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Ultrastructural and biomolecular detection of Rickettsiales-like organisms in tissues of rainbow trout with Red Mark Syndrome

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Abstract

Red mark syndrome (RMS) and US strawberry disease (US SD) are skin disorders affecting rainbow trout farmed in Europe and USA. The disease etiology has not yet been established. In spite of specific investigations, identifying *Rickettsia*-like organism (RLO)- and *Midichloria*-like organism (MLO)-related DNA in affected individuals, these pathogens have never been observed. We performed histological, ultrastructural and biomolecular analysis on skin and spleen samples of trout with RMS. Examination by TEM revealed the presence of intracytoplasmic microorganisms resembling Rickettsiales within macrophages, fibroblasts and erythrocytes. The microorganisms were oval or short rod shaped (400–800 nm in length and 100–200 nm in width) and often showed a cell wall similar to Gram-negative bacteria. PCR analysis for Rickettsiales supported these findings: 53% of affected trout were positive by both PCR and TEM The primers RiFCfw-RiFCrev were used to anneal both the RLO 16S DNA sequence and the MLO 16S DNA sequence. For this reason, and in agreement with previous studies confirming the presence of Rickettsiales-related DNA in trout with RMS, we assume that TEM detected microorganisms morphologically consistent with bacteria belonging to Rickettsiales order and could be considered as possible causative agents of RMS.

Keywords: PCR, rainbow trout, red mark syndrome, Rickettsiales-like organisms, TEM.

Introduction

Red mark syndrome (RMS) is a skin disorder affecting farmed rainbow trout (*Oncorhynchus mykiss*) that was reported in the U.K. (Ferguson *et al.* 2006; Verner-Jeffreys *et al.* 2006, 2008; Noguera 2008; Oidtmann & Noguera 2008). Cases of the disease were subsequently recorded in Finland (Bruno *et al.* 2007), Austria and Switzerland (Schmidt-Posthaus *et al.* 2009), Italy (Galeotti *et al.* 2011) and more recently in Turkey (Kubilay *et al.* 2014) and Iran (Sasani *et al.* 2016). The authors strongly correlate European RMS with two similar rainbow trout diseases reported in the USA, namely US strawberry disease (US SD) and US Rash (Olson *et al.* 1985; LaPatra *et al.* 1994; Lloyd *et al.* 2008) that have been well characterized according to the criteria of Oidtmann *et al.* 2013. Moreover, RMS shares some similarities with a warm water strawberry disease (WWSD), previously described in Europe (Fleury, Vuillaume & Sochon 1985; St-Hilaire & Jeffery 2004) even if RMS displays peculiar features related to water temperature and antibiotic responsiveness. RMS may spread from unit to unit within a single farm or among different farms (Verner-Jeffreys *et al.* 2006, 2008). The transmissible nature of the disease has been demonstrated

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and there are etiological speculations regarding the possible role of an infectious agent (Verner-Jeffreys *et al.* 2008). As potential pathogen, Ferguson *et al.* (2006) suggested *Flavobacterium psychrophilum*, and more recently, a *Rickettsia*-like organism (RLO) was proposed for US SD (Lloyd *et al.* 2008) and for RMS (Metselaar *et al.* 2010). Splenic impression smears revealed morula-like structures (*Rickettsia*-like organisms) in the cytoplasm of spleen macrophages of RMS-affected rainbow trout (Galeotti *et al.* 2013a). Recently, the positivity for *Midichloria*-like organism (MLO)-related DNA in trout affected by RMS in Scotland has been described (Cafiso *et al.* 2015). In spite of these investigations identifying RLO/MLO-related DNA in tissues of RMS and US SD-affected individuals, these pathogens have never been observed in histological and ultrastructural samples. This study was aimed at providing novel insights into the potential association of the disease with a transmissible infectious agent, by taking a histological, ultrastructural and biomolecular approach. Here, we present the ultrastructural and molecular evidence for the presence of Rickettsiales-like organisms in the macrophages, fibroblasts and erythrocytes populations of RMS-affected rainbow trout in Italy.

Materials and methods

Description of outbreaks and sampling

This study considered two rainbow trout farms located in Northern Italy, periodically affected by RMS episodes. They were surveyed throughout the spring–autumn period of 2013 and 2014, when the water temperature ranged between 9° and 10 °C. The percentage of affected fish in the farms was 10–15%, and their mean size was 500 g. They did not show other signs of disease except those ascribable to RMS (Oidtmann *et al.* 2013), and mortality was absent. Eight symptomatic fish from farm A and nine from farm B were sampled among those showing severe skin lesions and sacrificed for the investigation. They were submitted to histological, ultrastructural and biomolecular analysis. For comparison purposes, normal skin and underlying muscle tissues, as well as spleens, were also collected from five healthy fish from the aquaculture unit of the University of Udine (Pagnacco), which has never reported cases of RMS and is presumptively a RMS-free facility.

