Chapter 14
Transmission of Members of the "*Candidatus* Midichloriaceae" Family to Vertebrates and Possible Involvement in Disease Pathogenesis

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1 General Considerations of "*Candidatus* Midichloriaceae" Potential Pathogenicity

"*Candidatus* Midichloriaceae", a new family of the order *Rickettsiales*, was discovered only recently thanks to molecular characterization techniques. The first report of a "*Candidatus* Midichloriaceae" endosymbiont of *Acanthamoeba* was in 1999 (Fritsche et al. 1999), and formal taxonomic recognition as a family within *Rickettsiales* was proposed in recent years (Montagna et al. 2013). Hereafter, the taxonomic category name *Candidatus* will be abbreviated to *Ca.* (for the explanation on its meaning see Chap. 3: "Biodiversity of non-model *Rickettsiales* and their association with aquatic organisms").

As shown in detail in Chap. 3, "*Ca.* Midichloriaceae" display a variability both in terms of molecular divergence and of host spectrum at least comparable to the two widely investigated families of *Rickettsiales*, namely *Rickettsiaceae* and *Anaplasmataceae*, which are responsible for several pathological alterations in humans and animals (Dumler and Walker 2005). Moreover, several "*Ca.* Midichloriaceae" bacteria have been retrieved in association with pathogenic amoebae and ectoparasites (i.e. ticks, fleas), suggesting that these bacteria could also potentially be infectious to vertebrates and even responsible for human and animal diseases However, compared with *Rickettsiaceae* and *Anaplasmataceae*,

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the mechanisms of interaction between "*Ca*. Midichloriaceae" and eukaryotic hosts, and their ability to infect different host species, are still poorly studied.

Few studies on the interaction between midichloriaceae and their hosts included investigations of the tick *Ixodes ricinus* and its endosymbiont "*Ca*. Midichloria mitochondrii" (e.g. Sassera et al. 2011), and *Acanthamoeba* infected with "*Ca*. Jidaibacter acanthamoeba" (Schulz et al. 2016) (see Chap. 3 for details). Moreover, although the phylogenetic analysis of "*Ca.* Midichloriaceae" associated with different eukaryotic hosts clearly indicates that they were repeatedly able to move and become adapted from one host to another, this aspect was rarely investigated experimentally and only included protist hosts (Schulz et al. 2016; Senra et al. 2016).

Despite the limited number of dedicated studies, some convincing indications of potential pathogenicity to humans and other vertebrates are already available for representatives of "*Ca*. Midichloriaceae". At least three cases have been documented that clearly suggest some linkage between pathogenic or immunogenic effects in vertebrates and bacteria belonging to "*Ca*. Midichloriaceae", two of which involve humans:

- "*Ca*. Midichloria mitochondrii" or strictly related bacteria can be transmitted after tick bite in humans and other mammals;
- "*Ca*. Lariskella arthropodarum" can be transmitted after tick bite in humans, which manifest acute fever symptoms;
- The presence of a "*Ca*. Midichloria"-related organism is associated with pathogenic effects in the rainbow trout *Oncorhynchus mykiss*.

The pathological implications of "*Ca*. Midichloriaceae" bacteria will be the subject of this chapter.

2 Transmission of "*Ca*. Midichloria mitochondrii" and Strictly Related Bacteria to Vertebrates

The mechanisms of interaction and the relationship of "*Ca*. M. mitochondrii" with its tick host *I. ricinus* still need to be fully elucidated (Fig. 14.1) (see Chap. 3 for details). On the other hand, this bacterium has recently attracted attention because of direct and indirect evidence for a potential pathogenic role in humans and other vertebrates (Mariconti et al. 2012a; Bazzocchi et al. 2013).

In the "*Ca*. M. mitochondrii" genome a set of 26 flagellar genes was found, including components of hook, filament and basal body, which putatively encode for functional flagella (Sassera et al. 2011). Although complete flagella have never been observed by electronic microscopy, the expression of a subset of the flagellar genes was demonstrated at the RNA level at several stages of *I. ricinus* development. In particular, all flagellar genes of "*Ca*. M. mitochondrii" were simultaneously expressed in *I. ricinus* eggs and female adults (Mariconti et al. 2012b).

