related to the presence of an unculturable *Rickettsiales* organism that affects farmed rainbow trout, *Oncorhynchus mykiss*. This disease is responsible of economic losses for fish farmers in Europe. The aim of this work was to confirm the presence of *Rickettsiales* organisms in affected fish and to develop a specific and sensitive system that can detect the unculturable *Rickettsiales* organisms in farmed trout. Trout gathered from affected and unaffected farms were initially submitted to necropsy and subsequently tissue samples were collected from each fish. Specific primers for a nested PCR (RiFCfw and RiFC), and a DNA probe for dot blot assay were designed using the 16S rRNA sequence. The use of the DNA probe to target amplicons as the template increased the sensitivity to 0.5 pg/µl DNA. Fifteen trout were analysed, of which nine showed visible skin lesions. Eight trout out of the nine with skin lesions were positive for the presence of the unculturable *Rickettsiales* organism DNA sequence. The tests developed can help rainbow trout producers to quickly treat fish in order to reduce economic losses. Up to 30% of fish with skin lesions are downgraded and rejected at the market by consumers.

1. INTRODUCTION

Aquaculture is a growing production system in the world, especially for high protein food. Trout is one of the most important species for EU, and rainbow trout (*Oncorhynchus mykiss*) is one of the most commonly cultivated trout in freshwater environments. Diseases causing multiple skin lesions can make the product less appealing to consumers, leading to product downgrade and eventual product rejection, causing economic losses for trout farmers [1, 2]. Red mark syndrome (RMS) is a disease that affects farmed rainbow trout, *Oncorhynchus mykiss*. It is referred by rainbow trout farmers as the biggest problem affecting the market size fish.

The disease was reported in Scotland in the winter of 2003 and has spread throughout Great Britain [3, 4] and subsequently, through continental Europe, with reports of RMS outbreaks in Switzerland, Austria, Germany, France, and Italy [5, 6, 7, 8, 9]. Affected fish show not evident changes in behaviour or weight gain. It is not clear what predisposes fish to RMS, although stress is thought to aggravate the condition. Antibiotic treatment shortens the duration of infection, suggesting that a bacterial agent may be responsible for RMS [10]. However, many attempts to unequivocally isolate a candidate bacterial agent using conventional culture methods have been unsuccessful. Lloyd et al. [6,10] found that one particular DNA sequence that is present in most strawberry disease induced lesions, the RLO 16S rDNA sequence, is also present in RMS lesions and is not present in healthy skin samples. These authors demonstrated that the organism involved was closely related to a *Rickettsia* spp. and was therefore referred as a *Rickettsiales*-like organism (RLO). Phylogenetic studies of the RLO showed that the RLO sequence is most closely related to the 16S rRNA sequences of bacteria and may form a novel lineage within the order *Rickettsiales* [11, 6]. Members of the order *Rickettsiales* have an obligate intracellular lifestyle, and are generally susceptible to tetracyclines [12].

* *Corresponding Author*
Email: marisa.manzano @ uniud.it in

Thus the isolation of an RLO by conventional bacteriological culture methods is very challenging. Molecular methods have frequently facilitated studies on culture-independent microorganisms in fish and most of these methods are based on direct DNA extraction from samples and a subsequent study of the 16S rRNA genes.

Fluorescent *in situ* hybridization (FISH) [13], denaturing gradient gel electrophoresis [14] and DNA clone libraries for the study of microbial communities have been satisfactorily used [15, 10]. Moreover, specific microorganisms can be detected with probes that anneal to specific DNA sequences [16, 10, 6, 17], and by immunohistochemistry [2].

In the present research a molecular approach and the anatomo-pathological evaluation were used to investigate RMS etiology. Symptomatic trout were collected from fish farms and subjected to necropsy in order to record and describe the macroscopic and histological lesions prior to utilise specific primers and a DNA probe in the detection of the unculturable *Rickettsiales* bacterium. Both PCR and dot blot assays were used in order to confirm the presence of *Rickettsiales* organisms, the potential aetiological agent of RMS. There are no specific preventive/therapeutic procedures able to limit the spread of RMS but it seems relevant to provide insights on the potential agent responsible for this disease.

The methods proposed in the present study might be useful for the detection of RMS at early stages so as to limit the spread of the disease and reduce economic losses in farms where trout are affected.

