Title: ~~Evidence~~ Molecular evidence for ~~the presence of a~~ a bacterium of the family Midichloriaceae (order Rickettsiales) in skin and organs of the rainbow trout ~~(~~*Oncorhynchus mykiss* ~~)~~ (Walbaum) affected by ~~the~~ red mark syndrome

Running title: Midichloriaceae in rainbow trout organs

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ABSTRACT

Red mark syndrome (RMS) is a skin pathology affecting farmed rainbow trout~~s~~ ~~(~~*Oncorhynchus mykiss*~~)~~ (Walbaum) that consists of bright red skin lesions on the flanks of ~~the trunk of~~ affected fish. The economical loss caused by RMS can be important. ~~Multiple lines of~~PCR and histopathological evidence indicate that this pathology could be caused by an ~~single~~ infectious agent, possibly a bacterium of the family Midichloriaceae (order Rickettsiales). Here we present a novel Real Time PCR method, useful to quantify the presence of this bacterium in fresh skin and organs but also in samples fixed on glass slides. The method was used to investigate the presence of the Midichloriaceae bacterium in samples from 14 fish individuals, obtained from three farms in Scotland. Positivity was obtained for all the examined skin lesions, but also for some samples of apparently healthy skin from affected fish, and from different organs of ~~affected~~ pathological fish. We were thus able to conclude that the putative aetiological agent of RMS is not only localized in the skin lesion, but can be detected in different organs of affected fish, suggesting a possible diffusion during the course of the pathology.

KEYWORDS

Red mark syndrome**;** *Oncorhynchus mykiss*, Midichloriaceae family; Real Time PCR

SHORT COMMUNICATION

Red mark syndrome (RMS) is a chronic skin disease of unknown aetiology affecting farmed rainbow trout ~~(~~*Oncorhynchus mykiss*~~,~~ (Walbaum ~~1792~~) in Europe. It consists of single or multiple skin lesions usually localized on the trunk of fish approaching market size. RMS was first reported in Scotland in 2003 but has subsequently been observed in other European countries, including Italy (Verner-Jeffreys *et al.* 2008; Schmidt-Posthaus *et al.* 2009). This disease is generally associated with ~~breeding~~ water temperatures below 16°C and can still be present at 2°C (Verner-Jeffreys *et al.* 2008; McCarthy *et al.* 2013). RMS causes bright red lesions that tend to heal spontaneously, with ~~within 6-8 weeks, with~~ no effects on weight or behaviour and the healing time seems to be associated with the water temperature (Ferguson *et al.* 2006). Histologically, severe lymphohistiocytic dermatitis is reported with scale resorption (Ferguson *et al.* 2006; Noguera, 2008; Verner-Jeffreys *et al.* 2008; Schmidt-Posthaus *et al.* 2009). In some RMS affected individuals, acute necrotizing myocarditis, inflammation of intestinal muscle, splenic congestion, peritonitis and perivascular lymphocyte infiltrate can be observed (Oidtmann *et al.* 2013). Although RMS is not lethal, its morbidity can reach up to 60% in fish culture and can thus cause important economic losses, due to downgrading of the product (Schmidt-Posthaus *et al.* 2009; Oidtmann *et al.* 2013). In the United States a similar ~~pathology~~ condition of *O. mykiss* is known as strawberry disease (SD) and the skin ~~symptoms~~ pathology associated with it resembles ~~those~~ that of RMS.

No aetiological agent has been unequivocally identified for RMS or for SD, ~~however~~ although different authors suggest that a single transmissible agent could be responsible for both (Lloyd *et al.* 2011). Oman (1990) reported experimental infection through inoculation with SD lesion homogenate (Lloyd *et al.* 2008). Ferguson *et al.* (2006) suggested a possible association with *Flavobacterium psychrophilum* but no data confirmed this hypothesis. Experimental cohabitation between fish affected by RMS and naïve ones demonstrated the transmissibility of this pathology (Verner-Jeffreys *et al.* 2008). Moreover, histological analysis showed acute inflammation in the area of the skin lesion with the presence of neutrophils and protein oedema (Metselaar *et al.* 2010) suggesting a bacterial infection. ~~Galeotti *et al.* (2011) reported the presence, in the cytoplasm of macrophages, of morula-~~

type structures (typical in some Rickettsiales) in the spleen of rainbow trout individuals affected by RMS.

Lloyd *et al.* (2008) presented PCR evidence for the presence of a *Rickettsia*-like organism (RLO) associated with SD skin lesions; subsequent studies on RMS detected the same DNA sequence in the lesion (Metselaar *et al.* 2010). Later a positive correlation between the quantity of RLO bacteria and the severity of the SD skin lesions has been reported (Lloyd *et al.* 2011).