Histology

Samples of skin with RMS lesions, muscle and spleen of the 17 trout displaying RMS gross lesions, and the five healthy trout, were fixed in 4% neutral-buffered formaldehyde for 48 h and Bouin's solution at 4 °C overnight. After fixation, samples were equilibrated at room temperature and processed by an automatic histoprocessor (TISBE tissue processor, Diapath) to embed the tissue in paraffin (ParaplastPlus, Diapath). Serial 5-μm sections were obtained by a programmable microtome (Reichert-Jung 2050) and then stained with haematoxylin–eosin, Grocott, Giemsa, Brown-Hopps and Macchiavello. The specimens were examined by light microscope (Leica DMRB), and digital images were acquired by a Nikon system.

Transmission electron microscopy

Samples (about 1 mm) of skin/muscle and spleen of 17 RMS and five RMS-free fish were fixed for 3 h in 2.5% glutaraldehyde in 0.1 M cacodylate buffer at pH 7.2. After rinsing in the same buffer, samples were post-fixed for 1 h in 1% osmium tetroxide in 0.1 M cacodylate buffer at pH 7.2, dehydrated in a graded sequence of alcohols and embedded in Durcupan AcM resin. Semithin sections were stained with toluidine blue and examined by light microscopy to locate skin areas characterized by severe inflammation. Selected semithin sections from RMS-free fish including the matching skin layers were similarly processed. Ultrathin sections were cut from each specimen and stained with uranyl acetate and lead citrate. Ultrastructural observation was performed using a Zeiss EM 109 transmission electron microscope (TEM) (Carl Zeiss AG) operated at 80 kV.

Molecular biology

Samples of skin/muscle with lesions and spleen of 17 symptomatic trout, and skin/muscle and spleen without lesions of five RMS-free trout were immediately frozen in liquid nitrogen (−196 °C) and cryopreserved at −80 °C before the analysis. The DNA was extracted from spleen (sp) (1 g) and skin (sk) (1.5 g), using Wizard Genomic DNA Purification kit (Promega) following the animal tissue mouse and

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liver brain protocol. The DNA was then stored at −20 °C for further analysis.

PCR assays for the detection of Rickettsiales *sequence.* The first step of a nested PCR assay for the detection of RLO 16S rRNA in RMS-affected and not affected tissues (control fish) was performed using the primers RLO1 and RLO2 (Lloyd *et al.* 2008) with some modifications. An aliquot of 49 μL of the master mix instead of 24 μL was used, including water, containing the following reaction mixture for each sample: 1.25 U of GoTaq® DNA Polymerase (Promega) instead of 0.5 U of Taq polymerase (Fisher Scientific), 1X PCR buffer, $MgCl_2$ concentration was increased from 1 mM to 1.5 mM, each dNTPs was increased from 0.1 mM to 0.2 mM and each primer was reduced from 0.4 mM to 0.2 μM.

The mix was distributed in each tube and added with 1 μL of template DNA (250 ng μL^{-1}) added to obtain a final volume of 50 μL. The amplification protocol: the time of denaturation at 95 °C was increased from 2 to 5 min, 35 cycles of 95 °C for 30 s, the annealing temperature was increased from 57 °C to 69 °C for 30 s, 72 °C for 30 s instead of 60 s and a final extension at 72 °C for 10 min in a thermal cycler (DNA Engine Dyad peltier Thermal Cycler, Bio-Rad). For each PCR, water has been used as a blank (negative control) to verify that no contaminations were present during the amplification step.

To increase the specificity and the sensitivity of the protocol, a second PCR assay of the nested PCR was developed by designing a new primer pair, RiFCfw 5′-AAGGCAACGATCTTTAGTT GG-3′ and RiFCrev 5′- CCGTCATTATCTT CCCCACT-3′, annealing within the amplicon obtained by the first step using the primers RLO1 and RLO2. The RiFCfw and RiFCrev primers anneal both MLO and RLO sequences. After the alignment of the sequences retrieved from GenBank using the 'Multiple sequence alignment with hierarchical clustering' function (Corpet 1988), and tested with Blast (http://blast.ncbi.nlm.nih.gov/Blast.cgi?CMD=Web&PAGE_TYPE=BlastHome; Blast.h), the primers were designed and synthetized. The amplification was obtained using 1 μL of the first step as template following the protocol: 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.