2. MATERIALS AND METHODS

2.1 Sample collection

Nine RMS trout of approximately 500 g each with skin lesions (named with the letters from A to I) were sampled from commercial farms in the North of Italy with repeated RMS outbreaks, from October 2011 to January 2012 (water temperature ranging from 9 to 10°C). Six healthy trout (without skin lesions, named with the letters from L to Q) were sampled from a RMS-free fish farm and were analysed as negative controls.

2.2 Anatomopathology

Prior to the sampling for the bio-molecular analyses, individual fish were submitted to necropsy in order to describe the macroscopic lesions and confirm the RMS diagnosis. Then, tissue samples were collected from skin lesions and fixed in 4% neutral buffered formaldehyde. After fixation, the samples were equilibrated at RT and processed by an automatic histoprocessor (TISBE tissue processor, Diapath) to be embedded in paraffin (ParaplastPlus, Diapath). Serial 5 µm sections were obtained using a programmable microtome (Reichert-Jung 2050) and stained with haematoxylin–eosin (H.E.). The specimens were examined by light microscopy (Leica DMRB) and digital images were acquired with a Nikon system. The skin macroscopic lesions were classified according to the criteria stated by Galeotti et al. [18].

2.3 Biomolecular analysis

The fish both showing RMS skin lesions, named from A to I, and the healthy, named from L to Q, were submitted to sampling for biomolecular analysis. Three tissue samples (one from the centre of the RMS skin lesion, one from the spleen and one from the liver), were collected separately from each individual using a sterile scalpel, and each sample was stored in a sterile plastic vial. The average weights of the collected samples were 1.5 g (skin), 1 g (spleen) and 2.5 g (liver). Tissue samples from skin, liver and spleen of control fish were collected with the same procedure. All the samples were maintained on ice during the sampling and were stored at -20°C within a few hours until analysis.

2.4 DNA extraction

The DNA was extracted from the liver (liv), spleen (sp), and skin (sk) using the Wizard Genomic DNA Purification kit (Promega, Milan, Italy) following the Animal Tissue (Mouse liver and brain) protocol. Then, the DNA was stored at -20°C within few hours post-sampling, until analysis. The DNA extraction for the twenty-five reference strains listed in Table 1, and used to check the specificity of the primers and the probe, was performed as described by Manzano et al. [19]. The DNAs were standardized at 100 ng μl^{-1} when used as templates in the PCR protocols.

2.5 PCR assays for the detection of the *Rickettsiales* sequence

A first PCR assay for the detection of *Rickettsiales* 16S rRNA in RMS-affected and unaffected tissues was performed using the RLO1 and RLO2 primers [10]. A 49 µl aliquot of the master mix included water and contained the following reaction mixture for each sample: 1.25 U of GoTaq® DNA Polymerase (Promega, Milan, Italy), 1X PCR buffer, 1.5 mmol l^{-1} $MgCl_2$, 0.2 mmol l^{-1} dNTPs, and 0.2 µmol l^{-1} of each primer. The mix was distributed into each tube and 1 µl of DNA (250 ng μl^{-1}) was added to obtain a final volume of 50 µl. The amplification protocol was performed in a Thermal Cycler (DNA Engine Dyad Peltier Thermal Cycler, BioRad, Milan, Italy): 5 min denaturation at 95°C, 35 cycles of 95°C for 30 s, annealing at 69°C for 30 s; 72°C for 30 s; and a final extension was carried out at 72°C for 10 min. The PCR assay was run on the DNA extracted from the skin, spleen, and liver tissues. Moreover, for each PCR a blank was added to ensure that the samples were uncontaminated.

Because of the unculturable nature of *Rickettsiales* bacterium a nested PCR (second PCR assay) was used to increase the specificity and the sensitivity of the protocol. The primer pair, RiFCfw 5'-AAGGCAACGATCTTTAGTTGG-3' and RiFC 5'-CCGTCATTATCTTCCCCACT-3', were used to further amplify the amplicon obtained using the RLO1 and RLO2 primers in the first step. RiFCfw and RiFC were designed after alignment of various 16S rRNA gene sequences of microbial flora from fish, such as Candidatus *Arthromitus* AY007720, *Enterobacter* spp. HQ179966, *Salmonella* Enteritidis FJ465088, *Vibrio* spp. DQ451212, *Proteus mirabilis* HQ259935, *Yersinia enterocolitica* HM007567, *Moraxella* spp. HE575924, *Leuconostoc lactis*

AB680284, *Weissella cibaria* HQ009757, *Listeria monocytogenes* FJ774249, *Clostridium botulinum* HQ328061, *Escherichia coli* HQ615933, *Bacillus subtilis* JQ309828, and *Pseudomonas aeruginosa* HQ712124, and uncultured *Rickettsiales bacterium* EU555284, AF322443 and JF421148. The software "Multiple sequence alignment with hierarchical clustering" [20] and Blast [23] were used for the purpose.

