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Department of Veterinary Science and Public Health (DIVET)

PhD Course in Veterinary Hygiene and Animal Pathology
(Cycle XXV)

Doctoral Thesis

Midichloria mitochondrii as an emerging infectious agent: molecular and immunological studies on the intra-mitochondrial symbiont of the hard tick *Ixodes ricinus*

(SSD: Vet/06)

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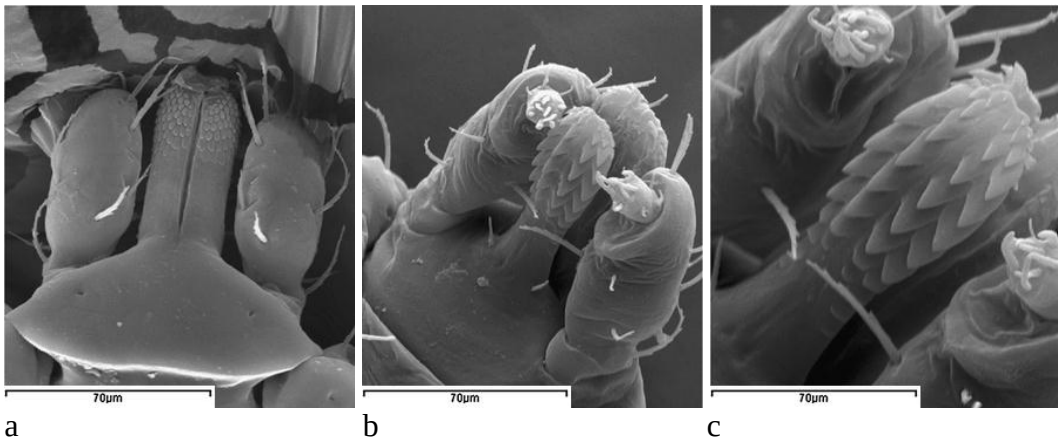
1. GENERAL INTRODUCTION

1.1 TICKS

1.1.1 General description

Ticks are members of the subclass Acari, which is the largest subclass in the class Arachnida of the suborder Ixodida within the order Parasitiformes. Ticks are divided into three families: the Ixodidae (703 spp), or "hard ticks", so called because of their sclerotized dorsal plate, which are the most important family in numerical terms and in medical importance; the Argaside (194 spp) or "soft ticks" so called because of their flexible cuticle; and the Nuttalliellidae (1 sp) that are represented by only a single species that is confined to southern Africa (Olivier, 1989; Horak et al., 2003; Apanaskevich et al., 2011; Dantas-Torres et al., 2012).

Ticks have a large body (2-30 mm) with no antennae, and their bodies are not divided into distinct head, thorax and abdomen parts. The mouthpart is localized in the anterior part of the body (capitulum), and includes sensory organs, cutting organs and the hypostome, a median immobile organ with numerous recurved teeth that penetrate the host skin (Fig. 1). During the tick bite, the salivary glands produce a various molecules such as enzymes, vasodilators, anti-inflammatory and immunosuppressive. These salivary secretions facilitates successful blood feeding; in particular the anesthetic makes the bite usually painless.



a b c

Figure 1. Capitulum of *Hyalomma impressum* larva: (a) dorsal view, (b) ventral view, (c) hypostome (Abdel-Shafy et al., 2011).

Ticks are able to find their hosts using sensory organs located on the dorsal surface of the tarsus of the legI, which also contain a cluster of olfactory and gustatory receptors (Haller's organ) that allow to perceive chemical stimuli, phenols, humidity and aromatic chemicals (Parola and Raoult, 2001). Ticks use typically two host-seeking strategy: the ambush strategy in which the ticks climb up the vegetation and wait for passing hosts (e.g. *Rhipicephalus sanguineus*, *Ixodes ricinus*, *Ixodes scapularis* and *Dermacentor variabilis*); and the hunter strategy in which ticks attack hosts (e.g. *Amblyomma hebraeum* and *Amblyomma variegatum*).

Ticks have three basic life stages: larval, nymphal and adult (female and male) and typically each feeding stage have a single host (Spickett, 1994). Some ticks species are host-specific, feeding on only a specific variety of animals, while others have different hosts for each feeding stage, and host specificity may vary between the different stages in the same species (e.g. *R. sanguineus* feeds principally on dogs; *I. ricinus* usually feeds on different hosts such as small and big mammals).

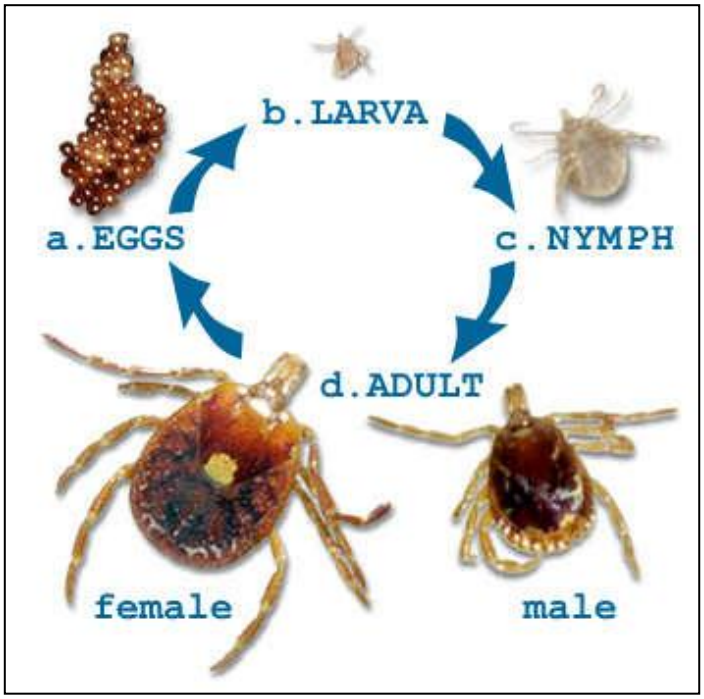


Figure 2. Developmental stages of tick.

Females have the ability to expand their body during the feeding, and some of them, like the Ixodide, may increase their body weight up to 120-fold. Environmental conditions such as photoperiod, temperature and relative humidity can influence the length of the life cycle: usually it is completed in 2-3 years, but it may take from 6 months to 6 years.

1.1.2 Bacteria and ticks

Ticks are the main arthropod vectors of disease agents for humans and domestic animals, and they are also considered the first arthropods to be established as vectors of pathogens (Jongejan and Uilenberg, 2004; Colwell et al., 2011). It is known that species belonging to the genera *Ixodides*, *Dermacentor*, *Rhipicephalus*, *Amblyomma*, *Hyalomma*, *Haemaphysalis* and *Ornithodors* are the main carriers of pathogens for vertebrates. Bacteria infect ticks by feeding on bacteremic hosts, by transstadial and transovarial transmission or by cofeeding in which several ticks feed in close proximity on the host (bacteria pass from an infected tick to an uninfected one; Raoult and Roux, 1997).

Some bacteria such as rickettsiae, are able to multiply in almost all tick organs, in particular ovary and salivary glands which enables transmission of organism during the feeding. Rickettsiae also are transmitted both transtadially and transovarially; in this way ticks are also a reservoir of bacteria and increase the efficiency of their distribution. Other bacteria may be transmitted transovarially but do not infect the salivary glands of ticks and cannot then be transmitted to susceptible vertebrate hosts where they might cause a disease (Niebylski et al., 1997). Moreover each stage of ixodid tick feeds only once, and bacteria acquired during feeding can then be transmitted to another host only when the tick has molted to its next developmental stage. Ticks transmit bacteria to vertebrates when their feeding sites are contaminated with infected salivary secretions (e.g., spotted fever group rickettsiae, *B. burgdorferi,* relapsing fever borreliae), regurgitated midgut contents (e.g., *B. burgdorferi*) and feces (e.g., *Coxiella burnetii*) (Raoult and Roux, 1997). The increased risk of pathogens transmission is correlated to the duration of the blood meal. Ixodide feed for several days (2-15 days) during which they stay firmly attached to the host and usually do not cause pain while feeding. During blood meal there are alternating periods of sucking

blood and salivation, in which pathogens could be transmitted to the host (Parola and Raoult, 2001).

Tick-borne diseases (TBDs) has a worldwide distribution, and the spectrum of TBDs affecting domestic animals and humans is increased in recent years (Nicholson et al., 2010; Dantas-Torres, 2007; Piesman and Eisen, 2008). Ticks and wildlife are the main reservoirs of tick-borne pathogens of medical and veterinary concern. Wildlife may serve as reservoirs or as hosts that amplify several human pathogens; the vast majority of TDBs are from wildlife reservoirs (Colwell et al., 2011; Dantas-Torres, 2007; Piesman and Eisen, 2008). Most cases of human parasitism are related to hard ticks that are the main vector of tick-borne rickettsial pathogens (Jongejan and Uilenberg, 2004; Piesman and Eisen, 2008; Labruna, 2009; Breitschwerdt et al., 2011). Dogs and people are exposed and susceptible to infection by many of the same tick-borne bacterial pathogens in the order Rickettsiales. The global distribution of tick-borne rickettsial pathogens varies according to the density and distribution of the predominant tick vectors and the population density of reservoir hosts (Shaw et al., 2001; Beugnet and Marie, 2009; Neer and Harrus, 2006; Greig and Armstrong, 2006; Greene and Breitschwerdt, 2006).

In the recent years an increasing number of novel rickettsial organisms has been described since the advent of molecular detection and characterization (Parola et al., 2005). Some of these organisms are often described from amplicons and without cultivation of isolates, and are often detected only in arthropod populations, while other organisms were also detected in vertebrates blood samples, as the case of *Midichloria* and like organisms (hereafter: MALOs). There is circumstantial evidence that MALOs could be transmitted to terrestrial vertebrates during the tick bite: (i) 16S rRNA gene sequences related with *M. mitochondrii* have been amplified from roe deer during a screening for tick-borne bacteria (Skarphedinsson et al., 2005); (ii) phylogeny of ticks and their respective MALOs are not congruent, with distantly related ticks harboring MALOs that are identical at the 16S rRNA gene level (Epis et al., 2008); this implies that MALOs could be transmitted horizontally among ticks, and a simple mechanism that could be hypothesized to explain horizontal transmission is the infection of an host parasitized by different tick species (or co-feeding with bacterial transmission

without true infection); (iii) MALO 16S rRNA gene sequences have been amplified from human patients parasitized by ticks (Mediannikov et al., 2004).

1.2. *MIDICHLORIA MITOCHONDRII*

M. mitochondrii appears as a Gram-negative, non sporeform intracellular bacterium with a bacillus shape of 0,45 μm in diameter and 1,2 μm in length. This bacterium was observed for the first time in 1970 by Lewis during an electron microscopical (EM) study on *I. ricinus* collected in England. In 1992, highly similar bacteria with this same peculiarity were found in *I. ricinus* from Switzerland (Zhu et al., 1992), and later in 2004 in the same tick species in Italy by EM study and in situ hybridization (Beninati et al., 2004; Sacchi et al., 2004). *M. mitochondrii* was principally observed within various cell types (luminal cells, funicular cells and oocytes) of the reproductive tissue of *I. ricinus* female, free in the cytoplasm or included in a host-derived membrane. In addition, in luminal cells and oocytes, a high amount of these bacteria are observed within the mitochondria, in the space between the two membranes of the organells. The number of *M. mitochondrii* observed within mitochondria is variable, ranging from 1 to over 20 in single thin sections. Mitochondria that harbor a high load of bacteria appear swollen, with a dramatic reduction of the matrix (Sacchi et al., 2004). Current data indicate that the prevalence of *M. mitochondrii* in wild-collected *I. ricinus* females is 100 %, while in males it is lower (44 %, Lo et al., 2006). Recent screening studies showed the presence of this bacteria in 7 species of hard ticks, in which there is evidence that the prevalence of *M. mitochondrii* is lower than 100%. In *I. ricinus, M. mitochondrii* is vertically transmitted to the progeny, as indicated by PCR evidence on eggs and newly-emerged larvae and by the presence of these bacteria in the oocytes of this tick (Sassera et al., 2006; Sacchiet al., 2004).

From a taxonomic point of view *M. mitochondrii* represents a divergent lineage of the order Rickettsiales, bacteria belonging to the class of the alpha-proteobacteria (Sassera et al., 2006). Phylogenetic comparisons between the *M. mitochondrii* 16S rRNA gene sequence and near full-length sequences (>1200 bp) in the databases

revealed the existence of a strongly supported monophyletic group containing various unclassified bacteria from ticks and from A*canthamoeba* spp. (Fig. 3). These results are in agreement with previous studies (Parola et al., 2005; Beninati et al., 2004; Mediannikov et al., 2004) in which bacteria of this group were found to be the sister group of the family Anaplasmataceae (comprising the genera *Anaplasma*, *Ehrlichia*, *Neorickettsia* and *Wolbachia*).