Results

Case studies and necropsy findings

The gross skin lesions included small, flat, pale red areas, or pink spots, characterized by the absence of desquamation and rare haemorrhages, or protruding areas with great extension, a round/oval shape, covered by serous/fibrin exudate, displaying various intensity of reddening, haemorrhage and slight desquamation (Fig. 1). In some individuals, larger, slightly raised lesions with variable degrees of redness, evident desquamation and central ulceration were observed. Necropsy included the examination of internal organs and did not reveal further lesions. According to the standardized method proposed for the description of rainbow trout skin disorders of uncertain aetiology (including the RMS cases definitions) (Oidtmann *et al.* 2013), the authors asserted that the macroscopic signs were ascribable to RMS.

Histology

The skin lesions observed in all specimens were characterized by an inflammation involving all the layers from epidermis to subcutis, including the underlying muscular tissue (Fig. 2). The epidermis was often present, or partially missing. In some specimens, a moderate intra-epidermal necrosis was detectable and this layer was also oedematous (with peculiar thorn shaped cellular expansions). The stratum spongiosus (SS) of the derma was affected by a mild or severe infiltration of lymphocytes and monocytes, and occasionally recruited neutrophil granulocytes. Scales and their pockets were severely affected by an inflammatory reaction, showing a strong infiltration of lymphocytes and monocytes/macrophages within the pockets, particularly below the scales. The stratum compactum (SC) was thickened and slightly infiltrated by lymphocytes/macrophages, but still maintaining its structure. The subcutis was heavily infiltrated by lymphocytes, plasma cells and macrophages. A slight fibroplasia was also detectable. The underlying muscular tissue appeared often subjected to moderate/severe cellular

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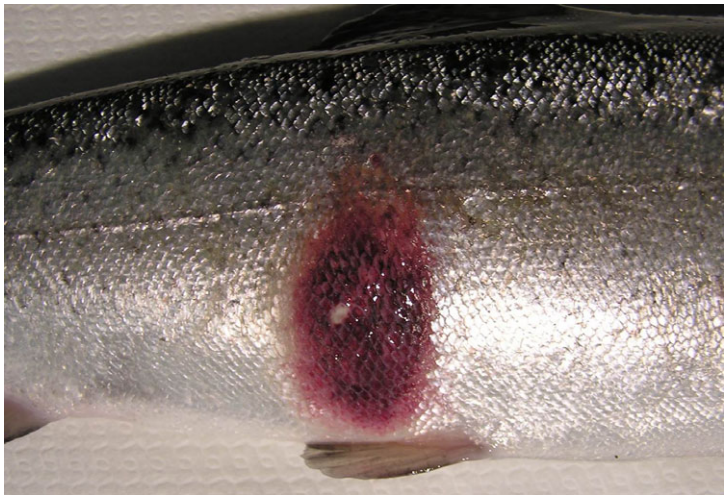

Figure 1 Typical gross skin lesion including a large, flat, red area, oval shaped, displaying haemorrhage and slight desquamation. [Colour figure can be viewed at wileyonlinelibrary.com].

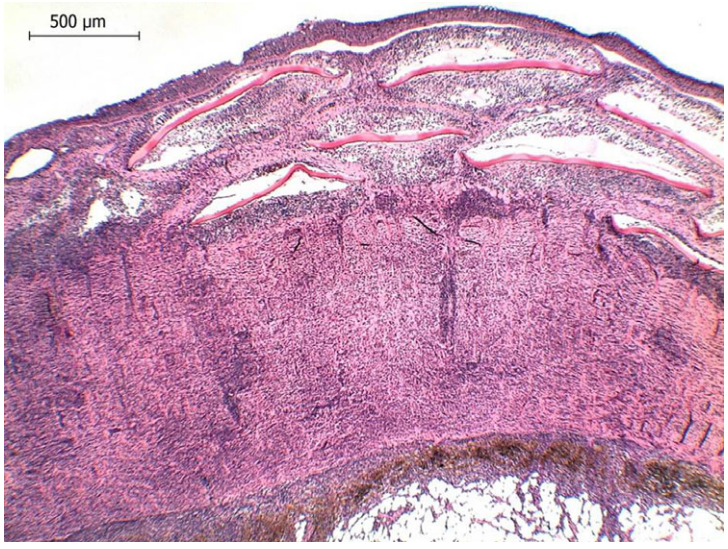

Figure 2 Skin lesion characterized by inflammation involving all layers from epidermis to subcutis, including the underlying muscular tissue. The stratum spongiosus of the derma is affected by a severe infiltration of lymphocytes and monocytes. Scales and their pockets are severely affected by infiltration of lymphocytes and monocytes/macrophages both internal to the pockets and below the scales. The stratum compactum is thickened and heavily infiltrated by lymphocytes/macrophages. The subcutis is heavily infiltrated by lymphocytes, plasma cells and macrophages (H&E). [Colour figure can be viewed at wileyonlinelibrary.com].

infiltration of lymphocytes, plasma cells and macrophages. These cells were mainly localized in muscle areas underlying the infiltrated subcutis and tended to penetrate trough the myosepta. Grocott, Giemsa, Brown-Hopps and Macchiavello staining did not allow the detection of microorganisms in the tissues under examinations. In the five RMS-free trout, no lesions and microorganisms were observed.