The RiFCfw and RiFC primers were first tested in silico using the AmplifX 1.5.4 [21], and then synthesized by MWG-Biotech (Ebersberg, Germany) and tested for specificity on the microorganisms reported in Table 1, which were considered as negative samples (the expected amplicon was of 188 bp).

The concentration of the reagents used in the second PCR assay master mix was the same of first PCR assay, except the primers. One µl of the first PCR product was used as the template, and 1 µl of sterile distilled water was used as a blank to ensure that there was no contamination. The second PCR reaction was performed in a Thermal Cycler (DNA Engine Dyad Peltier Thermal Cycler, BioRad) with the following conditions: 95°C denaturation for 5 min, 35 cycles of 95°C for 45 s, 54°C for 45 s, 72°C for 45 s, and a final extension at 72°C for 7 min. To evaluate the sensitivity of the second array of the nested PCR, the amplicon obtained from the amplification of the E_{liv} sample with the RLO1 and RLO2 primer pair was purified with the DNA Enzyme-free Isolation Spin-Kit (AppliChem, Gatersleben, Germany), measured using a NanoDrop 2000c spectrophotometer (Thermo Scientific, Wilmington, USA), and diluted to different concentrations ranging from 50 ng μl^{-1} to 0.25 pg μl^{-1}. All the PCR assays were performed in triplicate. The amplicons were visualized by an electrophoretical separation in a 2% agarose gel (Sigma, Milan, Italy) stained with ethidium bromide (0.5 mg ml^{-1}) in 0.5X TBE buffer (tris-borate-EDTA, 0.045 mol l^{-1} Tris-borate; 0.001 mol l^{-1} EDTA, pH 8) of 5 µl aliquots, and compared with a 100 bp DNA Ladder (Promega, Milan, Italy).

2.6 Sequencing

Amplicons obtained from the nested PCR using the RiFCfw and RiFC primers were purified using the QIAquick PCR Purification Kit (Qiagen, Milan, Italy), dried and sent to the MWG sequencing centre (Eurofins MWG GmbH, Martinsried, Germany) for sequencing as reported by Cecchini et al. [16].

2.7 Probe design to detect the RLO sequence

A 63 bp probe (Rick probe) specific for unculturable *Rickettsiales* bacterium was designed to anneal within the sequence of the amplicon obtained by the RiFCfw and RiFC primers, and was tested for specificity using the same sequences and softwares previously reported in section 2.5.

The sequence of the probe synthesised by MWG-Biotech (Ebersberg, Germany) and labelled at 5' and 3' ends to increase the resolution was as follows: 5'- AAT ATT GGA CAA TGG GCG CAA GCC TGA TCC AGC GAC GCC GCG TGA GTG ATG AAG GCC TTA GGG –3'. An ssDNA sequence (Anti-Rick probe) complementary to the Rick probe sequence was used as positive control in the blotting protocols to optimise the hybridisation conditions and as a positive control. The specificity of the probe was tested on the DNA extracted from the microorganisms reported in Table 1.

Table 1: List of the reference microorganisms used to test the sensitivity and specificity of the DNA probes.