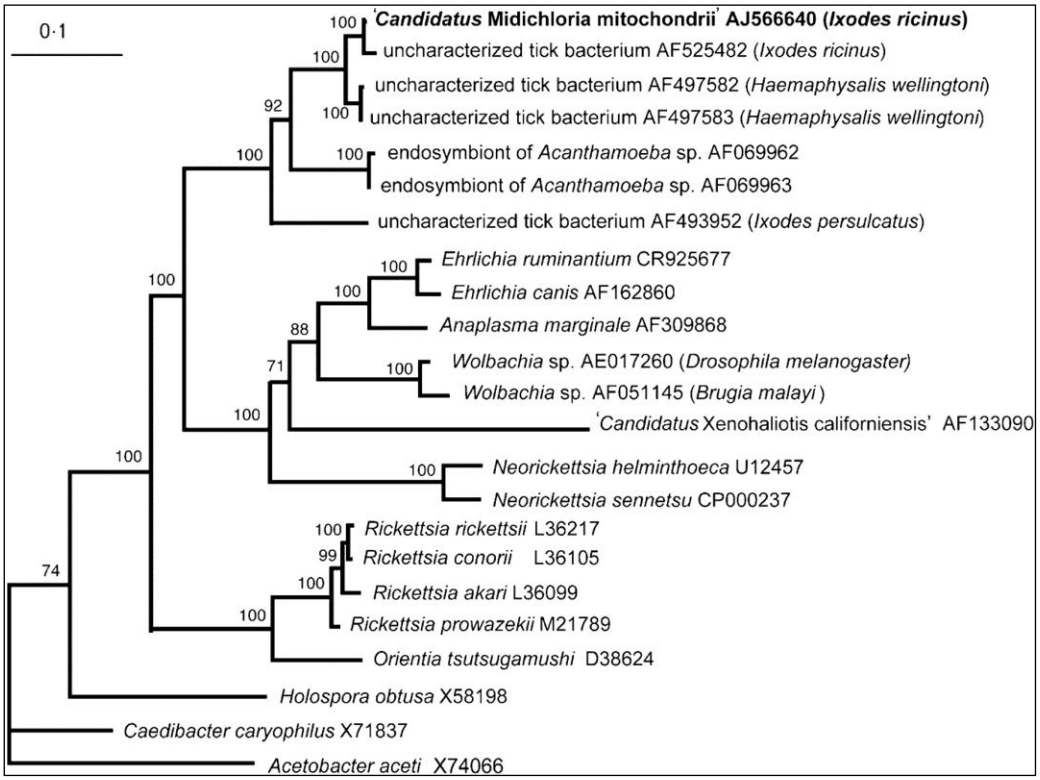


Figure 3. Phylogenetic tree based on near full-length 16S rRNA gene sequences (> 1200 bp) showing the position of *M. mitochondrii* and selected members of the *Rickettsiales* (Sassera et al., 2006).

Separate analyses, including partial 16S rRNA gene sequences (<1000 bp), showed that this phylogenetic branch includes other sequences obtained from ticks, humans and a microbial consortium (Fig. 4). *M. mitochondrii* is closely related to an uncharacterized bacterium from the tick *Haemaphysalis wellingtoni* (Figs 3 and 4). Since these ticks are distantly related (Black and Piesman, 1994), this suggests that *M. mitochondrii* and its relatives may undergo horizontal transfer.

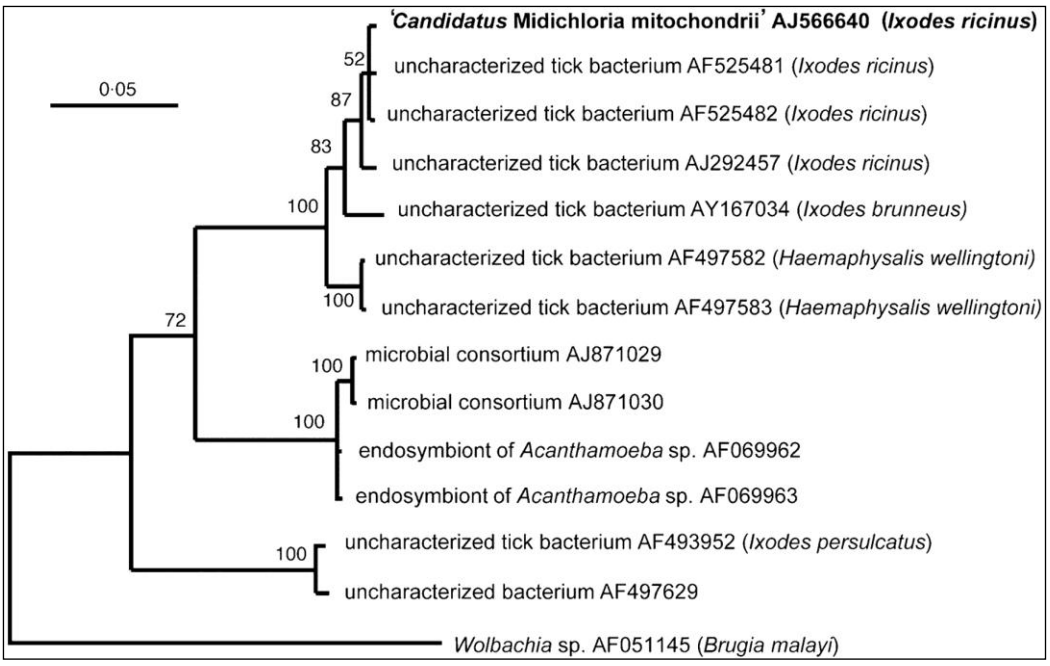


Figure 4. Phylogenetic tree showing the position of *M. mitochondrii* in relation to closely related and short uncharacterized sequences. The tree is based on a 524 bp16S rRNA gene sequence alignment (Sassera et al., 2006).

The high prevalence of *M. mitochondrii* in wild collected adult females of *I. ricinus* seems to suggest a beneficial role of this bacterium toward the host ticks. There is evidence that *M. mitochondrii* is vertically transmitted in *I. ricinus*, from adult females to the offspring. The bacteria then follow the development of the females host, with an efficient trans-stadial transmission. The population dynamics of this bacteria has been followed by quantitative real-time polymerase chain reaction (qPCR): bacterial numbers increase after the blood meal and decrease after the molts (Sassera et al., 2008). The symbiont also can be transmitted horizontally, indeed it was detected that ticks belonging to different species harbor identical *M. mitochondrii* 16s rRNA gene sequences (Epis et al., 2008). This could suggest that horizontal transmission occurs through a passage in a vertebrate host. PCR analysis detected the presence of bacteria with a high gene sequence similarity with *M. mitochondrii* in blood of roe deer (Skarphedinsson et al., 2005), and in samples from human patients that presenting acute febrile symptoms (Mediannikov et al., 2004): this may support the idea that this bacterium is able to circulate among ticks and passing through the infection of a vertebrate host.

Bacteria closely related to *M. mitochondrii* have also been detected in other tick species (Epis et al., 2008; Beninati et al., 2009); in addition, 16S rRNA gene sequences that cluster with *M. mitochondrii* have been amplified from a variety of sources, including fleas, bed bugs, tabanid flies and cnidarians (Erickson et al., 2009; Richard et al., 2009; Hornok et al., 2008; Matsuura et al., 2012; Sunagawa et al., 2010).

The biology of *M. mitochondrii* is still unexplored. Major questions are open: is *M. mitochondrii* beneficial to the host tick? How can the tick cope with destruction of mitochondria? How does the bacterium move among mitochondria? There are only hypotheses about possible roles of *M. mitochondrii* in the host moulting, in haem detoxification and in conferring protection toward different types of pathogens/parasites (Pistone et al., 2011). It is clear that specific tools for the study of this bacterium (e.g. antibodies for immunostaining and purification) would be of great help to address these issues.

2. PURPOSE OF THE PHD PROJECT

M. mitochondrii is an alpha proteobacteria belonging to the order Rickettsiales. It is an intracellular bacterium present in different tick species and in particular it is studied in the hard tick *I. ricinus*. The prevalence of *M. mitochondrii* in *I. ricinus* is 100% in females and around 40% in males. *M. mitochondrii* was principally observed in the reproductive tissue of *I. ricinus* female, free in the cytoplasm or included in a host-derived membrane. In addition, in luminal cells and oocytes, a high amount of these bacteria are observed in the space between the two membranes of mitochondria. There is evidence that *M. mitochondrii* is vertically transmitted in *I. ricinus*, from adult females to the offspring; the symbiont can also be transmitted horizontally. The horizontal transmission could occur through a passage in a vertebrate during the co-feeding of different tick species on the same host; ticks belonging to different species show, indeed identical 16S rRNA gene sequences related to *M. mitochondrii.* In addition, bacteria with an high similarity of 16S rRNA gene sequences with *M. mitochondrii* have been detected in blood of roe deer and in samples from human patients that present acute febrile symptoms. This may support the idea that these bacteria are able to circulate among ticks and passing through the infection of a vertebrate host.

Major questions are open: how does *M. mitochondrii* move among mitochondria? Is *M. mitochondrii* transmitted to the vertebrate host during the ticks blood meal? It is clear that specific tools for the study of this bacterium (e.g. antibodies for immunostaining and immunoblotting) would be of great help to address these issues.

The principal aims of my PhD project are: 1) study the presence of a flagellar structure in *M. mitochondrii*, and its possible function; 2) investigate if *M. mitochondrii* is present in the salivary glands of the tick *I. ricinus* and whether it is transmitted to vertebrates during the tick blood meal.

1) The recently sequenced genome of *M. mitochondrii* revealed the presence of 26 putative flagellar genes. *M. mitochondrii* appears to be the first described

rickettsiales that posses a putative flagellar structure. Open questions are whether these genes are expressed and whether they possess the domains expected for the flagellar function. The flagellar apparatus, if present, could plays a function in *M. mitochondrii* biology. Since nothing is currently known about how this bacterium is able to invade the mitochondria of *I. ricinus* cells, is possible to speculate about a potential role of a flagellar structure in the invasion of tick mitochondria by *M. mitochondrii*. Indeed, the flagellum has previously been indicated to be the means of interaction between bacterial and symbionts. To answer to this issue, a putative flagellar protein has been produced in recombinant form, purified and used for the production of polyclonal antibodies. These can be used for immunostainig and immunogold detection of *M. mitochondrii* in *I. ricinus* tissue.

2) There are circumstantial evidences that *Midichloria* and like organisms (MALOs) could be transmitted to terrestrial vertebrates during the tick bite. To find out whether *M. mitochondrii* could be inoculated to the vertebrate host during the tick bite, we designed a study to investigate whether *M. mitochondrii* is present in the salivary glands of the host tick *I. ricinus* and whether vertebrates parasitized by *I. ricinus* develop antibodies against *M. mitochondrii*. To address these questions, we used a recombinant antigen from *M. mitochondrii* (for circulating antibodies detection in humans and dog sera by using an ELISA assay) and a polyclonal antibody raised against this antigen (for immunofluorescence assays on salivary glands). These results could provide evidence that *M. mitochondrii* is released with the tick saliva and raise the possibility that *M. mitochondrii* is infectious to vertebrates.

3. A STUDY ON THE PRESENCE OF FLAGELLA IN THE ORDER RICKETTSIALES: THE CASE OF *MIDICHLORIA MITOCHONDRII*

3.1 PURPOSE

According to Bergey's Manual of Systematic Bacteriology, the Rickettsiales are '...bacteria with typical Gram-negative cell walls and no flagella'. The recently sequenced genome of *M. mitochondrii* revealed the presence of 26 putative flagellar genes. Open questions in relation to this observation are whether these genes are expressed and whether they possess the domains expected for the flagellar function. Here we show that: (i) the putative flagellar proteins of *M. mitochondrii* actually possess the conserved domains and structural features required for their function in a model bacterium; (ii) the seven flagellar genes of *M. mitochondrii* that have been tested are expressed at the RNA level; and (iii) the putative flagellar cap gene of this bacterium (FliD) is expressed at the protein level, and can be stained within the bacterium and at its surface. Beside the specific questions that relate to the first evidence, to our knowledge, for a flagellar apparatus in a member of the order Rickettsiales, we present here novel tools (recombinant protein and antibodies) that will facilitate the study of *M. mitochondrii*.