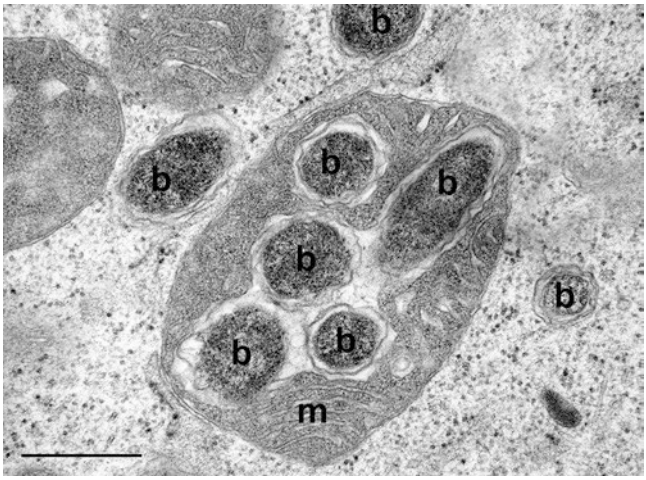

Fig. 14.1 "*Candidatus* Midichloria mitochondrii" bacteria in an oocyte of the tick *Ixodes ricinus*. *b* indicates the bacteria, *m* indicates a mitochondrion. A group of five bacteria is clearly visible within one organelle, while other "*Ca*. M. mitochondrii" appear to be in the cytoplasm, engulfed by a host-derived membrane. Bar = 0.7 μm

By applying an immunofluorescence technique using polyclonal antibodies against a fragment of the flagellar protein FliD of "Ca. M. mitochondrii", it became evident that "Ca. M. mitochondrii" is present both in ovaries and in salivary glands of the adult female tick I. ricinus. The presence of bacteria in salivary glands was also confirmed by PCR (Mariconti et al. 2012a). Such findings led to the hypothesis of transmission of "Ca. M. mitochondrii" bacteria to humans or animals by means of the tick bite, as occurs with other tick-borne bacteria such as Borrelia, Anaplasma and Rickettsia (Pesquera et al. 2015).

Serological analysis, using the recombinant FliD protein as antigen, confirmed the presence of an immunological response in humans and dogs after the tick bite (Mariconti et al. 2012a; Bazzocchi et al. 2013). Moreover, circulating DNA of "*Ca*. M. mitochondrii" and related bacteria has been found in the blood of sheep, horses, dogs and roe deer exposed to ticks (Skarphédinsson et al. 2005; Bazzocchi et al. 2013).

Taken together, these data clearly indicate that "*Ca*. M. mitochondrii" can be considered at least as a "package of antigens and DNA" that is transmitted to vertebrates through bite of its tick host. It is not clarified, however, whether the transmission involves live bacterial cells, nor if they are actually able to multiply within the vertebrate host. Nevertheless, according to the authors' interpretation, replication in the vertebrate is probable, as the small initial quantity of bacterial cells (or cell components) transferred during the tick bite would have been most likely in sufficient for detection, especially in large animals such as horses (Bazzocchi et al. 2013).

3 Transmission of "*Ca*. Lariskella arthropodarum" to Vertebrates

"*Ca*. Lariskella arthropodarum" is another member of the "*Ca*. Midichloriaceae" for which at least transient transmission to humans was observed. Mediannikov and co-authors (2004) reported that some patients affected by an acute febrile disease after a tick bite, presented, in their blood or near the area of tick bite, a 16S rRNA gene sequence corresponding to a midichloriacea retrieved in association with ticks collected in the same area (District of Khorovsk, East Russia). The authors called this organism "Montezuma", and considered it the suspected etiological agent of the disease. Later, this bacterium was found to be evolutionarily closely related to endosymbionts of other arthropods, such as stinkbugs and fleas, which were all subsequently included in the new species "*Ca*. Lariskella arthropodarum" (Matsuura et al. 2012).

According to the description by the authors, the reported symptoms of the disease resembled those of rickettsiosis and anaplasmosis, namely respiratory disorders, left shift of leukocyte formula, increased erythrocyte sedimentation rates, and increased level of serum transaminases (Mediannikov et al. 2004). However, PCR tests did not detect DNA of the etiological agents of monocytic and granulocytic ehrlichiosis, tick-borne rickettsiosis, nor other tick-borne transmissible diseases such as Lyme disease and babesiosis. On the other hand, while "*Ca*. Lariskella arthropodarum" DNA was found only in 4 out of the 22 patients examined, this could be due to assay detection limits (for example, in one patient it was found in a biopsy of the area of tick bite but not in the blood).

Nevertheless, it was not possible to establish a direct causal link of the organism to the disease although the available data are in part comparable to those on "*Ca*. Midichloria mitochondrii", as evidence of transmission of molecules to humans from "*Ca*. Midichloriaceae" bacteria after tick bites. On one hand, the case of "*Ca*. Lariskella arthropodarum" is even more relevant, because disease symptoms were also found but, on the other hand, the interaction with the vertebrate was less definite, since no specific test for host immune response against "*Ca*. Lariskella arthropodarum" was possible.