N°	microorganism	source
1	*Aeromonas sobria* 19176	DSM°
2	*Bacillus cereus* 2301	DSM°
3	*Bacillus coagulans* 2308	DSM°
4	*Bacillus subtilis* 1092	DSM°
5	*Citrobacter freundii* 15979	DSM°
6	*Enterobacter cloacae* 30054	DSM°
7	*Escherichia coli*	DISTAM§
8	*Kokuria kristinae* 20032	DSM°
9	*Lactobacillus plantarum* 20174	DSM°
10	*Listeria monocytogenes* 7644	ATCC*
11	*Leuconostoc lactis* 4173	CECT°°
12	*Morganella morganii*	DISTAM§
13	*Pediococcus pentosaceus* 20336	DSM°
14	*Proteus vulgaris*	DISTAM§
15	*Pseudomonas aeruginosa*	DISTAM§
16	*Pseudomonas bremerii*	DISTAM§
17	*Pseudomonas fluorescens*	DISTAM§
18	*Pseudomonas migulae*	DISTAM§
19	*Saccharomyces cerevisiae* 36024	ATCC*
20	*Salmonella enterica* 4883	DSM°
21	*Serratia marcescens* 6067	DISTAM§
22	*Shewanella putrefaciens*	DSM°
23	*Vibrio ruber* 14379	DSM°
24	*Weissella cibaria* 14295	DSM°
25	*Yersinia enterocolitica*	DISTAM§

°Deutsche Sammlung von Mikroorganism und Zellkulturen GmbH, Braunschweigh, Germany.
°°Colección Española de Cultivos Tipo, Valencia, Spain.
§Dipartimento di Scienze e Tecnologie Alimentari e Microbiologiche, Milan, Italy.
*American Type Culture Collection, Manassas, VA, USA.

2.8 dot blot assay

The following samples were used: (a) 1 µl of DNA (100 ng μl^{-1}) extracted from each strain reported in Table 1 (negative control for specificity); (b) 1 µl of DNA (250 ng μl^{-1}) extracted from the tissues of the trout (DNA extraction section, 2.4); (c) 1 µl of the forty-five amplicons obtained from the second step of the nested PCR, independent of whether the amplicon was visible in the agarose gel; and (d) 1 µl of various dilutions (from 50 ng/µl to 0.25 pg μl^{-1}) of the liver of sample E (E_{liv}) amplicon obtained in the second step of the nested PCR.

One µl of Anti-Rick probe (100 ng μl^{-1}) was added as a positive control to each blotting assay. The dsDNAs were denatured at 95°C for 10 min and the ssDNA (DNA probes) were kept at 95°C for 5 min.

Then the probes were chilled in ice immediately before spotting them onto the nylon membranes and cross-linked to the air-dried membranes by UV light for 10 min. One µl of the DNA samples was spotted onto the positively charged nylon membrane.

The nucleic acids were cross-linked to the air-dried membranes by exposure to UV light for 10 min and subjected to hybridization as reported by Cecchini et al. [16]. For each sample the dot blot analysis was repeated three times.

3. RESULTS

3.1 Anatomopathology

Skin lesions occupying the lateral and/or ventral body areas were macroscopically detectable in 9 examined fish. The lesions displayed various shape and extension. They were single or multi-focal and were often well circumscribed (Fig.1).

Fig. 1: Single well delimited, raised, with bright red colour RMS skin lesion in adult rainbow trout.

In few subjects they appeared as small pink spots or wider areas that were bright red in colour (Fig.1), with diameter ranging from 5 mm to 5 cm. Some lesions were raised and had several levels of scale loss. In rainbow trout healthy skin, collected from a control subject, no lesions were histologically observed (Fig.2).

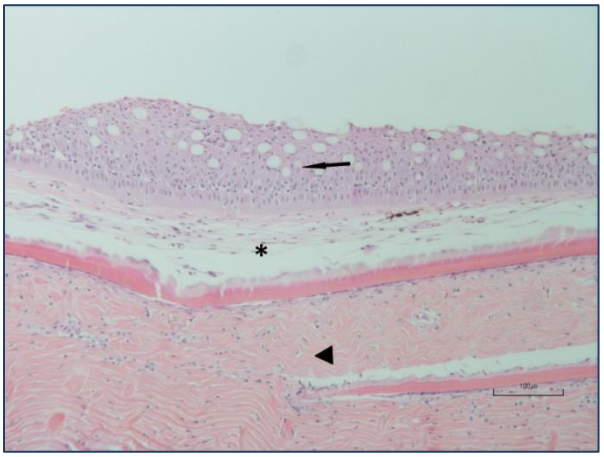

Fig. 2: Rainbow trout healthy skin, collected from a control subject. Detail of skin layers: epidermis (arrow); scale pocket (asterisk); stratum compactum of the dermis (arrow head) (H&E).

In the skin collected from the 9 RMS affected rainbow trout, the histology revealed an inflammatory status involving all the skin layers from epidermis to subcutis (Fig.3). The epidermis was often present, or partially missing. In the stratum spongiosum of the dermis, a mild to severe lympho-monocyte infiltration could be observed (asterisk) (Fig.3).