3.2 MATERIAL AND METHODS

3.2.1 Overexpression and purification of the flagellar protein FliD

A DNA fragment coding for 325 aa of the flagellar protein FliD of *M. mitochondrii* was amplified by PCR. The locations of the forward (5'-ACTCGAGGATCCGATGTGCTAGCTAGAAG-3') and reverse (5'-CGATCAAAGCTTTTACCGATTAAATAAAG-3') primers on the FliD full-length gene sequence available for *M. mitochondrii* (GenBank accession no. NC_015722) are 1716–1733 and 2674– 2691, respectively. These primers include

restriction sites (underlined) at their 5'-ends for ligation into the pQE30 expression vector (Qiagen), which are BamHI and HindIII, respectively. The reverse primer also includes a stop codon. The recombinant plasmid obtained after ligation was named FliDpQE30. The expected fusion protein includes FliD and nine additional amino acids encoded by the vector at the 5' end (MHHHHHHGS). The plasmid was amplified in *Escherichia coli* M15pRep4 and, after sequencing a sample of clones using ABI technology, a clone with the correct gene sequence was selected. *E. coli* M15pRep4 cells containing FliDpQE30 were grown at 37 °C in 50 ml Luria–Bertani (LB) medium containing ampicillin (100 mg/ml) and kanamicin (25 mg/ml) to OD_{600} 0.5 before induction of recombinant FliD expression by addition of 1 mM IPTG. Cells were harvested after 3 h by centrifugation at 4000 g for 20 min. Collected cells were disrupted by freeze–thawing and sonication, and recombinant FliD was collected as an insoluble aggregate by centrifugation at 10 000 g for 10 min. The recombinant FliD was solubilized in 0.1 M NaH_2PO_4, 0.01 M Tris/HCl, 6 M urea (pH 8.0) and was purified by using HPLC (Poros MC/M 2.1630 mm) using Cu^{2+} as a ligand. The protein was then eluted in one step by adding 100 mM imidazole in solubilization/loading buffer. The molecular mass of the recombinant protein (rFliD) is 38 kDa.

3.2.2 Antibody production

Purified rFliD was used for the production of polyclonal antibodies in two rabbits (PRIMM, srl). At the end of the immunization protocol, sera were collected and titred by ELISA. Sera specificity were tested by Western blot on a total extract of proteins of *I. ricinus* (which harbours *M. mitochondrii*) and *Ixodes hexagonus* (which does not harbour *M. mitochondrii*) adult females under standard conditions (1:5000 of anti-rFliD; 1:2500 of secondary anti-rabbit antibody).

3.2.3 Sample collection

Six *I. ricinus* and two I. *hexagonus* semiengorged adult tick females were collected from goats and hedgehogs in Northern Italy. All ticks were identified by using standard taxonomic keys (Manilla, 1998). Tick ovaries were extracted in sterile conditions and divided in four parts for: (i) DNA extraction to confirm *M.*

mitochondrii infection; (ii) transmission electron microscopy (TEM); (iii) immunogold staining; and (iv) indirect immunofluorescence assay for *M. mitochondrii* detection and staining. In addition, two pools of 50 eggs from two wild-collected fully engorged females, two pools of five wild-collected larvae, two pools of five wild-collected nymphs and the ovaries from two semiengorged adult females of *I. ricinus* were processed for RNA extraction and cDNA synthesis to the determine the expression of seven flagellar genes (*fliC, fliD, flgL, flgK, flgE, fliG, motA*) in different tick developmental stages.

3.2.4 PCR for *M. mitochondrii* detection

DNA was extracted using the DNeasy blood and tissue kit (Qiagen), eluted in 100 µl of sterile water, quantified by spectrophotometer and stored at -80 °C before using. PCR screening for *M. mitochondrii* detection was performed using two sets of primers: Midi-F (5'-GTACATGGGAATCTACCTTGC-3') and Midi-R (5'-CAGGTCGCCCTATTGCTTCTTT-3'); Midi-F2 (5'-CAACGAGCGCAACCCTTAT-3') and MidiR2 (5'-CAGTCGTCAACCTTACCGT-3'). These primers, targeted the 16S rRNA gene, amplify fragments of ~1100 (Midi-F –R) and ~350 bp (Midi-F2 –R2) and were designed to be conserved between the sequence of *M. mitochondrii* from *I. ricinus* (AJ566640) and the closely related sequences available in the data bases. Amplifications were performed, with both primer sets, in 20 µl of buffer [10 mM Tris-HCl (pH 8.3), 50 mM KCl, 1.5 mM $MgCl_2$, 0.001% gelatine] with 0.2 mM each deoxynucleoside triphosphate, 10 pmol of each primer, 0.5 U of *Taq* Polymerase (Eppendorf) and 2 µl of DNA sample. The thermal profile was: 2 min at 95 °C; 40 cycles of 95 °C for 30 sec, 56 °C for 30 sec and 68 °C for 45 sec; the elongation was completed at 10 min at 68 °C. After gel electrophoresis, PCR product was purified with the Wizard DNA Clean-Up System (Promega), and sequenced. Analysis of nucleotide sequence data was performed with BLAST algorithms and databases from the National Center for Biotechnology (http://www.ncbi.nlm.nih.gov).

3.2.5 Transmission Electron Microscopy (TEM)

The collected samples were fixed in 0.1 M cacodylate buffer (pH 7.2) containing 2.5% glutaraldehyde for 12 h at 4 °C, washed in the same buffer and post-fixed in 1% OsO_4 in the same buffer for 1.5 h at 4 °C. All samples were successively dehydrated in ethanol and embedded in Epon 812. The semi-thin sections (1 mm) for light microscopy were stained with 0.5% toluidine blue; thin sections (80 nm) were stained with uranyl acetate and lead citrate and examined under a EM900 transmission electron microscope (Zeiss).

3.2.6 Indirect immunofluorescence assay

Samples were incubated at room temperature with MitoTracker Red CMXRos (Invitrogen) for 30 min, fixed in 4% (w/v) paraformaldehyde in PBS for 20 min at 4 °C, permeabilized with 0.5% (v/v) Triton X-100 (10 min at room temperature), and then washed three times in PBS. Ovaries were incubated with anti-rFliD at 1:5000 dilution for 1 h at 37 °C, washed three times in PBS and incubated at the same condition with antirabbit secondary antibodies conjugated with FITC (Sigma Aldrich) at 1:40 dilution plus 6 mM TOTO-3 for nucleus detection. Tissues were mounted with glycerol on glass slides. Observations were recorded with a Leica (Leica TCSNT).

3.2.7 Immunogold staining

Samples were fixed by immersion in 4% paraformaldehyde in PBS for 2 h at 4 °C and washed in PBS. Free aldehydes were blocked in 0.5 M NH_4Cl in PBS for 45 min at 4 °C; samples were washed in PBS, dehydrated through graded concentrations of ethanol and embedded in LR White resin (Electron Microscopy Sciences) overnight at 4 °C. Resin samples were polymerized for 24 h at 60 °C. Ultrathin sections were placed on grids coated with a Formvar-carbon layer and then processed for immunocytochemistry. Ultrathin sections were floated for 3 min on normal goat serum (NGS) diluted 1:100 in PBS and then incubated overnight at 4 °C with anti-rFliD diluted with PBS containing 0.1% BSA (Fluka) and 0.05% Tween 20. After rinsing, sections were floated on NGS and then reacted for 20 min at room temperature with secondary 12 nm gold-conjugated antibodies (Jackson Laboratories) diluted 1:20 in PBS. Finally, the sections were

rinsed and air-dried. As controls, some grids were treated omitting the primary antibody from the incubation mixture and then processed as described earlier. The specimens were observed on a Philips Morgagni transmission electron microscope operating at 80 kV and equipped with a Megaview II camera for digital image acquisition.

3.2.8 RNA extraction, cDNA synthesis and expression of flagellar genes

RNA was extracted from different samples of *I. ricinus* using the mirVANA kit (Ambion). The samples were two pools of eggs, two pools of larvae, two pools of nymphs and the ovaries from two adult females. Samples were treated with DNase I RNase free (New England Biolabs) for DNA contamination removal and quantified by spectrophotometry; 500 ng each RNA was retrotranscribed to cDNA by using the QuantiTect Reverse Transcription kit (Qiagen). The amplification of *M. mitochondrii* gyrB was performed as described by Sassera et al., (2008) to confirm the presence of the symbionts and the correct cDNA synthesis. Based on the available gene sequences, primers for the amplification of fragments of *M. mitochondrii fliC, fliD, flgL, flgK, flgE, motA* and *fliG* genes were designed. Primers sequences are given in Table 1. The expression of these genes in different tick development stages was verified by qualitative PCR under standard conditions (250 nM primers concentration, 58 °C annealing temperature, 40 amplification cycles). The expression of the *gyrB* gene was used for sample normalization.

Forward primer		**Reverse primer**		**PCR product size (bp)**
Name	**Sequence (5'–3')**	**Name**	**Sequence (5'–3')**	
FliD-F	CTTATCACTAATGCGTTGAAGT	FliD-R	GGATATTGTAACAGTTTCTTCAAGC	117
FliC-F	TCGCTAGTGCTTCCTATGCT	FliC-R	GATTTAGTTTCTGCATTCAAGGCT	148
FlgL-F	AGACTATACCAACCCTGATTCA	FlgL-R	TTCTCGATATCACACAGTTTGC	146

FlgK-F	TTGCTCGCCGATGCACTTA	FlgK-R	GAGATTGAAATTTACGCTTCCACT	131
FlgE-F	TTACTCCAGGATTCTCATGAAG	FlgE-R	AGGCTAACTGCTCTTGTATTG	149
MotA-F	TGTATGATATTACTAAGCAGG	MotA-R	AGTATCGCAGGTTAGCTCC	172
FliG-F	GGCTCTTGAGGATTCTGAG	FliG-R	TCATATTGACCTTATCATCG	194

Table 1. Sequences of oligonucleotide primers used for reverse transcriptase PCR on seven flagellar genes.

3.3 RESULTS AND DISCUSSION

The flagellar protein FliD of *M. mitochondrii*, i.e. the external flagellar cap, was chosen for production in recombinant form due to its variability (Beatson et al., 2006), and thus for the expected specificity of the antiserum. Based on the available gene sequence, the expected molecular mass of *M. mitochondrii* FliD was 100 kDa. We decided to produce a fragment of this protein, in recombinant form, as a fusion protein with an His tag useful for the successive purification from *E. coli* contaminant proteins. The molecular mass of rFliD is 38 kDa and the protein is produced in *E. coli* cells as inclusion bodies. After solubilization in 6 M urea buffer and purification by using a metal chelate column, rFliD was used for the immunization of rabbits for the production of polyclonal antibodies (anti-rFliD). Fig. 1 shows the specificity of anti-rFliD in Western blot analysis performed on total extracts of proteins from individual females of *I. ricinus* (harbours *M. mitochondrii*) and of *I. hexagonus* (does not harbour *M. mitochondrii*); the recombinant protein was also loaded on the gel. Bands of the expected size were only observed for the *I. ricinus* extract and rFliD. We predict that the generated polyclonal rFliD antibodies will be useful for: (i) the specific staining of *M. mitochondrii*, avoiding cross-reaction with other micro-organisms that *I. ricinus* could harbour and (ii) investigating the presence of a flagellar structure.