4 "*Ca*. Midichloria"-Like Organism in Rainbow Trout, *Oncorhynchus mykiss* Walbaum, Affected by Red Mark Syndrome

Red mark syndrome (RMS) is a non-debilitating condition affecting farm-reared rainbow trout (Fig. 14.2). It is characterized by the presence of red, pleomorphic, slightly raised lesions, typically on the flanks of affected fish, and generally occurs in fish > 100g. Although the disease does not cause mortality and fish can recover completely, with full healing of the lesions, the morbidity level can reach up to 90 %

Fig. 14.2 Rainbow trout (*Oncorhynchus mykiss* Walbaum) with lesions of red mark syndrome (RMS)

of an affected stock (Verner-Jeffreys et al. 2008). Fish behavior and appetite appear unaffected by the disease, but the lesions are unsightly and the negative economic impact can be severe if an outbreak occurs in stocks which are of market size, through downgrading of affected fish.

The condition was first recognized in Idaho, USA, during the 1950s, and by the 1970s was endemic in some hatcheries in the western and Pacific north-western regions (Erickson 1969; Olson et al. 1985). In the USA, the disease is referred to as strawberry disease (SD) which reflects the bright red coloration of the lesions resulting from dilation and congestion of the microvasculature (Olson et al. 1985). In 2003, a disease of rainbow trout, with lesions resembling those of SD, was detected in Scotland, UK (Noguera et al. 2007). This disease, referred to as RMS, had spread to rainbow trout farms across the UK by 2007 (Verner-Jeffreys et al. 2008). The occurrence of RMS at colder water temperatures (usually below 16 °C) and similarities in clinical signs suggested that SD from the USA and RMS were the same (Ferguson et al. 2006; Verner-Jeffreys et al. 2008). This has now been verified by pathologists from Europe and the USA, based on the case definitions of the two diseases (Oidtmann et al. 2013). RMS has also been reported from Switzerland, Austria, Italy, Turkey, Chile, and Iran (Schmidt-Posthaus et al. 2009; Galeotti et al. 2011; Kubilay et al. 2014; Sandoval et al. 2016; Sasani et al. 2016).

RMS is characterized at the histological level by dermal infiltration of lymphocytes and mononuclear cells, causing deformation and swelling of the laminar collagen structure of the stratum compactum. Edema of the scale pocket and osteoclastic resorption of the scales can be observed. Infiltration into the subcutaneous adipose tissue and skeletal muscle becomes more severe. In the initial stages, there is no involvement of the epidermis but, as the lesion develops, inflammation can extend into the epidermis and detachment of the epidermis may follow in advanced lesions, probably due to mechanical abrasion. However, it is clear that the lesion develops "from the inside out". A histological investigation of RMS lesion development in July, at a water temperature of 15 °C, suggested that the initial host cellular response is targeted toward the stratum spongiosum, with lymphocyte accumulation immediately below and above the scale pocket (McCarthy et al. 2013). A slight infiltration to the region surrounding the scale pocket was detected also in apparently healthy skin from affected trout. This observation from RMS-affected trout in Scotland is consistent with the report from SD-affected fish in the USA by Lloyd

et al. (2008), of inflammation beginning in the stratum spongiosum, which was also observed in healthy skin from affected fish and in some cohabitating unaffected fish. During the healing process, scales are regenerated; at the gross level, this is visible as a flat, grayish mark with no swelling, redness, or excess mucus. Once the fish is recovered, it is thought that there is no recurrence of the lesions in that individual (Olson et al. 1985; McCarthy et al. 2013).

Despite evidence from epidemiological studies and cohabitation trials that RMS is transmissible, numerous investigations have failed to uncover or confirm an etiological agent (Erickson 1969; Olson et al. 1985; Verner-Jeffreys et al. 2008). *Flavobacterium psychrophilum* has been suggested as one possible candidate (Ferguson et al. 2006). Using a nested PCR assay, Lloyd et al. (2008) reported a statistically significant association between SD lesions and the presence of 16S rDNA sequence similar to members of the order *Rickettsiales*. Phylogenetic analysis of the *Rickettsia*-like organism (RLO) 16S rDNA suggested that the most closely related organism was "*Ca.* M. mitochondrii", isolated from ixodid ticks. This relationship was confirmed by Montagna et al. (2013). Using the same nested PCR assay, a product of identical sequence has been amplified from rainbow trout affected by RMS in the UK (Metselaar et al. 2010; McCarthy unpublished). Subsequently, Cafiso et al. (2015) provided quantitative molecular evidence for the presence of the *Midichloria*-like organism in the organs (heart, liver, spleen, intestine, kidney) as well as the skin of RMS-affected fish.