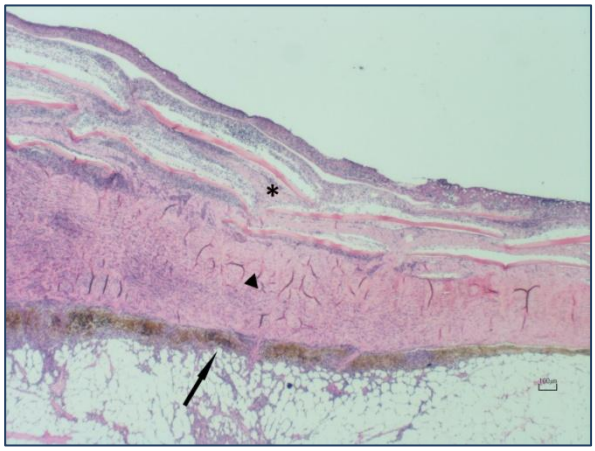

Fig. 3: Skin collected from RMS affected rainbow trout. Inflammatory condition involving all the skin layers, from epidermis to subcutis. The epidermal layer is still present. The underlying stratum spongiosum of the dermis shows scale pockets with slight oedema and cellular infiltration (asterisk). The stratum compactum appears strongly thickened and infiltrated by inflammatory cells (arrow head), invading also the subcutis (arrow) (H&E).

The scale pockets showed a cellular infiltration and the presence of multinucleated osteoaclasts in the phase of resorption of the scales (arrow) (Fig. 4). The stratum compactum often appeared thickened and infiltrated by lymphocytes and macrophages (arrow head); the cellular infiltration involved also the subcutis (arrow) (Fig.3).

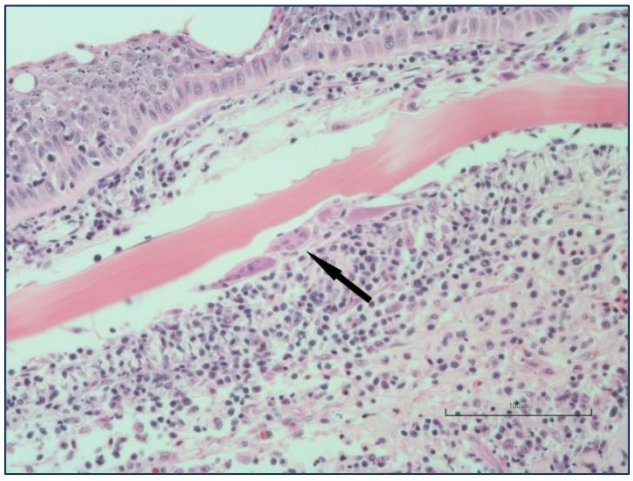

Fig. 4: Detail of Figure 3. Lymphocyte and monocyte infiltration of a scale pocket. Few multinucleated osteoaclasts in the phase of resorption of the scale (arrow).H.E.

3.2 PCR assay

The RiFCfw and RiFC primers showed specificity toward unculturable *Rickettsiales* bacterium: no PCR products were obtained using DNA extracted from the strains listed in Table 1, whereas the expected amplicon of 188 bp was obtained from eight out of the nine symptomatic trout analysed (Table 2, Fig. 5). The samples I, L, M, N, O, P and Q tested negative for RLO, as no visible amplicons were produced by nested PCR. All of these samples except I were expected to be negative, as they were gathered from the site without RMS infection.

Table 2: Results of molecular assays conducted on the DNA extracted from the trout tissue samples. Trout organ description: liv= liver; sp= spleen; sk= skin. PCR: production of the amplicon by the nested-PCR using primers RiFCfw - RiFC on the DNA extracted from the tissues. Dot blot on DNA: probe hybridization on the DNA extracted from the tissues. The presence of the 188 bp amplification product, and the blue spot due to the positive hybridization reaction are indicated by: +. Absence of the 188 bp amplification products and of the blue spot are indicated by: -. Results of samples that tested negative by using both methods in all tissues analysed (I, L, M, N O, P and Q) are not reported.