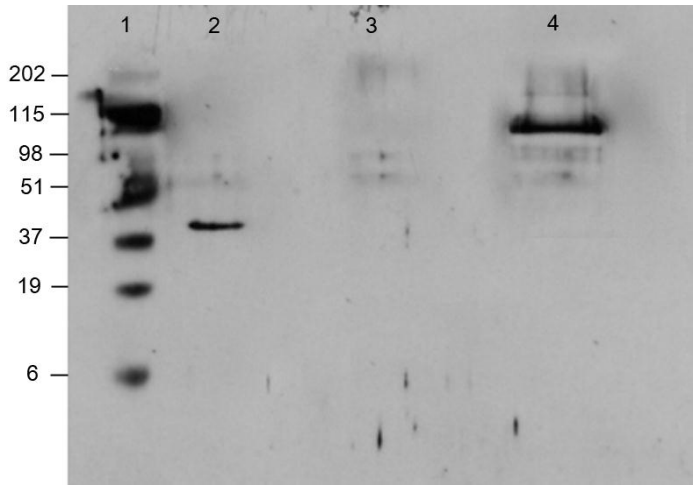
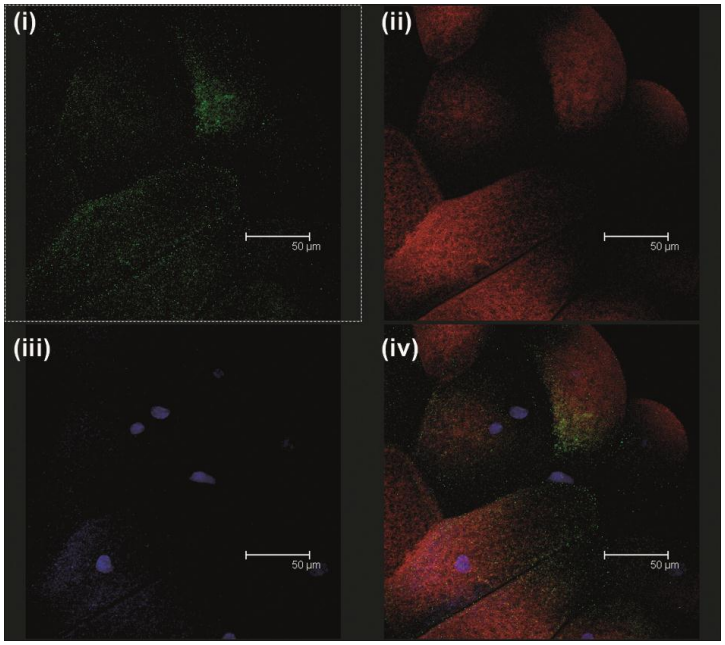
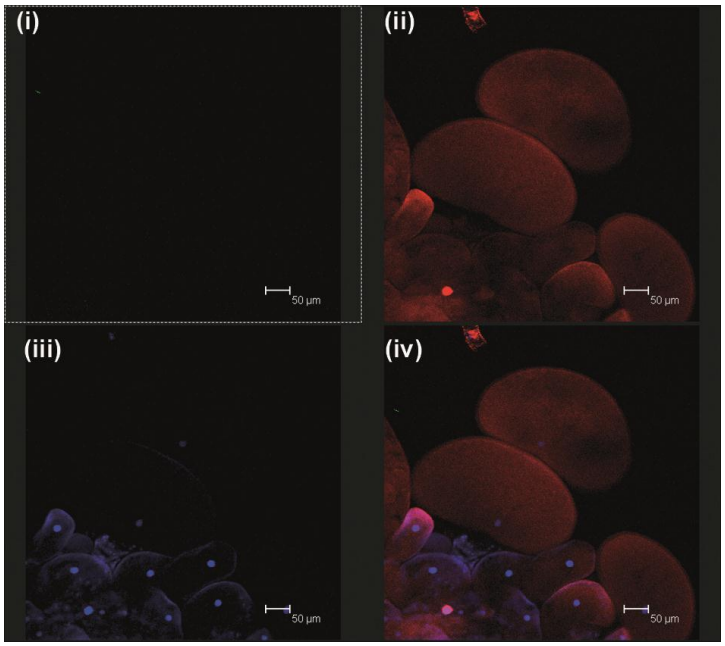


Figure 1. Western blot analysis on rFliD and total extracts of proteins from *I. ricinus* and *I. hexagonus* adult females using anti-rFliD antibodies. Lanes: 1, prestained SDS-PAGE standards broad range (Bio-Rad; sizes in kDa); 2, recombinant protein rFliD (38 kDa); 3, total extract of proteins from *I. hexagonus*; 4, total extract of proteins from *I. ricinus*. The expected molecular mass of native FliD is 100 kDa.

The indirect immunofluorescence assay on adult *I. ricinus* ovaries using a polyclonal FITC-conjugated anti-rFliD antibody led to the observation of clusters of bacteria that were shown by MitoTracker staining to be associated with mitochondria (Fig. 2a). These bacteria can thus be assumed to be *M. mitochondrii*. No staining was observed in the ovaries of *I. hexagonus*, a tick closely related to *I. ricinus* that does not harbour *M. mitochondrii* (Fig. 2b). There are thus overall data that show that anti-rFliD antibodies specifically stained *M. mitochondrii*.



a



b

Figure 2. Indirect immunofluorescence assay of *I. ricinus* (a) and *I. hexagonus* (b) semi-engorged adult tick female ovaries. (i) *M. mitochondrii* stained with polyclonal FITC-conjugated anti-rFliD antibodies (green); (ii) live mitochondria stained with MitoTracker Red CMXROS (red); (iii) cellular nuclei stained for cell viability with TOTO-3 iodide; (iv) merging of images (i)–(iii). Yellow spots indicate overlap between the green *M. mitochondrii* and the red MitoTracker signal.

Moreover, anti-rFliD immunogold staining on *I. ricinus* ovaries revealed a specific pattern of colloidal gold deposit inside bacteria-like bodies and on the

surface of these bacterial bodies (Fig. 3). Standard TEM (i.e. without immunogold staining) did not lead to the observation of flagella (data not shown; see previous work, e.g. Sacchi et al., 2004). This is not surprising considering that bacterial flagella in standard Epon inclusions can be expected to be twisted/coiled. Thin sections for TEM would thus reveal essentially transverse sections of the flagella, i.e. electron-dense spots of 10–20 nm, which would be difficult to identify as flagella.

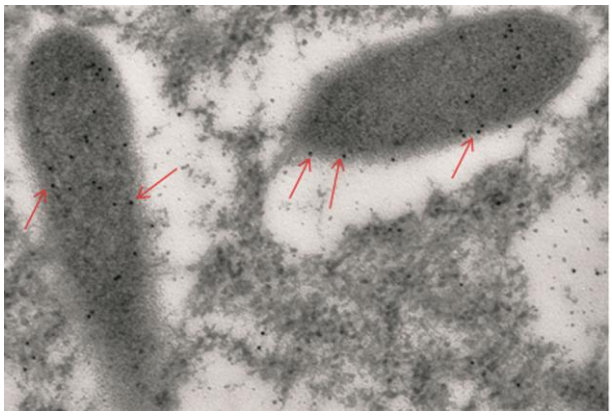


Figure 3. Immunogold staining of the ovary of *I. ricinus* using antirFliD antibodies. Arrows indicate the gold particles present inside bacteria-like structures.

Beside the expression of FliD at the protein level, we investigated the expression of this and a further six genes at the mRNA level. Reverse transcriptase PCR assays on these seven *M. mitochondrii* flagellar genes (*fliC*, filament; *fliD*, filament cap; *flgL* and *flgK*, hook filament junction; *flgE*, hook; *fliG*, C ring; *motA*, motor) in eggs, larvae, nymphs and adults of *I. ricinus* revealed variable levels of expression. As shown in Fig. 4, the seven analysed genes are co-expressed in eggs and adults, while larvae and nymphs present variable patterns of gene expression. The expression of seven out of 26 flagellar genes is obviously not enough to prove that the flagellum is assembled. However, the fact that genes that encode for proteins of the external part of the flagellum (*FliD*, *FliC*, *FlgL*, *FlgK* and *FlgE*) are co-expressed in the same tick stages (eggs and adults; see Fig. 4) is consistent with the production of a flagellar structure.

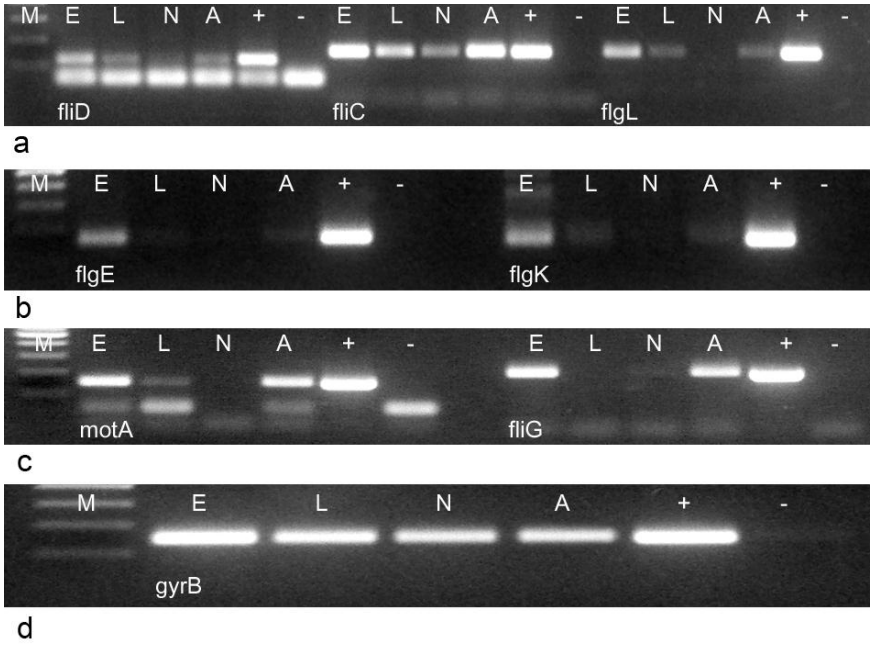


Figure 4. Reverse transcriptase PCR assays on seven *M. mitochondrii* flagellar genes (*fliD, fliC, flgL, flgE, flgK, motA* and *fliG*) and one housekeeping gene (*gyrB*) in eggs, larvae, nymphs and adults of *I. ricinus*. Representative results are reported, from one of the two samples examined for the different life stages (pools of individuals or ovaries; see Material and methods). Lanes: M, molecular mass marker (100 bp, Promega); E, eggs; L, larvae; N, nymphs; A, adult ovaries; +, positive control (DNA extracted from an *I. ricinus* adult female); negative control (no template). (a) *fliD, fliC* and *flgL*; (b) *flgE* and *flgK*; (c) *motA* and *fliG*; (d) *gyrB.*

M. mitochondrii is the sole member in the order Rickettsiales that is so far described to possess a complete set of genes coding for a putative flagellar apparatus. We thus decided to perform an in silico analysis of the 26 predicted flagellar proteins of *M. mitochondrii*, in order to determine whether conserved structural domains are present. We tested the 26 proteins with two online prediction software programs, SledgeHMMER (Chukkapalli et al., 2004) and CDD search (Marchler-Bauer et al., 2011) using default parameters. We included in the analyses the homologous proteins from *E. coli* and *Caulobacter crescentus*, a flagellated alphaproteobacterium. This approach allowed us to compare the selected amino acid sequences with two databases, the PFAM and the NCBI Conserved Domain Database. All of the 26 putative *M. mitochondrii* flagellar proteins were shown to present the corresponding flagellar protein domains by at least one of the two prediction tools used, with the exception of FlgL. Most of the proteins (23/26) were shown to present the corresponding flagellar domains by both softwares. These results are comparable with the ones obtained for the *E. coli* and *C. crescentus* homologous proteins, showing that the genes annotated as

flagellar in *M. mitochondrii* can actually be classified as such based on functional domain conservation of corresponding proteins.

Crystal structures have been solved for major portions of the FliG and FliN proteins in *E. coli* (Brown et al., 2002; Park et al., 2006). The structurally characterized part of FliG includes the C-terminal two-thirds of the protein and consists of two globular domains joined by an alpha-helix and a Gly-containing segment that is presumably flexible. The conserved charged residues that interact with the stator lie together on a ridge at the top of the FliG Cterminal domain. At the end of the domain opposite the charge ridge, FliG has a conserved surface hydrophobic patch that was shown to interact with FliM. The other domain of FliG in the crystal structure displays two conserved surface features, termed EHPQR motif and Gly- Gly motif, that also interact with FliM. The Gly-Gly motif is well conserved and just a few species have a single Gly and only certain species of the alphaproteobacteria (*C. crescentus, Rhodopseudomonas palustrus and Magnestospirillum megnetotactium*) have non-Gly residues in both positions (Brown et al., 2002). Fig. 5 shows the alignment of the middle and C-terminal domain of FliG proteins of *E. coli*, *M. mitochondrii* and *C. crescentus*. Residues that form the hydrophobic core of the two domains, residues important for motor rotation and the EHPQR motif, are conserved in all three sequences.