The 16S rDNA sequence of the RLO bacterium associated with RMS/SD lesions was included in a phylogenetic study that showed it to belong to the recently described family Midichloriaceae, within the order Rickettsiales (Montagna *et al.* 2013). In ecological terms, the Midichloriaceae family presents a wide range of hosts, from ciliate protists to parasitic arthropods and marine organisms (Cnidaria and Porifera). Furthermore, there is evidence that the type species of the family, *Midichloria mitochondrii* (symbiont of the tick *Ixodes ricinus*) is infectious to vertebrates (Mariconti *et al.* 2012; Bazzocchi *et al.* 2013). The presence of Midichloriaceae in aquatic hosts and the infectivity of *M. mitochondrii* suggest that *Midichloria*-like organisms (MLO) could be responsible for pathologies, such as RMS and SD in *O. mykiss* (Lloyd *et al.* 2008; Lloyd *et al.* 2011).

Here we present a novel specific method for absolute quantification of the MLO associated with RMS in *O. mykiss,* based on a quantitative Sybr-green Real Time PCR approach (qPCR - iQ5, BioRad). Furthermore, we show the first evidence of the presence of this MLO in organs (heart, liver, spleen, intestine, kidney) of affected fish.

In order to investigate the presence of the MLO in *O. mykiss*, we collected a set of samples from three farm sites (A, B and C) located in Scotland. The sites are operated independently and are well separated geographically, without any transfer of eggs, fish or equipment. A and B are pond sites while C is a loch site. Samples were i) a fragment of skin lesion from one individual from site A strongly affected by RMS and used as positive control (F1); ii) sections of tissues from two RMS positive individuals (F2 and F3) from site A with different severity in lesions; iii) skin and organ fragments from one *O. mykiss* individual from a RMS free site (named D), used as negative control

(F4); iv) skin (healthy and with lesion, when present) and organ samples from six individuals from site B (F5-F10), four of which were visibly affected by RMS (F7-F10); v) skin (healthy and with lesion, when present) and organ samples from four individuals from farm C (F11-F14), three of which were visibly affected by RMS (F12-F14). ~~(~~See Table 1 for a description of all skin and organ samples~~)~~. Skin and organ samples from fish F2 and F3 were fixed in formalin for 24 h. Following routine processing for histology, 3 µm sections were placed on Polysine slides and dried overnight at 45°C before use. The severity of lesions and the health of the skin were evaluated after hematoxylin and eosin staining of histological sections (Fig. 1).

In normal rainbow trout skin, the stratum compactum of the dermis was seen as a dense layer of collagen fibres underlying the stratum spongiosum (Fig. 1b)~~, which is loose connective tissue containing the scale pockets~~. ~~The epidermis, a non-keratinised, stratified, squamous epithelium, was separated from the dermis by the basement membrane. Between the dermis and the subcutaneous muscle was the hypodermis, containing loose connective tissue and fat cells (Fig. 1b). Chromatophores (pigmented cells) were visible in the stratum spongiosum and the hypodermis.~~ Early lesions appeared as lymphocytic infiltration in the stratum spongiosum immediately surrounding the scale pockets, becoming more pronounced as the lesion progressed, with infiltration directly below the dermis and spreading into the stratum compactum (Fig. 1c). In more advanced lesions, infiltration was seen throughout the dermis and sometimes extending into the epidermis (Fig. 1f). In some fish sampled during the summer, evidence of infiltration was apparent even in skin which appeared grossly normal (Fig. 1e).

DNA from fresh samples (F1 and F4-F14) was extracted using the DNeasy Blood and Tissue kit (Qiagen). DNA from glass slides (F2 and F3) was extracted using the QIAmp DNA Investigator kit (Qiagen). A previously obtained alignment of Midichloriaceae 16SrDNA (Montagna *et al.* 2013) was used to design a specific primer set to amplify the 16S rDNA of the MLO previously detected in lesions of RMS/SD affected fish, targeting a variable region of the gene (16SrDNA-F: GCGGTTATCTGGGCAGTC and 16SrDNA-R: TGCGACACGAAACCTAAG; amplification size: 127 bp). Qualitative PCR was performed (95 °C for 15 s and 60 °C for 30 s for 40 times; final primers