Sample	PCR	Dot blot on DNA	Sample	PCR	Dot blot on DNA
A_{liv}	+	-	E_{liv}	+	-
A_{sp}	+	+	E_{sp}	+	+
A_{sk}	+	+	E_{sk}	+	-
B_{liv}	-	-	F_{liv}	-	-
B_{sp}	+	+	F_{sp}	-	-
B_{sk}	-	-	F_{sk}	+	+
C_{liv}	-	-	G_{liv}	-	-
C_{sp}	-	-	G_{sp}	-	-
C_{sk}	+	-	G_{sk}	+	-
D_{liv}	-	-	H_{liv}	+	-
D_{sp}	+	+	H_{sp}	-	-
D_{sk}	+	+	H_{sk}	+	-

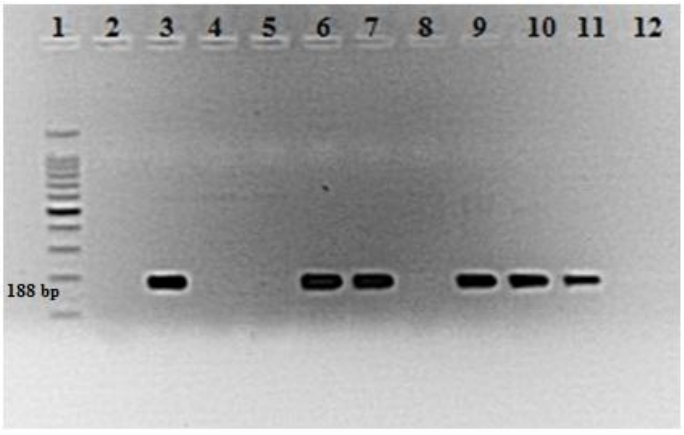

Fig. 5: Examples of PCR products obtained for liver, spleen, and skin samples from affected trout using primers RiFCfw and RiFC. Lane 1, molecular weight (100 bp, Promega); lane 2, negative control; lane 3, A spleen; lane 4, B liver; lane 5, C spleen; lane 6, D skin; lane 7, E liver; lane 8, F spleen; lane 9, G skin; lane 10, H liver; lane 11, H skin; lane 12, H spleen.

Only two subjects, A and E, resulted positive for *Rickettsiales* in all the three tissues analysed by PCR. The other six positive subjects, B, C, D, F, G and H, showed positivity only for one or two tissue samples. Totally 14 samples out of the 45 analysed resulted positive by PCR. Using various dilutions of the E_{liv} amplicon obtained with the RLO1 and RLO2 primers in the first step of the nested-PCR as the template, the sensitivity of the RiFCfw and RiFC primer pair was found to be 1 pg μl^{-1} DNA (Fig. 6).

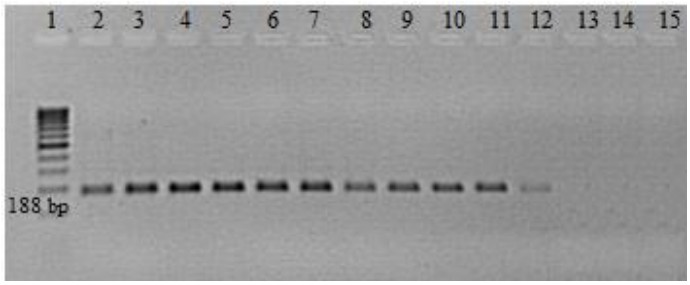

Fig. 6: Results of sensitivity of the primers RiFCfw - RiFC using as a template 1 µl of the amplicon obtained with the RLO1-RLO2 primers at various dilutions. Lane 1: 100 bp DNA Ladder (Promega, Milan, Italy); lane 2: 1 µl amplicon at 50 ng μl^{-1}; lane 3: 1 µl amplicon at 25 ng μl^{-1}; lane 4: 1 µl amplicon at 10 ng μl^{-1}; lane 5: 1 µl amplicon at 5 ng μl^{-1}; lane 6: 1 µl amplicon at 1 ng μl^{-1}; lane 7: 1 µl amplicon at 0.5 ng μl^{-1}; lane 8: 1 µl amplicon at 0.1 ng μl^{-1}; lane 9: 1 µl amplicon at 0.05 ng μl^{-1}; lane 10: 1 µl amplicon at 0.01 ng μl^{-1}; lane 11: 1 µl amplicon at 5.0 pg μl^{-1}; lane 12: 1 µl amplicon at 1.0 pg μl^{-1}; lane 13: 1 µl amplicon at 0.5 pg μl^{-1}; lane 14: 1 µl amplicon at 0.25 pg μl^{-1}; lane 15: negative control.