Transmission electron microscopy

From the skin and/or spleen specimens of 10 RMS trout (59%), in numerous fibroblasts and in a variable number of macrophages, neutrophils and erythrocytes, intracytoplasmic microorganisms were present (Table 1). In eight of 10 trout, the number of cells containing the microorganisms was always more than five in the skin or spleen tissue samples under investigation. The microorganisms appeared as oval or short rod shaped, displaying a size ranging from 400 to 800 nm in length and 100 to 200 nm in width with a finely granular electron-dense cytoplasm (Figs 3–9). Often, the microorganisms had a distinct thin cell wall of about 10 nm, arranged in a distinct trilaminar structure typical of Gram-negative bacteria, composed by an outer and an inner membrane (cellular envelope) and a cytoplasmic membrane (Figs 4, 6 & 7). Also a thread-like structure located at one pole of the body was present beneath the cell membrane (Fig. 4) and a

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Table 1 Transmission electron microscopy observations for the presence of intracellular Rickettsiales-like organisms in trout with RMS. Criteria for scoring positivity was considered as follows: a + was assigned when intracellular bacteria were observed in one to five cells/sample, a ++ if 5 to 10 cells/sample showed intracellular bacteria and a +++ when more of 10 cells/sample were positive

Trout no.	Rickettsiales-like organisms positive organ	Rickettsiales-like organisms positive cells	Positivity score
1	Skin	Macrophages, fibroblasts	+
2	Skin, spleen	Macrophage, fibroblasts, erythrocytes	++
3	Spleen	Macrophages, erythrocytes	+++
4	Skin, spleen	Macrophages, fibroblasts	+++
5	Skin	Macrophages, fibroblasts	++
6	Spleen	Macrophages, erythrocytes	++
7	Skin	Fibroblasts	++
8	Negative	–	–
9	Negative	–	–
10	Negative	–	–
11	Negative	–	–
12	Negative	–	–
13	Skin, spleen	Macrophages, erythrocytes, neutrophils	+++
14	Skin	Macrophages	++
15	Negative	–	–
16	Spleen	Erythrocytes	+
17	Negative	–	–
1 Control	Negative	–	–
2 Control	Negative	–	–
3 Control	Negative	–	–
4 Control	Negative	–	–
5 Control	Negative	–	–

prominent slime layer obscuring the underlying cell wall was present in numerous microorganisms (Fig. 5). These microorganisms were mainly localized free within cytoplasm, frequently surrounded by an adjacent clear zone (Fig. 6) and only rarely delimitated by a host cell membrane (Fig. 7). In the spleens, microorganisms were often detectable within erythrocytes and macrophages. Those observed within erythrocytes were included in a clear vacuole, rarely delimitated by a distinct cell membrane and did not possess a clear cell wall organization (Figs 8, 9), as observed in microorganisms present within skin macrophages or fibroblasts. Often, skin neutrophils, macrophages and fibroblasts containing the microorganisms showed degenerative changes, and sometimes, the infected cells were not recognizable due to severe degeneration. Degenerative changes consisted of widened and rarefied cytoplasm, containing electron-lucent material (oedema/cell swelling), swollen degenerated mitochondria, with partial loss of cristae (cristolysis), amorphous poorly electron-dense flocculent debris and irregularly distended profiles of endoplasmic reticulum and nuclear envelope. Occasionally, plasma membrane disruption and blebbing were detectable. Neither microorganisms nor ultrastructural lesions were observed in any of the five RMS-free trout.