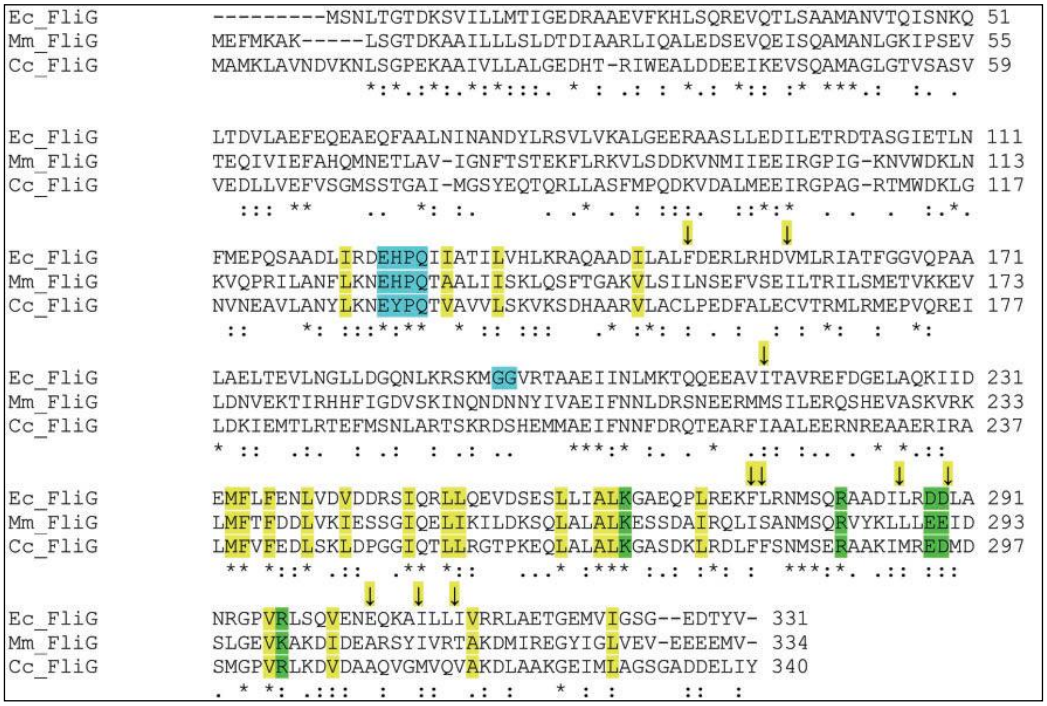


Figure 5. Alignment of *E. coli* (AAB60186; Ec), *M. mitochondrii* (AEI88716; Mm) and *C. crescentus* (ACL94416; Cc) FliG proteins. Blue, conserved EHPQ and GG motifs (see Results and discussion); yellow, conserved residues of the cores of domains I and II; yellow arrows, non-conserved residues of domains I and II after comparison with *E. coli*; green, conserved residues, important for motor rotation. *, Identical; :, conserved substitution; ., semi-conserved substitution.

In *M. mitochondrii* and *C. crescentus*, the Gly-Gly motif is not conserved as described above. The structure of FliN is known for the C-terminal two-thirds of the protein. FliN interacts with FliG and FliM to form the rotor-mounted switch complex that controls clockwise–counterclockwise switching of the motor. In addition to its functions in motor rotation and switching, FliN is thought to have a role in the export of proteins that form the exterior structures of the flagellum (the rod, hook and filament). Fig. 6 shows the alignment of the C-terminal domain of FliN proteins of *E. coli*, *M. mitochondrii* and *C. crescentus*. For this protein, the core of the domain and the surface hydrophobic patch are also conserved in the three sequences.

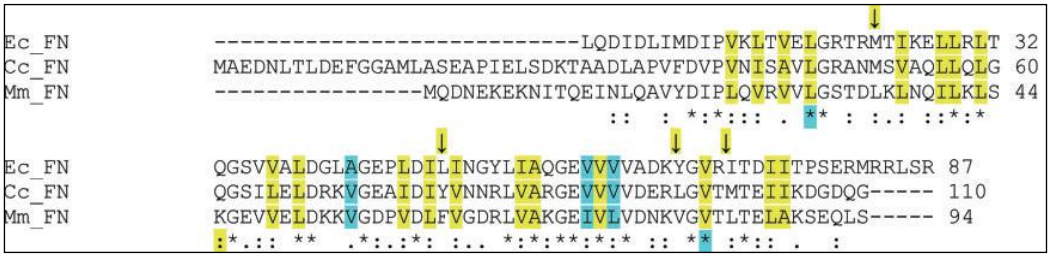


Figure 6. Alignment of *E. coli* (EGT69941; Ec), *M. mitochondrii* (AEI88718; Mm) and *C. crescentus* (ACL94418; Cc) FliN proteins. Blue, conserved surface hydrophobic patch; yellow, conserved residues of FliN core; yellow arrows, non-conserved residues of FliN core after comparison with *E. coli*. *, Identical; :, conserved substitution; ., semi-conserved substitution.

Whether *M. mitochondrii* uses a flagellar apparatus for motility is yet to be determined. However, considering the conservation of the flagellar genes that we have analysed, their expression at the RNA and protein level and the evidence for their ancestral origin (Sassera et al., 2011), we suggest that these genes have maintained their original function along the phylogenetic lineage leading to *M. mitochondrii*, and possibly in other lineages. We could thus also suggest that, in the future, other members of the order Rickettsiales will be discovered to possess flagella/ flagellar genes, at least in the *M. mitochondrii* lineage. Considering other arthropod-associated intracellular bacteria, there are at least two cases where flagellar genes have been discovered: the symbiont of aphids, *Buchnera aphidicola* (Maezawa et al., 2006) and the symbiont of tse-tse flies, *Wigglesworthia* spp. (Rio et al., 2012). Both of these bacteria are related to flagellated, free-living and motile prokaryotes, and the discovery of the flagellar genes in their genome was not surprising, differently from *M. mitochondrii* that belongs to the order Rickettsiales. In the case of *Wigglesworthia glossinidia*, there is evidence that flagellar genes are expressed at the RNA level (and at the protein level for one gene) during phases of the bacterial cycle in which motility is probably needed (Rio et al., 2012). Our results for *M. mitochondrii* are thus similar to those reported for *W. glossinidia*, in that levels of mRNA expression for the seven genes examined vary during the life cycle. It is reasonable to assume that intracellular bacteria such as *M. mitochondrii* and *W. glossinidia* alternate trophic phases (in which flagella and motility are not required) with phases of the cycle in which a flagellar apparatus is used for motility or for other functions. This might explain the differences in the mRNA expression levels because in eggs and

adults, the examined flagellar genes display a higher mRNA expression compared with larvae and nymphs. The adults that we examined were semi-engorged females close to the end of the blood meal. We could thus assume that during this phase, in which the oogenesis is about to complete, and then in the newly deposited eggs, the flagellar apparatus plays a function in *M. mitochondrii* biology. In the case of *B. aphidicola*, the flagellar apparatus is thought to have a secretory function. Since nothing is currently known about how *M. mitochondrii* is able to invade the mitochondria of *I. ricinus* cells, is it possible to speculate about a possible role of a flagellar structure in the invasion of tick mitochondria by *M. mitochondrii*? Indeed, the flagellum has previously been indicated to be the means of interaction between bacterial symbionts (Shimoyama et al., 2009).

4. HUMANS PARASITIZED BY THE HARD TICK *IXODES RICINUS* ARE SEROPOSITIVE TO *MIDICHLORIA MITOCHONDRII*: IS *MIDICHLORIA* A NOVEL PATHOGEN, OR JUST A MARKER OF TICK BITE?

4.1 PURPOSE

It is known that *M. mitochondrii* is present in the oocytes and in other cells of the ovary of the hard tick *I. ricinus*, but no studies have so far investigated whether this bacterium is present in the salivary glands of the tick and whether it is transmitted to vertebrates during the tick blood meal. To address the above issues, we developed a recombinant antigen of *M. mitochondrii* (to screen human sera) and antibodies against this antigen (for the staining of the symbiont). Using these reagents we decided to investigate (i) the presence of *M. mitochondrii* in the salivary glands of *I. ricinus* and (ii) the seropositivity against *M. mitochondrii* in humans parasitized by *I. ricinus*. These results could provide evidence that *M. mitochondrii* is released with the tick saliva and raise the possibility that *M. mitochondrii* is infectious to vertebrates. Besides this, *M. mitochondrii* should be regarded as a package of antigens inoculated into the human host during the tick bite. This implies that the immunology of the response toward the saliva of *I. ricinus* is to be reconsidered on the basis of potential effects of *M. mitochondrii* and poses the basis for the development of novel markers for investigating the exposure of humans and animals to this tick species.

4.2 MATERIAL AND METHODS

4.2.1 Tick samples

Three semi-engorged adult females of *I. ricinus* and two of *I. hexagonus* were collected from naturally infected animals (sheep and hedgehog), in the counties of Varese and Bergamo (Northern Italy). Ticks were identified morphologically with standard taxonomic keys (Manilla, 1998). Salivary glands and rostra were

dissected in sterile conditions and prepared for indirect immunofluorescence assay (salivary glands) or for PCR analysis (salivary glands and rostra).

4.2.2 DNA extraction and PCR analysis

DNA from salivary glands and rostra was extracted using the DNeasy Blood & Tissue Kit (Qiagen), eluted in 50 µl of sterile water, quantified by a spectrophotometer and stored at -80 °C before use. PCR screening for *M. mitochondrii* detection was performed using a protocol described above in the paragraph 3.2.4.

4.2.3 Sera samples

A total of 249 samples of human sera were screened for the presence of anti-*M. mitochondrii* antibodies, using an experimental ELISA assay (see below). These sera were from two groups of subjects: 169 healthy blood donors and 80 subjects exposed to tick bite. In particular, the latter group was composed of persons parasitized by ticks, examined at the emergency services of the hospitals IRCCS Policlinico San Matteo (Pavia), Azienda Ospedaliera Universitaria S.Orsola-Malpighi (Bologna) and Spedali Civili di Brescia (Brescia), or with a reliable request for Lyme disease diagnosis, based on clinical signs and anamnesis (of these, 31 had actually been shown to be seropositive for *Borrelia burgdorferi* sensu lato before the enrollment in this study; in the context of this study, a further subject was then shown to be seropositive for *B. burgdorferi* – see below). Unfortunately, in most cases the emergency service physicians did not conserve the ticks removed from the patients for identification; when the tick was identified, it was in most cases *I. ricinus* (13 out of 15 ticks examined). Based on this information, on a previous study showing that 90% of the ticks removed from humans in Northern Italy are *I. ricinus* (Manfredi, et al., 1999), and on preliminary results of a parallel study in the same area (Bandi et al., unpublished results), we assume that most of the patients had been parasitized by *I. ricinus*. All of the above sera were collected after no less than six weeks from the removal of the tick. The screening on human sera was conducted under the regulation of the Ethical Committees of the S. Orsola Malpighi University Hospital (Bologna), Spedali Civili di Brescia and Fondazione IRCCS Policlinico San Matteo (Pavia);

all patients provided informed consent and the study protocol was approved by the Ethical Committees of the above hospitals.

4.2.4 Indirect immunofluorescence assay on tick salivary glands

Salivary glands were stained with MitoTracker Red CMXRos (Invitrogen) and with a polyclonal antibody raised against the recombinant flagellar FliD protein of *M. mitochondrii* (anti-rFliD) as previously described (Mariconti et al., 2012a). Observation was recorded with a Leica confocal microscope (Leica TCSNT).

4.2.5 Detection of anti-*M. mitochondrii* antibodies in human sera

The recombinant flagellar protein FliD of *M. mitochondrii* (rFliD) was produced in *E. coli* and purified as previously described (Mariconti et al., 2012a). Anti-rFliD antibody levels in human sera were determined using an enzyme-linked immunosorbent assay (ELISA), using 96-well microtiter plates coated with 0.1 mg/well of rFliD protein. Each sample was diluted 1:100 in phosphate buffered saline supplemented with 1% bovine serum albumin and 100 µl of each diluted sera were tested following the procedure previously described in Gaibani et al., (2012). Threshold value was established as the mean optical density (OD) $_{450/630}$ of the sera from the healthy blood donors plus three times the standard deviation (i.e. mean $OD_{450/630}$ + 3 standard deviations). Using this method the threshold was set at 0.793. Each sample was considered negative if its OD $_{450/630}$ was less than the threshold value, and positive if its OD $_{450/630}$ was higher than or equal to the threshold.

4.2.6 Serological screening for *B. burgdorferi*

Even though part of the sera used in this study had already been diagnosed for *B. burgdorferi* infection, we examined all of the sera for the presence of IgG antibodies specific for *B. burgdorferi*. This screening was performed using a commercial Western blot kit (Borrelia ViraStripe Test Kit IgG; Viramed Biotech). Western blot results were interpreted following the manufacturer's recommendations.

4.2.7 Experimental Western blot assays for *B. burgdorferi* and *M. mitochondrii*

A culture of *B. burgdorferi* was pelleted with centrifugation at 4000g for 5 minutes, and the pellet was suspended in 150 µl of Tris HCl 25 mM (pH 8); after sonication to promote the release of the proteins, the supernatant was recovered after centrifugation at 16 000g for 10 minutes at 4 °C. The soluble proteins in the supernatant of *B. burgdorferi* or the purified rFliD were then used as antigens for Western blot assays, performed according to standard procedures (Sambri et al., 2001), with dilution of secondary antihuman antibodies at 1:5000. To test the possible cross-reactivity of rFliD in patients infected by *B. burgdorferi* s.l., the following sera were examined, at 1:1000 dilution: five sera positive for *B. burgdorferi*; five sera positive for *M. mitochondrii*; five sera positive for both *B. burgdorferi* and *M. mitochondrii*. Serological positivity to *M. mitochondrii* and *B. burgdorferi* were determined using respectively the ELISA method and the Western blot kit described in the above paragraphs.