3.3 Sequencing

The amplicons of 188 bp obtained using the primers RiFCfw and RiFC, and reported in Fig. 5, that were sent to the MWG sequencing centre, completely matched (100%) (Supplementary Material S1) (Fig. 7) with the uncultured *Rickettsiales* bacterium sequence retrieved from GenBank (EU555284). This result demonstrated that the DNA products obtained by the nested PCR belonged to an uncultured *Rickettsiales* bacterium.

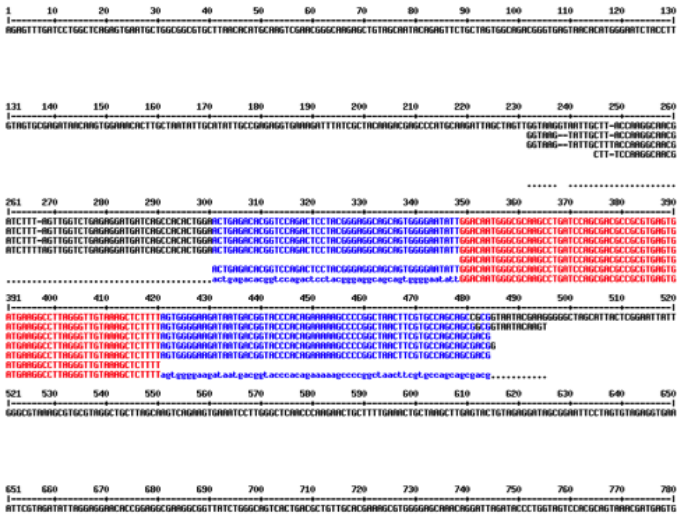

Fig. 7: Allignment of some amplicons obtained with RiFCfw-RiFC primers. Row 1: *Rickettsiales bacterium* EU555284; row 2: sample A skin; row 3: sample A spleen; row 4: sample D spleen; row 5: sample D skin; row 6: sample G skin; row 7: consensus

3.4 dot blot system

No hybridization was achieved between 10 pmol ml^{-1} of the Dig-Rick probe specific for uncultured *Rickettsiales* bacterium and 100 ng μl^{-1} DNA from the bacteria reported in Table 1 (data not shown). The sensitivity of the Dig-Rick probe was tested by hybridizing the probe to different concentrations of the amplicon obtained with nested PCR using as template DNA from the liver sample E (E_{liv}).

Blue spots were visible from 50 ng μl^{-1} to 0.5 pg μl^{-1} indicating that the sensitivity was 0.5 pg μl^{-1}. The Dig-Rick probe used on the DNA extracted from tissues produced seven positive samples (A_{sp}, A_{sk}, B_{sp}, D_{sp}, D_{sk}, E_{sp}, F_{sk}) out of forty-five analysed by dot blot (Table 2). The number of positive samples increased to twenty using the Dig-Rick probe on one µl of each amplicon obtained with the RiFCfw and RiFC primers (Fig. 8), and confirmed the positive results obtained with the nested PCR. Moreover, six samples that tested negative in the PCR assay (C_{liv}, E_{sk}, F_{liv}, G_{liv}, G_{sp}, and H_{sp}) as determined by the absence of visible amplicons on the agarose gel when electrophoresed, tested positive in the dot blot assay using the Dig-Rick probe on the amplicons (Fig. 8, spots A7, B3, B4, B7, B11). This result can be explained as: i) agarose gels are not very sensitive (they require about 10 ng DNA to produce a clear visible band) and the labelled probe produces a visible spot using 0.5 pg μl^{-1} DNA as template; ii) the not "normal" condition of some tissues that can affect PCR, as reported by Chen et al. [22], not giving a PCR product after the first step of PCR.