Molecular biology

Thirteen of 17 symptomatic trout (76%) were positive for the presence of Rickettsiales by the

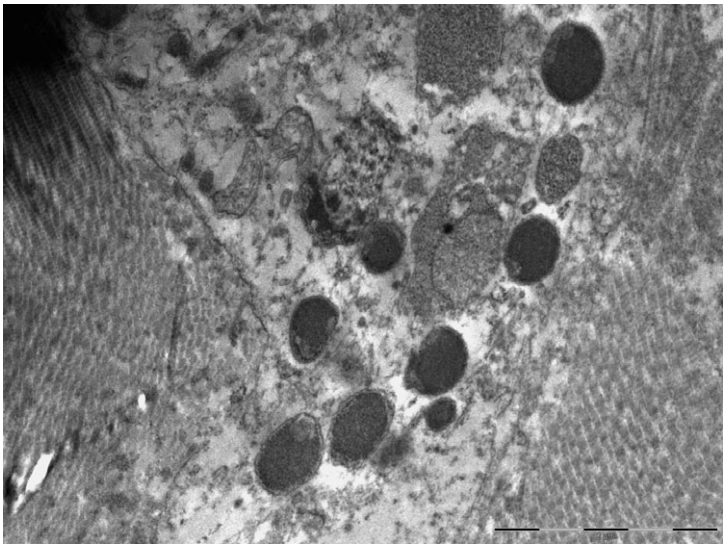

Figure 3 Skin. Transmission electron micrograph of a degenerated fibroblast with numerous microorganisms within the cytoplasm (Bar: 2 μm).

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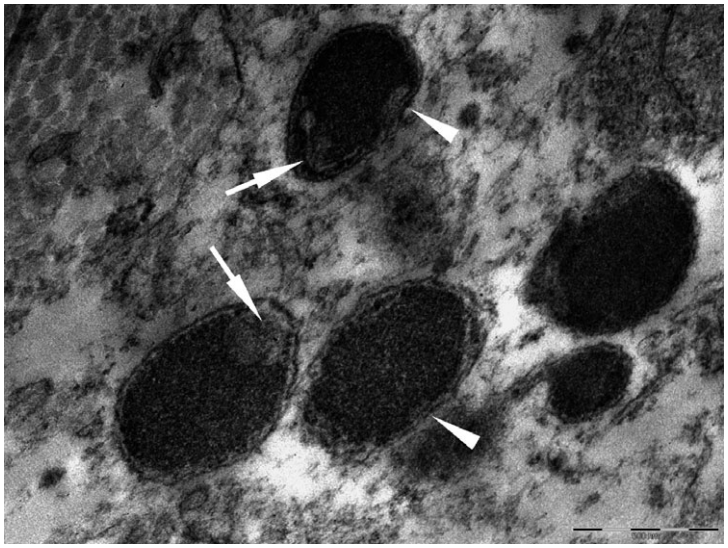

Figure 4 Magnification of Fig. 3. Some intracytoplasmic microorganisms with distinct inner and outer membrane (arrow heads) and thread-like structure (arrows) located at one pole of the bacterial body (arrows) (Bar: 500 nm).

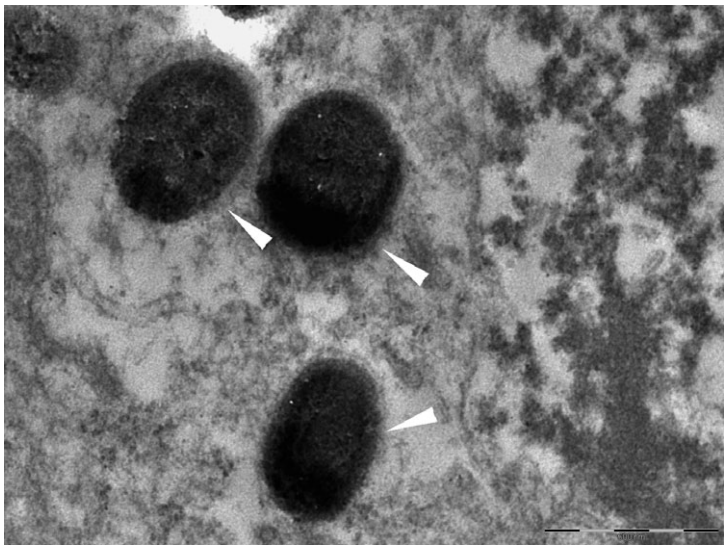

Figure 5 Skin. Degenerated mononuclear cell containing some microorganisms with distinct external slime layer (arrow heads) (Bar: 500 nm).

nested PCR assay (Table 2). The primers RiFCfw-RiFCrev amplified a band of 188 bp using as target an amplicon produced by the RLO1–RLO2 primers designed by Lloyd *et al.* (2008). No contamination was detected for the blanks used in the nested PCR steps. Only samples showing the expected band of 188 bp by the second step of the nested PCR were considered positive. The specificity of the primers induces to restrict the identification to a *Rickettsia*-like organism or *Midichloria*-like organism. In seven trout, both skin and spleen were positive, and the remaining six trout were positive only in skin. All RMS-free trout samples were negative for RLO/MLO DNA in nested PCR using RLO1/RLO2, as they did not produce any amplicon using RiFCfw-RiFCrev primers. The prevalence of positive RMS trout for MLO was 76% with PCR and 59% with TEM. In skin samples, the prevalence was 76% with PCR and 41% with TEM, respectively. In spleen samples, the prevalence was 41% with PCR and 41% by TEM, respectively.