Despite the strong statistical association with RMS, the RLO has not been confirmed as the etiological agent, and many questions remain to be answered about disease mechanisms and routes of transmission. The pathogenesis has yet to be elucidated; it has been postulated that RMS may represent a hypersensitivity response to a subclinical bacterial infection, in combination with precipitating risk factors (Olson et al. 1985; Noguera 2008). However, the belief that the manifestation of RMS lesions in an individual fish is a single, non-recurring event does not fit with the pattern of many hypersensitivity reactions, which are usually recurring or chronic (McCarthy et al. 2013). It does appear that there may be a host immunity component to the disease, as development of lesions can sometimes be triggered by stressful handling procedures, such as grading, and while outbreaks typically last 8 weeks, healing of lesions can occur very rapidly once temperatures rise toward 16 °C. It is not known if individual fish which have recovered from an outbreak are carriers of the putative etiological agent. The principle route for introduction of the disease appears to be movement of fish or ova onto a site (Adam 2009) and RMS is known to be transmissible via fertilized eggs, although disease outbreaks have not occurred at all sites stocked with fish grown from these eggs (Verner-Jeffreys et al. 2008).

RMS has commonly been managed through the use of antibiotics, particularly oxytetracycline, though the lesions will resolve without treatment (RMS Meeting 2009; Oidtmann et al. 2013). It has sometimes been possible for larger producers to maintain a supply of marketable trout through sourcing fish from a number of different production sites, which are at different stages of the disease, or by harvesting for fillet production. In the absence of definitive information about the etiological

agent, disease triggers and routes of transmission, the utilization of good biosecurity procedures is recommended to reduce the introduction and spread of disease through a site (Adam 2009). It appears that some sites have been able to eradicate the condition through systematic clearance of livestock, and disinfection or liming of ponds (Rodger 2008; Verner-Jeffreys et al. 2008). However, the continual emergence of RMS in new countries and the persistent recurrence of the disease on production sites in the UK underline the importance of identifying the etiological agent(s), so that detection and preventive measures can be targeted more effectively.

5 Conclusions and Future Perspective

As presented above in detail, after less than 20 years from the detection of the first representatives of "*Ca*. Midichloriaceae", several lines of evidence convincingly indicate that this family includes agents transmissible to humans and other vertebrates, and possibly involved in the development of pathological changes. These data clearly underline the need for further investigations to evaluate the propagation and dissemination of these bacteria inside vertebrates and the possible pathogenic effects, considering also that a formal proof of their association is lacking even in the three reported cases.

For example, human or animal diseases suspected to be linked with ticks or other ectoparasites could be reinterpreted and further investigated, in particular those clinical cases compatible with rickettsiosis or ehrlichiosis, but where there is an absence of antibody titers for known pathogenic *Rickettsiales* (Mediannikov et al. 2004). The involvement of tick-borne "*Ca*. Midichloriaceae" bacteria in modulating the immune response after the tick bite could also have consequences for the establishment of infections by other tick-borne pathogens (Mariconti et al. 2012a).

Similarly, considering that amoebae hosting "*Ca*. Midichloriaceae" are known to parasitize humans (Fritsche et al. 1999; Schulz et al. 2016), the direct involvement of bacteria in the amoeba-induced pathogenic effects as well as their infectivity could be investigated in more detail. Indeed, the role of amoebae and other protists as natural reservoirs and potential vectors for pathogenic bacteria has already been outlined (Barker and Brown 1994; Gao et al. 1997; Molmeret et al. 2005).

Aside from the aspects related to human health, the presence of several "*Ca*. Midichloriaceae" in aquatic hosts, in particular different ciliates (Vannini et al. 2010; Boscaro et al. 2013a, b; Szokoli et al. 2016; Senra et al. 2016) also calls for further investigations. These protists could be involved as reservoirs of bacteria infectious for aquatic invertebrates and vertebrates, in particular in aquaculture. As exemplified by the case of RMS in trout, the potential impact of such infectious diseases could be relevant in terms of economic consequences.

Finally, the importance of obtaining genomic sequences from other representatives of "*Ca*. Midichloriaceae" should be emphasized, as few are currently available (see "Chap. 3" for details). As in the case of "*Ca*. M. mitochondrii", genome analysis

could help in elucidating new aspects of the biology of the bacterium potentially involved in pathogenesis, as well as providing a basis for new diagnostic tools, such as the production of specific synthetic antibodies and specific tests for immune reactivity of sera (Mariconti et al. 2012a, b; Bazzocchi et al. 2013).

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