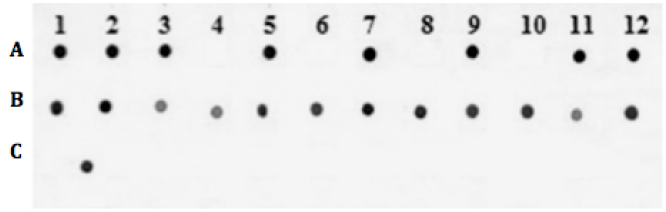

Fig. 8: Dot blot results obtained by using the Dig-Rick probe specific for RLO (at 10 pmol ml^{-1}) on the amplicons obtained using the RiFCfw - RiFC primers; liv= liver; sp= spleen; sk= skin.
Row A, spotA1: A_{liv}; spot A2: A_{sp}; spot A3: A_{sk}; spot A4: B_{liv}; spot A5: B_{sp}; spot A6: B_{sk}; spot A7: C_{liv}; spot A8: C_{sp}; spot A9: C_{sk}; spot A10: D_{liv}; spot A11: D_{sp}; spot A12: D_{sk};
Row B, spot B1: E_{liv}; spot B2: E_{sp}; spot B3: E_{sk}; spot B4: F_{liv}; spot B5: F_{sp}; spot B6: F_{sk}; spot B7: G_{liv}; spot B8: G_{sp}; spot B9: G_{sk}; spot B10: H_{liv}; spot B11: H_{sp}; spot B12: H_{sk};
Row C, sequence complementary to Dig-Rick probe at 100 ng μl^{-1} as positive control.

4. DISCUSSION

Due to the anatomo-pathological findings it was possible to recognize the disease affecting rainbow trout as RMS according to the description reported by Galeotti et al., [18] and Oidtmann et al. [8]. Apart from the typical RMS lesions, the fish submitted to biomolecular analyses showed no other disease signs. As hypothesized by Lloyd et al. [10], RLO is a primary or concurrent cause of RMS in rainbow trout, thus the goal of this study was to develop a method for an early detection of specific DNA sequences related to *Rickettsiales* in RMS affected trout. Because of the unculturable nature of this microorganism, isolation procedures using plate counting methods are not useful in its detection [10, 6, 12]. Moreover, culturing methods for the detection of obliged intracellular bacteria, require about one month when relevant, and they are not practical for routine diagnosis.

The application of the described molecular methods, RiFCfw and RiFC primers and the labelled DNA probe, is important for the early detection of *Rickettsiales* in affected trout as they show high sensitivity.

The RiFCfw and RiFC primers produced a highly specific single amplicon of 188 bp, which matched 100% with the DNA sequence of the uncultured *Rickettsiales* bacterium present in GenBank (Supplementary Material S1) going down to a detection limit of 1 pg μl^{-1} of DNA, as shown in Fig. 6. This value increased the sensitivity, as it is approximately 10^4 times lower than the value of 20 ng μl^{-1} reported by other authors [10] allowing for an early detection of the disease-causing agent. The negative results obtained for samples gathered from the disease-free farm (L, M, N, O P and Q) confirmed the specificity of the assay.

The presence of one subject (I) that tested negative for *Rickettsiales* in the molecular methods but still showed the typical skin lesions (red marks) on the flank may be justified by the assumption that the inflammatory response might persist during the regression phase of the disease even if the *Rickettsiales* has been eliminated by the macrophages.

The utilization of the probe on the amplicon as a template lead to the confirmation of the positive results obtained by PCR and increased the sensitivity that is 0.5 pg μl^{-1} of DNA, lower than 1 pg μl^{-1} of DNA that the primers could detect. The assays proposed in this work are more simple and easy to use than the methods for detecting DNA sequences proposed in previous studies like cloning methods, that require the utilization of specific plasmids for competent cell transformation as well as the construction of a DNA library for sequencing [10]. Other authors used cell lines to isolate the supposed intracellular pathogen, and because these pathogens can be associated with insects hosts, insect cell lines are required [2].

The methods mentioned above are more difficult and laborious than the amplification of DNA extracted from the matrix with the RiFCfw and RiFC primers even if coupled with the dot blot as proposed in this work. The methods proposed takes about 24-30 hours to produce results including DNA extraction, PCR steps and also dot blot if added, thus we can consider this methods rapid, especially when compared to plate culture methods.

5. CONCLUSIONS

The PCR and dot blot assays proposed in this work can be used alone or together to decrease the detection limit, and can therefore be helpful in improving the RMS etiological diagnosis. Both methods are simple and rapid and thus they can facilitate the processing of a large number of samples in a short time. They can be used in the early diagnosis of the disease, allowing an early treatment of the farmed fish, aimed to reduce the spread of an

infection responsible for a low rate of mortality but causing substantial economic losses for trout farmers.

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