Discussion

Based on the approach proposed by Oidtmann *et al.* (2013) for the diagnostics of rainbow trout skin disorders, and by considering water temperature, clinical signs, gross lesions distribution and histopathological features, as reported in the

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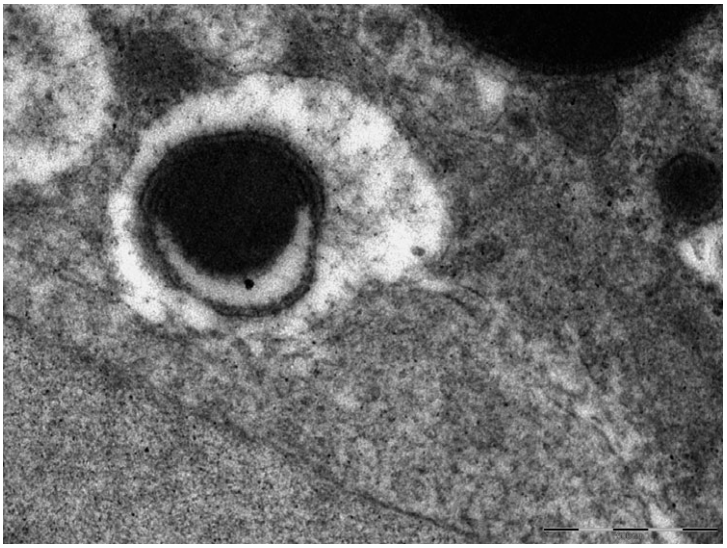

Figure 6 Skin. Macrophage. Microorganism with trilaminar cell membrane included within host cell cytoplasm surrounded by an adjacent clear zone (Bar: 500 nm).

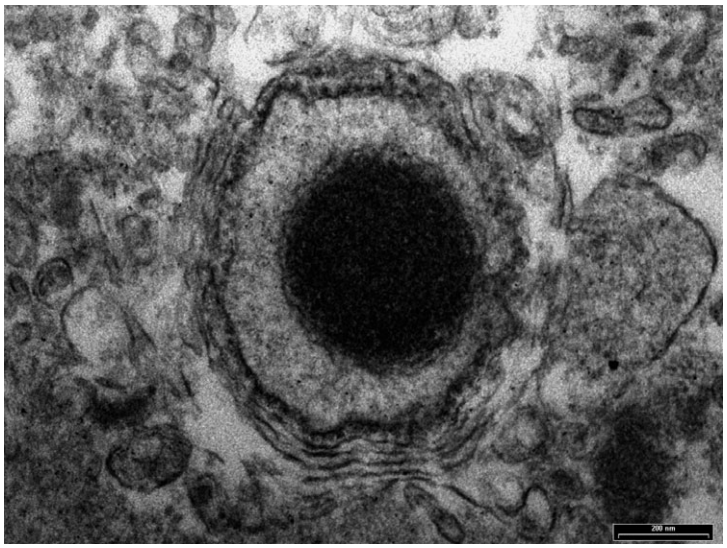

Figure 7 Skin. Macrophage containing a microorganism included within a vacuole delimitated by a host cell membrane (Bar: 200 nm).

diagnostic tree for RMS/CWSD, it is possible to argue that the episodes under investigation can be referred as RMS.

The histological evaluation made it possible to describe a typical pattern of RMS lesions that was easily comparable to that already reported by several authors (Ferguson *et al.* 2006; Verner-Jeffreys *et al.* 2006; Noguera 2008; Schmidt-Posthaus *et al.* 2009; Galeotti *et al.* 2013b; Oidtmann *et al.* 2013; Kubilay *et al.* 2014).

Moreover, in this study, we integrated the histological approach with ultrastructural and biomolecular investigations, with the aim of supporting the identification of a potential etiological agent. In 10 of 17 trout investigated by TEM, ultrastructural observations revealed the presence of intracytoplasmic oval to short rod-shaped electron-dense microorganisms (400 to 800 nm in length and 100 to 200 nm in width) that often showed a typical cell wall organization of Gram-negative bacteria comparable to other ultramicroscopical descriptions of intracellular bacteria belonging to the order Rickettsiales (Silverman & Wisseman 1978; Silverman 1991; Vannini *et al.* 2010; Vellaiswamy, Campagna & Raoult 2011). These microorganisms were mainly localized free within host cell cytoplasm as observed by other authors for many Rickettsiales, including various *Midichloria* species or various *Rickettsia* species (Fritsche *et al.* 1999; Dumler *et al.* 2001; Duh