4.3 RESULTS AND DISCUSSION

The indirect immunofluorescence assay on salivary glands of adult *I. ricinus* females using a primary antibody directed against the rFliD of *M. mitochondrii* revealed the presence of intensely green-stained bodies (Fig. 1a–d); these were generally collected in clusters, and closely associated with mitochondria (as revealed by the staining using MitoTracker Red). No staining was observed using the anti-rFliD antibody on the salivary glands of *I. hexagonus* (Figure 1e–h), a tick closely related to *I. ricinus* that does not harbor *M. mitochondrii*. No stained bodies were observed in *I. ricinus* when the primary anti-rFliD antibody was not used (not shown). The above observations were obtained on all the samples examined for *I. ricinus* (three positive, out of three adult females) and for *I. hexagonus* (two negative, out of two adult females).

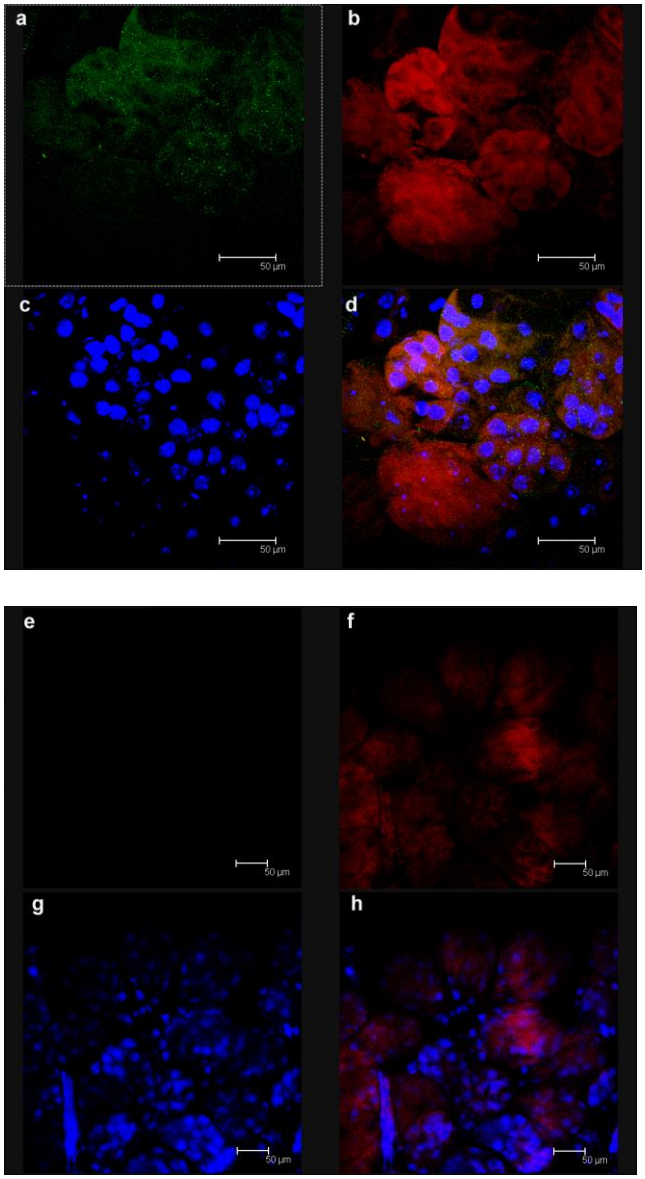


Figure 1. Indirect immunofluorescence assay (FITC-conjugated secondary antibodies) on salivary glands from *I. ricinus* (a–d) and *I. hexagonus* (e–h) semi-engorged adult females; (a, e) staining using polyclonal antibodies raised against the rFliD from *M. mitochondrii* (green); (b, f) live mitochondria stained with MitoTracker Red CMXROS (red); (c, g) cellular nuclei stained for cell viability with TOTO-3 iodide; (d, h) merging of the images. In d the yellow spots indicate the overlap between *M. mitochondrii* (green) and mitochondria (red).

Based on the above observations we assume that the bodies recognized by the anti-rFliD antibody in the salivary glands of *I. ricinus* are *M. mitochondrii* bacteria, or aggregates of the FliD protein from this bacterium. PCR analysis with primers specific for *M. mitochondrii* was also congruent with the above results: amplification of *M. mitochondrii* DNA was obtained from all three salivary gland samples from *I. ricinus*. In addition, PCR on the rostra was positive for *M.*

mitochondrii in two of the three adult females of *I. ricinus* examined (all of the PCR products obtained were sequenced, and matched the 16S rRNA sequence of *M. mitochondrii*). PCRs on the salivary glands and rostra were negative on all of the samples from *I. hexagonus*. Taken together, the results of immunostaining and PCR on the salivary glands show that *M. mitochondrii* (or proteins and DNA from *M. mitochondrii*) is present in the salivary glands of *I. ricinus*; PCR positivity on the rostra indicates that this bacterium (or DNA from this bacterium) could be released with the saliva. The above results prompted us to investigate whether humans parasitized by *I. ricinus* are seropositive for *M. mitochondrii*. To this purpose, we used the flagellar rFliD protein from *M. mitochondrii* as an antigen, in an ELISA screening on healthy blood donors and on subjects exposed to tick bite (Fig. 2). In tick-exposed subjects, the average OD values for IgG antibodies reacting with rFliD was 0.845 (SD=0,422); in healthy blood donors, OD values were significantly lower (U-MannWitney test; $P<0.001$), with an average of 0.373 (SD=0,140). After setting a threshold at an OD value of 0.793 (see Material and methods paragraph 4.2.5), we could then estimate that the seroprevalence for *M. mitochondrii* was 58.75% in subjects exposed to tick bite (47 out of 80), and 1.18% in the healthy blood donors (2 out of 169). These prevalence values are significantly different between the two groups (U-MannWitney test; $P<0.001$). The above results clearly indicate that subjects exposed to tick bite produce antibodies that react with an antigen from *M. mitochondrii*, and indicate that this bacterium is inoculated into the human host during the tick bite.

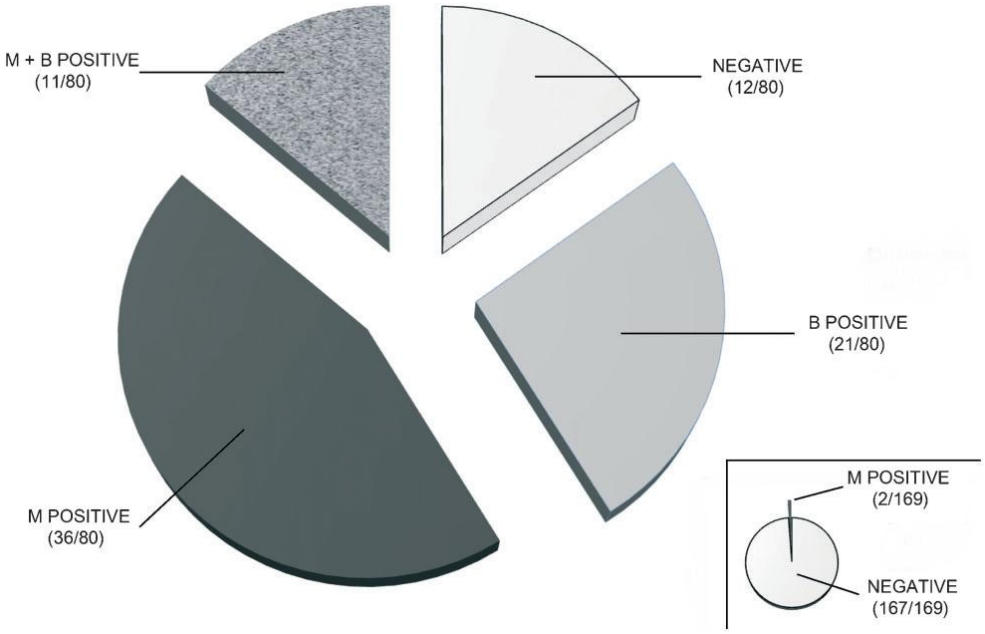


Figure 2. Results of the serological screening for *M. mitochondrii* and *B. burgdorferi* on sera from 80 tick exposed subjects (main figure) and from 169 healthy blood donors (inset). M: sera positive to *M. mitochondrii*; B: sera positive to *B. burgdorferi*; M+B: sera positive to both *M. mitochondrii* and *B. burgdorferi*. Sera negative to both bacteria (NEGATIVE) are also indicated.

The antigen that we used for the above ELISA screening (rFliD) corresponds to a portion of a flagellar protein of *M. mitochondrii*. Thus, we did not expect any cross-reactivity towards antibodies generated during an infection caused by any other Rickettsiales. Indeed, *M. mitochondrii* is the sole member of the order Rickettsiales that has so far been shown to possess flagellar genes. However, *I. ricinus* is the main vector in Europe of the Lyme disease spirochetes (*B. burgdorferi* s.l.) (Stanek and Strle, 2003), bacteria that possess flagella, well known for their immunogenicity (Aguero-Rosenfeld et al., 2005). One could thus argue that production of IgG antibodies in tick-exposed subjects had been induced by a *Borrelia* infection, and that these antibodies cross-reacted with rFliD from *M. mitochondrii*. Considering the numerous amino-acid differences between the FliD proteins of the two bacteria, we consider cross-reactivity rather unlikely (Mariconti et al., 2012a). However, we decided to address this issue by screening all of the sera included in this study with a Western blot diagnostic kit for the detection of antibodies against *B. burgdorferi* s.l.. Using this kit, positivity for *B. burgdorferi* s.l. was revealed in 32 out of the 80 samples of sera from the subjects

exposed to tick bite; none of the healthy blood donors was positive to *B. burgdorferi* s.l. Among the 32 subjects positive to *B. burgdorferi* s.l., 11 were concurrently positive to *M. mitochondrii*; however, a total of 36 subjects positive to *M. mitochondrii* were negative to *B. burgdorferi*. Finally, 21 subjects were positive only to *B. burgdorferi* (Fig. 2). In summary, the above results show that a high proportion of the subjects positive to *M. mitochondrii* were not positive to *B. burgdorferi* (and vice-versa), indicating that the positivity to the former bacterium does not derive from the crossreactivity with the latter.

We further addressed the above issue using an experimental Western blot assay, using as antigens rFliD from *M. mitochondrii* and proteins from *B. burgdorferi* s.l.. This Western blot assay was carried out on five sera each from the following types of subjects: positive for *M. mitochondrii*, positive for *B. burgdorferi* s.l., and positive for both bacteria. The following results were obtained. Sera from the first five patient labeled one band at 38 kDa, corresponding to the molecular weight of rFliD; no band corresponding to the molecular weight of *B. burgdorferi* FliD (78 kDa) was observed in these subjects. The sera from the patients positive to *B. burgdorferi* s.l. reacted with a band at 78 kDa (corresponding to *B. burgdorferi* FliD), while no labeling was observed at 38 kDa. Sera from patients that were positive both for *B. burgdorferi* s.l. and *M. mitochondrii* labeled bands at both 78 and 38 kDa (results not shown). These results indicate that the antibodies raised against the FliD protein of *B. burgdorferi* did not react with the homologous protein of *M. mitochondrii*, and vice-versa.

4.4 CONCLUSIONS

Our work provides strong evidence for the transmission of *M. mitochondrii* to humans during the blood meal of *I. ricinus*. Based on the results here reported, we cannot conclude that *M. mitochondrii* replicates in the human host, determining a true infection. However, should we assume that *M. mitochondrii* does not replicate into the human host, we would have to conclude that the amount of bacteria (or bacterial antigens) inoculated is by itself sufficient for stimulating an antibody production. Overall, we are more prone to hypothesize that live *M.*

mitochondrii bacteria (and not just proteins/DNA) can be inoculated into the vertebrate host, and that some replication can occur therein. At any case, our work shows that *M. mitochondrii* is to be regarded not only as an important symbiont of *I. ricinus,* but also as a package of antigens that ticks can inoculate into vertebrate hosts, and as a potential tick-borne microorganism that deserves further investigations. Among the 80 tick-exposed patients that we examined in this study, 47 were seropositive to *M. mitochondrii*, according to the defined threshold value. We emphasize that we cannot be certain that all of the subjects had been parasitized by *I. ricinus*; in addition, for some of the subjects, the duration of the blood meal could have been insufficient for an effective inoculation of bacteria. Furthermore, some *I. ricinus* nymphs present a very low *M. mitochondrii* load (Sassera et al., 2008), thus possibly resulting in inoculation of a low amount of bacteria during their blood meal. These considerations could explain why not all of the parasitized subjects were seropositive for this bacterium. On the other hand, immunostaining and PCR for *M. mitochondrii* on the salivary glands of *I. ricinus* were positive in all three specimens examined.