concentration: 250nM) on F1 to evaluate the specificity of the amplification. PCR product was purified and cloned into the pGEM®-T Easy Vector (Promega). Ten resulting clones were purified and sequenced confirming the specificity of the amplification. A fragment of the *O. mykiss* insulin growth factor I (*igf1*) gene was amplified as described by Lloyd *et al.* (2011). This PCR product was cloned into the pGEM®-T Easy Vector also. One clone for each target (16S rDNA and *igf1*) was used as standard for setting up qPCR reactions. Plasmids containing the target genes were serially diluted from 10^9 copies μl^{-1} to 1 copy μl^{-1} to evaluate the efficiency (16S rDNA: 105%; *igf1*: 101%) and the detection limit of each PCR protocol (10 copies in both cases). PCR conditions for both genes were: 95 °C for 2 m, 40 cycles at 95 °C for 15 s and at 60 °C for 30 s, melt curve from 55 °C to 95 °C with increments of 0.5 °C per cycle; final primers concentration: 250nM. qPCRs were performed on each DNA sample in triplicate. Number of MLO 16S rDNA and host *igf1* gene copies were obtained through a comparison of the qPCR results of each sample with those of serial dilutions of purified plasmid (containing known copy numbers). Melting curves showed the presence of specific amplified fragments belonging to the target sequences confirming the specificity of the method. Results were expressed as the ratio of 16S rDNA/*igf1* x 1000.

This method was set up on F1 and validated on fixed tissue sections from early stage lesion (F2) and advanced stage lesion (F3). F2 and F3 skin lesions were positive for the presence of MLO bacteria as shown in Fig. 2. No detectable presence of MLO was observed in F2 and F3 healthy skin and in organ samples from F4 (negative control). Moreover, the amount of bacteria detected in the F3 skin lesion was higher than in F2 in accordance with histological results that showed a different severity of lesions in the two fish. All analysed organs from both F2 and F3 were positive, with an amount of bacteria higher in spleen and liver with respect to kidney, heart and intestine (Fig 2).
The qPCR method was then tested on healthy skin, on lesions when present, and on organ-samples from ten fish coming from two different farms. Table 1 shows the qPCR results expressed as 16S rDNA/*igf1* x 1000. qPCR on samples from fish F5, F6 and F11, considered healthy at initial inspection, did not detect presence of MLO. In 57% of pathological samples from sites B and C (4/7), spleen was positive for the presence of MLO and in 43% of affected individuals (3/7) liver was

positive. Kidney did not show any positivity for the presence of MLO in any analysed fish. Skin lesions were positive in all the affected fish while 28% skin samples (2/7) with no visible lesions (healthy skin) of pathological fish were positive. See Table 1 for all qPCR results.

We were not able to find a correlation in single fish, or among the samples, between the quantity of bacteria present in the lesions and the quantity detected in positive organs. This result may indicate that distribution of the MLO may not be homogeneous in the fish, or that the dynamics of the infection in the various organs are not simultaneous. The negativity to MLO of kidneys obtained from fresh samples could thus be attributed either to the above explanation or to the sensitivity of the qPCR method (10 copies μl^{-1} of 16SrDNA gene). Additional studies, focused on monitoring the different stages of the pathology, could give more clues on these~~is~~ issues. Positive results in apparently healthy skin, from diseased fish, could suggest a possible spread of the pathology and could represent the beginning of the infection in the analysed skin section.

Our work shows that the MLO ~~putatively responsible for RMS~~ is not only localized in skin lesions of RMS affected fish, but can invade various organs, and can be detected in skin sections that do not present pathological alterations. As spleen and liver in both samples showed the highest 16S rDNA/*igf1* ratio values, this can lead to the hypothesis that melano-macrophages in spleen and liver could be primarily involved in the elimination of the MLO. Even though our data show a molecular evidence to support an association of this bacterium with RMS, further studies are needed to prove if this bacterium is actually involved in the pathology.

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Table 1 Analyzed fish samples and presence/absence of MLO DNA based on