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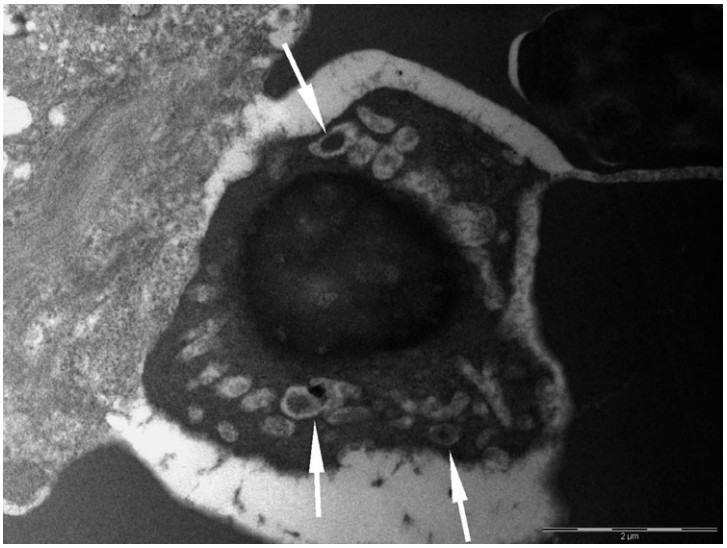

Figure 8 Spleen. Erythrocyte containing numerous microorganisms surrounded by a clear halo (arrows) (Bar: 2 μm).

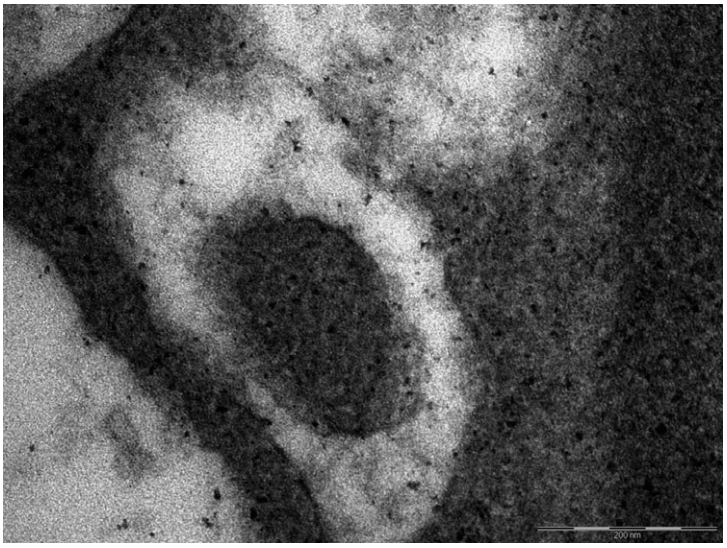

Figure 9 Spleen. High magnification of Fig. 8 (Bar: 200 nm).

et al. 2010; Vannini *et al.* 2010; Szokoli *et al.* 2016). Silverman (1991) demonstrated by ultrastructural analysis that members of the genus *Rickettsia* exist free within the cytoplasm whereas *Ehrlichia* and *Coxiella* are bound by a phagosomal or phagolysosomal membrane. This concept has been reconfirmed by Dumler *et al.* (2001) and by Vannini *et al.* (2010). The absence of a host-derived membrane delimitating the bacteria is considered a peculiar morphological feature of the family Rickettsiaceae (Dumler *et al.* 2001; Vannini *et al.* 2010) whereas the ultrastructural descriptions of bacteria belonging to the new family Midichloriaceae within Rickettsiales order reported variable presence of a host membrane (Sacchi *et al.* 2004; Sassera *et al.* 2006; Vannini *et al.* 2010; Szokoli *et al.* 2016). In our study, the microorganism observed sometimes showed an external slime layer as described in various *Rickettsia* species by Silverman *et al.* (1978) and also an intracellular thread-like structure was present, as observed by other authors (Wu & Pan 1999; Nilsson *et al.* 2002). The bacteria within spleen erythrocytes did not display a distinct trilaminar cell wall as observed in the skin fibroblasts or macrophages. The meaning of this difference is not clear and might be related to a different phase of development (Kocan *et al.* 2004), with a different morphotype of the organism as described in *Rickettsia prowazekii* and *Rickettsia rickettsii*

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Table 2 Results of the TEM and PCR assay performed on the skin and spleen tissues from symptomatic and control trout using the RIFCfw-RIFCrev primers on the amplicons obtained by RLO1–RLO2 primers with the first-step PCR