Since we still do not know whether *M. mitochondrii* replicates into the human host, it would be premature to discuss whether this bacterium could be responsible for any pathological alteration. For sure, considering the high seroprevalence for *M. mitochondrii* that we determined in tick-exposed subjects, we would conclude that this bacterium does not cause overt pathology in humans, at least in the vast majority of the cases. On the other hand, the high seroprevalence that we recorded in tick-exposed subjects (associated with the extremely low seroprevalence in healthy blood donors) raises the possibility that *M. mitochondrii* plays a role in the immune response and immune-modulation determined by the *I. ricinus* saliva, which is important both for the success of the tick blood meal and for the establishment of the infection by the pathogens vectored by the tick (Fontaine et al., 2011; Lieskovska and Kopecky, 2012). We emphasize that in the case of filarial nematodes, the discovery of *Wolbachia* bacterial endosymbionts in these parasites and of their immunological role led to a profound re-thinking of the immunology of filarial diseases (Bandi et al., 1998; Bazzocchi et al., 2000; Taylor et al., 2005). Finally, anti-*Midichloria* antibodies can now be considered as potential serological markers for *I. ricinus* bite. Such markers could be extremely

useful to determine the risk of infection by *I. ricinus*-borne pathogens in given areas, and for investigating the epidemiological association of a variety of pathological alterations with parasitism by this tick. The present study was focused on *M. mitochondrii*, but other MALOs could possibly be transmitted by ticks and other arthropods to a variety of vertebrates, including humans. Further studies are now urgent, to determine whether MALOs represent a novel class of emerging infectious agents.

5. SEROLOGICAL AND MOLECULAR EVIDENCE FOR THE CIRCULATION OF THE TICK SYMBIONT *MIDICHLORIA MITOCHONDRII* IN DIFFERENT ANIMAL SPECIES

5.1 PURPOSE

I. ricinus is a tick species with a low host specificity: its principal hosts are indeed domestic and wild ruminants at the adult stage, and sciuromorphes and lagomorphes at the larval and nymph stages. *M. mitochondrii* is an intacellular alphaproteobacterial symbionts within the order of Rickettsiales that live inside various cell type of the ovary of the hard ticks *I. ricinus*. The prevalence of the bacteria in the female of this tick is 100%, while in male is 44%. The circulation of bacteria related with *M. mitochondrii* and MALOs in roe deer and human samples parasited by ticks was confirmed by PCR analysis on 16S rRNA gene. Moreover, the detected antibody response against a protein of *M. mitochondrii* in human patients bited by *I. ricinus* could means that an amount of bacteria or bacterial antigens were inoculated in vertebrate hosts during tick blood meal. This led us to investigate the presence of *M. mitochondrii* DNA circulating in vertebrate blood samples parasitized by ticks and an immune response against a protein of *M. mitochondrii* in dogs exposed to tick bite that lived in an areas where sheep grazing and where a high density of wild ruminants were present.

5.2 MATERIAL AND METHODS

5.2.1 Samples collection

A total of 218 dog sera were collected from 16 kennels located in southern Italy (Table 1) and stored at -20 °C until use. Sera from 30 privately owned dogs, from the towns of Milano and Pavia (Northern Italy) and exposed at low risk of tick bite, were used as control samples. One-hundred and thirty two whole blood samples from different mammalian hosts exposed to the risk of tick bite (46 horses, 13 cattle, 62 dogs and 11 sheep) were also collected from farms in the

regions Lombardia and Lazio, Italy (Table 2). In addition, blood samples from the above 30 dogs and from cattle from an intensive dairy farms were included as controls. DNA from blood samples was extracted by using the QIAamp DNA blood minikit (Qiagen) according to the manufacturer's instructions, eluted into 100 µl of sterile water and stored at -20 °C until use.

Kennel site	n° of dogs	Dog positive	% of positivity	Average +/- SD
Campobasso, Molise	15	13	86,6	0,46 +/- 0,2
Napoli, Campania	5	0	0	0,12 +/- 0,06
Salerno, Campania	16	5	31,25	0,25 +/- 0,07
Bisaccia, Avellino, Campania	19	14	73,68	0,33 +/- 0,1
Pozzuoli, Napoli, Campania	12	1	8,3	0,14 +/- 0,08
Eboli, Salerno, Campania	16	0	0	0,13 +/- 0,2
Marcianise, Caserta, Campania	13	3	23	0,14 +/- 0,12
Mercogliano, Avellino, Campania	9	2	22,2	0,27 +/- 0,16
Savignano Irpino, Avellino, Campania	9	5	55,5	0,27 +/- 0,09
Montella, Avellino, Campania	13	8	61,53	0,27 +/- 0,08
Atripalda, Avellino, Campania	13	0	0	0,15 +/- 0,04
Luogosano, Avellino, Campania	15	3	20	0,20 +/- 0,09
Santa Maria Capua a Vetere, Caserta, Campania	19	1	5,26	0,14 +/- 0,06
Giuliano in Campania, Napoli (1), Campania	16	2	12,5	0,16 +/- 0,05
Giuliano in Campania, Napoli (2), Campania	16	0	0	0,12 +/- 0,03
Altavilla, Irpinia, Avellino, Campania	12	1	8,3	0,16 +/- 0,08
Urban areas (Pavia and Milano)	30	1	3,3	0,12 +/- 0.045

Table 1. List of the 16 kennels located in southern Italy used in the collection of the 218 dog sera for the detection of antibodies against *M. mitochodrii*. In yellow are highlighted the kennels that have an average above/equal than 0.25.

Vertebrate host	n° of animals	Animals positive
Horses (breeding 1), Frosinone, Lazio	18	1
Horses (breeding 2), Frosinone, Lazio	11	2
Horses (breeding 3), Frosinone, Lazio	8	0
Horses (breeding 4), Frosinone, Lazio	9	2
Cattle, Napoli, Campania	13	0
Dogs, Pantellaria, Sicilia	47	18
Dogs, Valtellina, Lombardia	15	0
Sheep, Bergamo, Lombardia	11	1
Cattle, Pavia, Lombardia	30	0
Dog, urban areas (Pavia and Milano, Lombardia)	30	1

Table 2. List of the 132 whole blood samples from different vertebrates exposed to the risk of tick bite collected from farms in differen regions of Italy. These samples were used for detect the presence of circulating DNA of *M. mitochondrii* by PCR analysys.

5.2.2 ELISA analysis

The recombinant flagellar protein FliD of *M. mithocondrii* (rFliD) was produced in *E. coli* and purified as described in Mariconti et al. (2012a). Wells of ELISA flat-bottom plates were coated with 0.1 μg/well of rFliD. Sera were analyzed in duplicate at a dilution of 1:100 and the anti IgG HRP-conjugated antibody was employed at 1:5000. The optical density (OD) was measured at 492 nm wave length. The cut-off was established at an OD of 0.25 which is the mean OD of the 30 control sera plus three times their standard deviation. Samples with OD less than of 0.25 were classified as negative and samples with OD greater than or equal to 0.25 were classified as positive.

5.2.3 PCR analysis

DNA extracted from blood samples was analyzed for the presence of circulating DNA of *M. mitochondrii* by PCR analysis. PCR screening for *M. mitochondrii* detection was performed using a protocol described above in paragraph 3.2.4. All of the DNAs from blood samples were also examined using universal mammalian PCR primers targeted on 12S rRNA gene (Wang et al., 2000). PCR products

obtained with *M. mitochondrii* primers were sequenced using ABI technology, and compared with the databases using BLAST (National Center for Biotechnology Information, Bethesda, Md). The obtained 16S rRNA sequences were deposited in the data bases.

Phylogenetic reconstruction

The 16S rRNA sequences generated were aligned with the corresponding sequences from *M. mitochondrii* and related bacteria, including *Rickettsia rickettsii* as an outgroup. In addition, an alignment was generated including sequences from various representatives of the Rickettsiaceae and Anaplamsataceae (order Rickettsiales). The alignment was generated using MUSCLE (Edgar, 2004) and manually checked. Phylogenetic analysis were effected using the neighbor joining method after Kimura 2-parameter correction, after either including or excluding the insertion/deletions, and using a maximum likelihood approach; both analyses were effected using SeaView (Gouy et al., 2010). The reliability of the nodes was estimated after 100 bootstrap replicates.

5.3 RESULTS AND DISCUSSION

In order to screen dog sera for indirect signs of *M. mitochondrii* infection we developed an ELISA test, using a recombinant flagellar protein (rFliD) of this bacterium. Using this test we analyzed 218 dog sera collected from 16 kennels located in southern Italy and 30 sera from personally-owned dogs, included as controls (Table 1). The cut-off of the test was determined on the basis of results obtained from the sera from the control dogs, and positioned at 0.25 OD. As shown in Table 1, the average OD values for IgG antibodies reacting with rFliD is above/equal to the cut-off in six kennels. In these six kennels the percentage of positive dogs ranges from 22.2% to 86.6%. In the remaining 10 kennels, the average OD value was below the 0.25 cut-off: dogs from four of these were all negative, while positive dogs were recorded in the remaining six (Table 1). Considering the whole population of the dogs examined from the 16 kennels, seroprevalence is 26.6%, which is significantly different from the 3.3%

seroprevalence of control dogs (P< 0.0099). The 218 dogs from the kennels can be assumed to be at risk of tick bite, while the 30 personally-owned dogs are assumed to have very limited risk of tick parasitism (even though we cannot exclude rare cases of tick bite). Considering the above information, the results here reported (i.e. 26.6% seroprevalence for *M. mitochondrii* in dogs at risk of tick bite, and 3.3% in dogs at low risk) are congruent with the idea that the tick symbiont *M. mitochondrii* (or its antigens) are inoculated into animals during the tick blood meal. As for the difference in the seroprevalence in dogs from the different kennels (Table 1), this could derive from management/sanitary/logistic differences among kennels, as well as from their geographic location, in relation with tick distribution in Italy.

The antigen used for the above ELISA screening (i.e. rFliD) is a component of the flagellum of *M. mitochondrii* from *I. ricinus*. This bacterium is rather peculiar in that it is the sole Rickettsiales so far shown to posses a flagellar structure: the well-established pathogenic Rickettsiales from the genera *Rickettsia*, *Ehrlichia*, *Anaplasma* and *Orientia* do not have flagella (Dumler and Walker, 2005). We would thus exclude that results of the above serological screening derived from cross-reactivity with antigens from other Rickettsiales. On the other hand, *B. burgdorferi* s.l., the main pathogen transmitted by I*xodes* ticks, possesses immunogenic flagella. However, published results have shown that, in humans exposed to tick bite, a high proportion of the subjects positive to *M. mitochondrii* are negative to *B. burgdorferi* (and vice-versa), indicating the absence of immunological cross reactivity among the FliD proteins of *Midichloria* and *Borrelia* bacteria (Mariconti et al., 2012b).

Even though we would exclude cross-reactivity with other bacteria, the detection of circulating antibodies against the rFliD of *M. mitochondrii* is only an indirect evidence of the circulation of this bacterium in animals. We thus performed a search for direct signs of *M. mitochondrii* circulation in animals, screening blood samples for *M. mitochondrii* DNA. A total 132 blood samples derived from horses, cattle, dogs and sheep at risk of tick bite were analyzed by PCR, using a previously described PCR protocol targeted on the 16S rRNA gene of *M. mitochondrii* (Epis et al., 2008). Blood samples from 30 cattle and 30 dogs that

are regarded to have no or limited risk of tick bite were included as controls. Details on the different groups of animals examined are listed in Table 2. The quality of the DNAs extracted was checked by a PCR using universal mammalian primers targeted on the mitochondrial 12S rRNA gene, that led to positive amplification from all samples. *Midichloria*-targeted primers led to positive amplification from 24 of the animals at risk of tick bite, and from none of the control animals (Table 2). The PCR products obtained from 8 of the 24 *Midichloria*-positive animals were recovered from the gel, purified and sequenced by ABI technology; the obtained sequences were compared with the databases using Blast, and included into an alignment with homologous sequences, for phylogenetic analyses. All of the sequences generated gave the best scores toward 16S rRNA sequences from *M. mitochondrii* and related bacteria. Phylogenetic analysis further confirmed that the sequenced PCR products derived from bacteria closely related with *M. mitochondrii*. Figure 1 presents an example of the phylogenetic trees obteined. The gene fragments generated from the blood samples here examined cluster with those of *Midichloria* bacteria amplified from ticks. It has already been shown that *Midichloria* bacteria harbored by different tick species are variable at the level of the 16S rRNA (Epis et al., 2008). The tree in Figure 1 presents the positionings of the *Midichloria* sequences obtained from the blood samples here examined, and highlights that these novel sequences are generally not identical with those so far generated from ticks. These results are not surprising, considering that only a minimal proportion of the ticks present in Italy have been screened for *Midichloria* bacteria, and the number of specimens so far examined for each species is generally very limited (Epis et al., 2008). In other words, we are still far from a precise knowledge of the prevalence of *Midichloria* bacteria in tick populations, and we are still very far from a knowledge of the molecular diversity of these bacteria. In addition other blood-sucking arthropods have been shown to harbour *Midichloria*-related bacteria. In summary, while we can affirm that DNA from *Midichloria* bacteria can be detected in the blood of different animal species, we can only propose some hypotheses on the origin of this DNA. For example, some of the DNA *Midichloria* sequences amplified from dogs are identical to those in *Rhipicephalus bursa*, and we can thus hypothesize that this tick transmitted the bacterium to the dog.