16SrDNA/*igf1*x1000 ratio

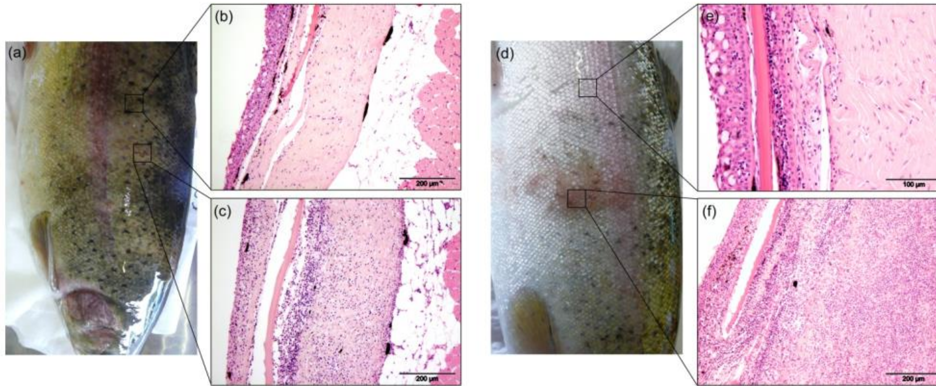
			16SrDNA/*igf1* x 1000						
Farm	**Sample**	**RMS signs**	**Healthy skin**	**Lesion**	**Liver**	**Spleen**	**Kidney**	**Intestine**	**Heart**
Site A	F1	++	0	7.85×10^{-1}	NA	NA	NA	NA	NA
	F2	+	0	8.46×10^{1}	9.40×10^{1}	5.28×10^{1}	5.15×10^{1}	5.53×10^{1}	1.59×10^{1}
	F3	++	0	5.73×10^{2}	1.71×10^{2}	1.16×10^{2}	7.19×10^{1}	4.80×10^{1}	4.25×10^{1}
Site D	F4	-	0	healthy	0	0	0	0	0
Site B	F5	-	0	healthy	0	0	0	NA	NA
	F6	-	0	healthy	0	0	0	NA	NA
	F7	+	0	1.29×10^{-1}	0	3.92×10^{-3}	0	NA	NA
	F8	+	0	5.72×10^{-2}	0	0	0	NA	NA
	F9	+	0	1.54×10^{-1}	0	4.55×10^{-2}	0	NA	NA
	F10	+	3.93×10^{-2}	3.40×10^{-1}	1.85×10^{-2}	1.31×10^{-2}	0	NA	NA
Site C	F11	-	0	healthy	0	0	NA	NA	NA
	F12	+	0	1.82	7.46×10^{-3}	2.63×10^{-2}	NA	NA	NA
	F13	+	0	7.10×10^{-3}	0	0	NA	NA	NA
	F14	+	1.50×10^{-1}	1.63×10^{-1}	2.72×10^{-3}	0	NA	NA	NA

NA - not available

NLD - no lesion detected

* samples from microscope slides

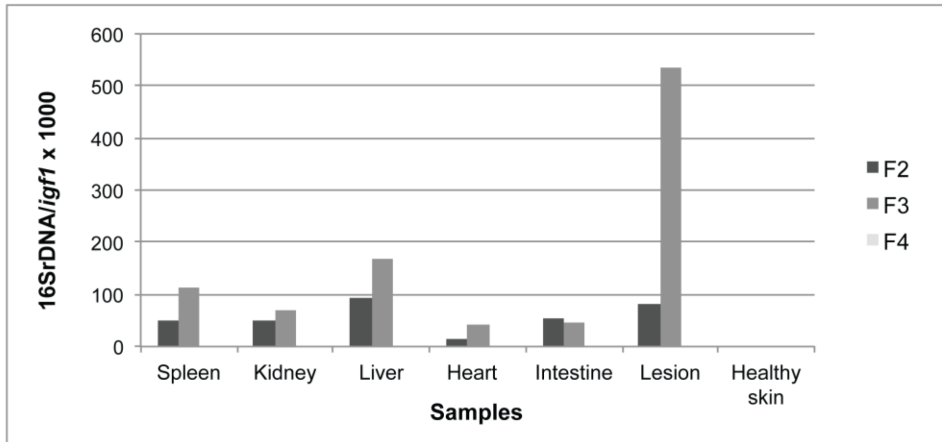
Figure 1



Gross signs and histological appearance of red mark syndrome in rainbow trout (*Oncorhynchus mykiss*).

(a) Fish 2, early stage lesion, gross appearance; (b) Fish 2 section of normal skin ~~showing the absence of leukocytes infiltration~~ (bar=200 µm); (c) F2 section of skin lesion~~, showing leukocytes infiltration below the scale pocket and moderate infiltration throughout the stratum compactum and in the hypodermis~~ (bar=200 µm); (d) F3 mid stage lesion, gross appearance; (e) F3 section of grossly normal skin~~, revealing the presence of infiltration below the scale pocket~~ (bar=100 µm); (f) F3 section of skin lesion ~~showing more advanced infiltration throughout the stratum compactum and into the epidermis~~ (bar=200 µm). All sections are stained with haematoxylin and eosin.

Figure 2



MLO quantity expressed as 16S rDNA/i*gf1* x 1000 in organs (spleen, kidney, liver, heart, intestine), healthy skin and lesion from F2 (with an early skin lesion) and F3 (with a mid-stage skin lesion) and in organs and skin from a RMS-free control (F4).