No.	Organs	TEM	Second-step PCR	No.	Organs	TEM	Second-step PCR
1	Skin	+	+	12	Skin	–	+
	Spleen	np	+		Spleen	–	–
2	Skin	+	+	13	Skin	+	+
	Spleen	+	+		Spleen	+	+
3	Skin	–	+	14	Skin	+	+
	Spleen	+	–		Spleen	+	+
4	Skin	+	+	15	Skin	–	–
	Spleen	+	–		Spleen	–	–
5	Skin	+	+	16	Skin	–	–
	Spleen	–	+		Spleen	+	–
6	Skin	–	+	17	Skin	–	–
	Spleen	+	+		Spleen	–	–
7	Skin	+	+	C	Skin	–	–
	Spleen	–	+		Spleen	–	–
8	Skin	–	+	C	Skin	–	–
	Spleen	–	–		Spleen	–	–
9	Skin	–	–	C	Skin	–	–
	Spleen	–	–		Spleen	–	–
10	Skin	–	+	C	Skin	–	–
	Spleen	–	–		Spleen	–	–
11	Skin	–	+	C	Skin	–	–
	Spleen	–	–		Spleen	–	–

np, not performed; C, control.

(Silverman 1991), in *Coxiella burnetti* (McCaul & Williams 1981) and in 'Candidatus *Megaira polyxenophila*' gen. nov., sp. nov. (Schrallhammer *et al.* 2013). Interestingly, the microorganisms we observed were detected in high numbers within the skin fibroblasts where the bacteria replication probably takes place, exploiting the proliferating stage of the fibroblast.

The data obtained by the molecular biology approach support the TEM findings. Indeed, 75% of the positive samples by TEM were also positive for Rickettsiales using PCR. In fact, the primers RiFCfw-RiFCrev annealed both the *Rickettsia*-like organism 16S DNA sequence and the Midichloriaceae 16S DNA sequence (*Midichloria*-like organism, MLO) limiting the identification of the unculturable microorganism present in the fish tissues to Rickettsiales.

The presence of RLOs in trout with RMS has been previously suggested by some authors (Lloyd *et al.* 2008; Metselaar *et al.* 2010), and more recently in RMS trout farmed in Scotland, *Midichloria*-like organisms (MLO) have been detected by PCR (Cafiso *et al.* 2015). Recent phylogenetic studies described a new clade–family within Rickettsiales *(Alphaproteobacteria),* named Midichloriaceae, comprising several bacterial symbionts and the bacteria *Midichloria mitochondri,* a symbiont of the hard tick *Ixodes ricinus* (Sassera *et al.* 2006; Vannini *et al.* 2010). To date, the only ultrastructural study performed in RMS trout failed to reveal the presence of intralesional microorganisms (Verner-Jeffreys *et al.* 2008), whereas in the present investigation intracellular bacteria displaying ultrastructural features similar to bacteria belonging to Rickettsiaceae and Midichloriaceae families, order Rickettsiales, regarding shape, dimension, cell wall organization and subcellular localization, have been observed (Fritsche *et al.* 1999; Sacchi *et al.* 2004; Sassera *et al.* 2006; Vannini *et al.* 2010; Szokoli *et al.* 2016).

Some reports in the literature have associated RLO with fish infectious diseases (Chen *et al.* 1994, 2000; Corbeil, Hyatt & Crane 2005). Most of these publications describe organisms close to *Piscirickettsia salmonis*, the causative agent of Piscirickettsiosis. This organism was erroneously considered a member of the Rickettsiaceae family, and in fact, phylogenetic studies confirmed the association of *P. salmonis* with the Gammaproteobacteria (Thiotrichales order) instead of Alphaproteobacteria (Fryer 2002; Fryer & Hedrick 2003).

The ultrastructural features, combined with the results obtained by primers used in the second-step PCR, annealing to RLO and MLO 16S rRNA

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sequences, allow us to assume that TEM detected microorganisms belonging to the order Rickettsiales. The ultrastructural morphology of the microorganisms reported in this study represents the first description of intralesional intracellular prokaryotic Rickettsiales-like (*Alphaproteobacteria*) bacteria in RMS trout, as confirmed by PCR. Taken together, the data obtained provide a strong support for the role of a Rickettsiales-like organism in the etiology of RMS.

This conclusion leads us to the need for further studies aimed at obtaining unchallengeable proof of the etiological agent both through the isolation of the microorganism in cell lines and *in vivo* transmission trials. Other epidemiological aspects should be investigated, including the possible involvement of a specific vector harbouring Rickettsiales-like bacteria, in the spread of the disease.

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