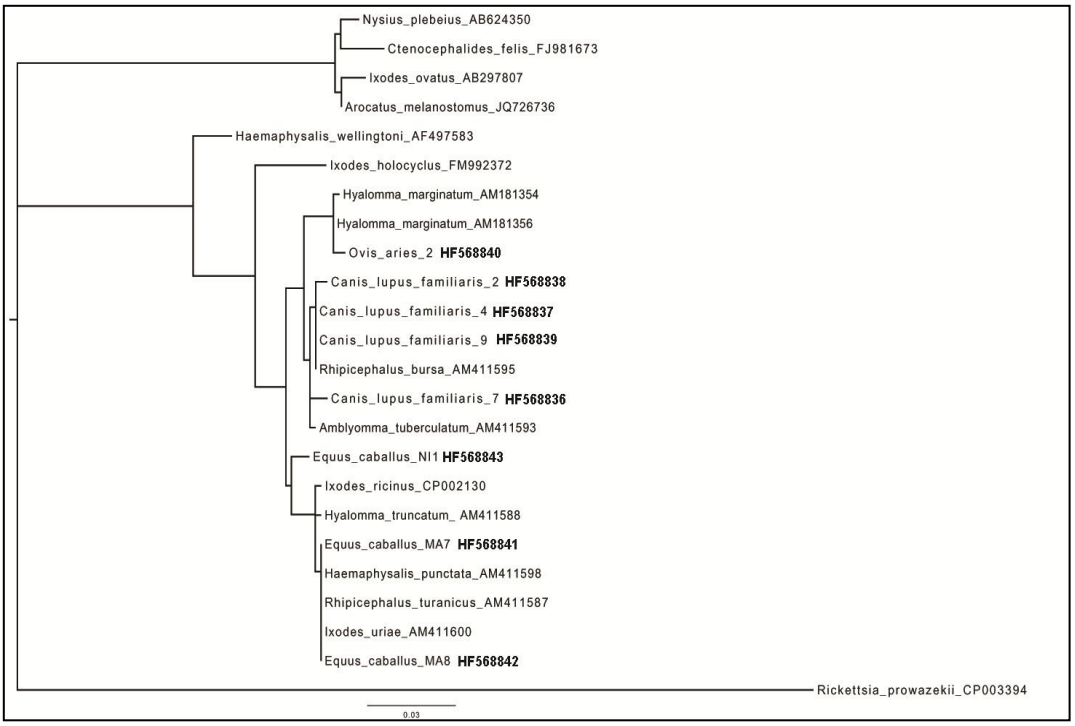


Figure 1. Phylogenetic tree based on 16S rRNA gene sequence showing the positions of *Midichloria* sequences. Sequences alignment was generated using MUSCLE (Edgar, 2004) and manually checked. Tree reconstruction was performed using SeaView (Gouy et al., 2010). *R. rickettsii* was included as an outgroup. The GeneBank accession number for each sequence is indicated.

The work here reported presents two lines of evidence that suggests that *Midichlori*a/*Midichloria*-related bacteria circulate in animals: (i) indirect signs of their presence, i.e. the detection of anti rFliD antibodies; (ii) direct signs of their presence, i.e. the detection of DNA gene fragments that cluster in this bacterial group. These results do not of course allow to derive any conclusion about whether *Midichloria* bacteria replicates in mammalians: ticks (or other arthropods) might simply inoculate *Midichloria* bacteria or DNA/proteins from these bacteria, in an amount sufficient to stimulate antibody production and for PCR detection. However, considering the amount of blood in the animals here screened (e.g. the horse), we believe it would be unlikely that bacterial DNA inoculated by a few ticks, and diluted into liters of blood, is then detected by PCR analysis of the DNA extracted from 100 µl of blood. We are thus more prone to consider the possibility that *Midichloria* bacteria multiplicates into the mammalian host. As for the antibody response toward rFliD, repeated inoculation

of *Midichloria* antigens by several ticks, as it might occur in dogs, could be sufficient to stimulate an antibody production. On the other hand, the sero-positivity for *Midichloria* recorded in humans parasitized by ticks is suggestive for a replication of these bacteria, considering that single ticks had generally been removed from the examined subjects, normally after a very short blood meal (Mariconti et al., 2012b).

In summary, our current work on animals, together with the previously published study on humans (Mariconti et al., 2012b), present overall evidence that ticks inoculate *Midichloria* bacteria to mammalian hosts, where these bacteria likely multiplicate, inducing an antibody response. Whether infection with *M. mitochondrii* determines pathological alteration is now a main research issue, as well as a main issue to be investigated is the role of the different ticks/arthropod species as vectors and reservoirs for *Midichloria* and related bacteria.

6. CONCLUSION OF THE PHD PROJECT AND FUTURE PROSPECT

M. mitochondrii is the sole member in the order Rickettsiales that is so far described to possess a complete set of genes coding for a putative flagellar apparatus. The usefulness and the function of the flagellar apparatus for this bacterium, is yet to be determined. We observed that the genes that we have analyzed are different expressed during tick life cycle, this could might that intracellular bacteria such as *M. mitochondrii* alternate trophic phases (in which flagella and motility are not required) with phases of the cycle in which a flagellar apparatus is used for motility or for other functions. Considering the conservation of the flagellar genes, their expression at the RNA and protein level and the evidence for their ancestral origin, we suggest that these genes have maintained their original function along the phylogenetic lineage leading to *M. mitochondrii*, and possibly in other lineages. We could suggest that in the future other members of the order Rickettsiales will be discovered to possess flagella/ flagellar genes, at least in the *M. mitochondrii* lineage.

In literature are reported circumstantial evidence that MALOs could be transmitted to terrestrial vertebrates during the tick bite as the presence of 16S rRNA gene sequences related with *M. mitochondrii* in roe deer and human patients parasitized by ticks, therefore we decided to investigate the presence of antibody against *M. mitochondrii* in human and dog exposed to or bite by ticks.
The positive results of the work raises the possibility that *M. mitochondrii* plays a role in the immune response and immune-modulation determined by the *I. ricinus* saliva, which is important both for the success of the tick blood meal and for the establishment of the infection by the pathogens vectored by the tick. Based on the results that we obtained, we cannot conclude that *M. mitochondrii* replicates in the vertebrate host, determining a true infection; nevertheless we would have to suggest that the amount of bacteria (or bacterial antigens) inoculated is sufficient to stimulate antibody production and for PCR detection. However, considering the amount of blood in the vertebrates that we have screened, we believe it would be unlikely that bacterial DNA inoculated by a few ticks, and diluted into liters of

blood, is then detected by PCR analysis of the DNA extracted from 100 µl of blood. We are thus more prone to consider the possibility that *Midichloria* bacteria multiplicates into the mammalian host. At any case, our work shows that *M. mitochondrii* is to be regarded not only as an important symbiont of *I. ricinus*, but also as a package of antigens that ticks can inoculate into vertebrate hosts, and as a potential tick-borne microorganism that deserves further investigations. For sure, considering the high seroprevalence for *M. mitochondrii* that we determined in tick-exposed vertebrate, we would conclude that this bacterium does not cause pathology in humans, at least in the vast majority of the cases.

Anti-*Midichloria* antibodies can now be considered as potential serological markers for *I. ricinus* bite. Such markers could be extremely useful to determine the risk of infection by *I. ricinus*-borne pathogens in given areas, and for investigating the epidemiological association of a variety of pathological alterations with parasitism by this tick.

My PhD project was focused on *M. mitochondrii*, but other MALOs could possibly be transmitted by ticks and other arthropods to a variety of vertebrates, including humans. Further studies are now urgent, to determine whether MALOs represent a novel class of emerging infectious agents.

7. OTHER COLLABORATION

During my PhD I have also participated in other research projects including:

- Mosquito cells stimulation with the *Wolbachia* Surface Protein (WSP). For this project I attended a COST STSM Application Form, which allowed me to make a work experience at the University of Oxford. In addition to the scientific aims of the project, the STSM to the lab. of Dr. Sinkins at the University of Oxford allowed me to acquire skills in mosquito manipulation and mosquito gene expression studies.

- Development of a Broad-range 23S rDNA real-time PCR assay for the detection and quantification of pathogenic bacteria in human whole blood and plasma specimens. This work has been developed in collaboration with colleagues of the S. Orsola Hospital (Bologna, Italia) and Policlinico S. Matteo (Pavia, Italia).

- Bacterial screening in *Hyalomma marginatum* (Ixodida, Ixodidae) from Socotra Island (Yemen), detection of new isolated *Francisella*-like tick endosymbiont. This work has been developed in collaboration with colleagues of the Faculty of Agraria (Università degli Studi di Milano, Italia).

8. *WOLBACHIA* SURFACE PROTEIN INDUCES INNATE IMMUNE RESPONSES IN MOSQUITO CELLS

8.1 INTRODUCTION

Wolbachia pipientis is a maternally inherited endosymbiotic bacterium that infects a wide range of nematodes and arthropods. It is responsible for the induction of several forms of reproductive manipulation in its arthropod hosts, all of which favour infected females at the expense of their uninfected counterparts. Cytoplasmic incompatibility, classically seen in its unidirectional form in crosses between uninfected females and infected males where there is high embryo mortality, provides a powerful insect population invasion capacity. Recently, the presence of *Wolbachia* has been associated with the inhibition of viral (Bian et al., 2010; Hedges et al., 2008; Moreira et al., 2009; Osborne et al., 2009; Teixeira et al., 2008) filarial nematode (Kambris et al., 2009) and *Plasmodium* (Moreira et al., 2009; Kambris et al., 2010) pathogens. In addition, *Wolbachia* is capable of inducing the production of anti-oxidant enzymes and reactive oxygen species (ROS) (Brennan et al., 2008), innate immune effectors (Kambris et al., 2009; Kambris et al., 2010; Hughes et al., 2011) as well as increasing haemocyte densities (Braquart-Varnier et al., 2008). However the molecular nature of the interactions between this symbiotic bacterium and the insect immune system are not well characterized. If *Wolbachia* is to be used optimally in applied strategies to disrupt pathogen transmission in mosquitoes and other pest insects, it is important to gain a better understanding of what *Wolbachia* molecules are involved in eliciting insect immune responses, and whether responses to these molecules differ between naturally *Wolbachia* infected and uninfected hosts. *Wolbachia* and its products have been shown to evoke strong innate immune responses in mammals and are very important in establishing and augmenting inflammatory pathogenesis of the diseases caused by filarial nematodes (Brattig et al., 2000; Cross et al., 2001; Taylor et al., 2000). In particular the *Wolbachia* Surface Protein (WSP) has been shown to elicit innate immune induction via

TLR2 and TLR4 activation in both humans and mice (Brattig et al., 2004) and to inhibit apoptosis in neutrophils through inhibition of caspase-3 activity (Bazzocchi et al., 2007).

8.2 PURPOSE

The aim of this study was to investigate whether WSP can also induce innate immune responses in insects, using mosquito cell lines originating from both naturally *Wolbachia*-uninfected and *Wolbachia*-infected mosquito species. An additional aim was to identify PAMPs (pathogen associated molecular patterns) that can elicit strong immune responses in mosquitoes, which could be useful for novel disease control strategies; thus in order to avoid the complications of possible strain-host co-adaptations, we have initially used WSP derived from a nematode *Wolbachia* rather than from an insect derived *Wolbachia* strain.

8.3 MATERIAL AND METHODS

8.3.1 Cell cultures

Two cell lines were used: 4a3A derived from the naturally *Wolbachia*-uninfected mosquito species *Anopheles gambiae* (Muller et al., 1999) and Aa23 from the naturally *Wolbachia* infected mosquito species *Aedes albopictus* (O'Neill et al., 1997). wAlbB strain infection present in Aa23 was cured via tetracycline treatment (100 μg/ml) for 5 days. *Wolbachia* absence after drug treatment was confirmed using PCR and the derived cell line was subsequently called Aa23T. Cell lines were maintained at 27 °C and grown in Schneider medium (Promo Cell) supplemented with 10% heat-inactivated FCS, 1% penicillin-streptomycin (Gibco).

8.3.2 WSP and bacterial cell challenges

Prior to cell challenges, cultures were re-suspended in growth medium and counted using a heamocytometer. For all experiments, approximately 2 million

cells were seeded per well in 6-well plates. Varying concentrations (0.5-10 μg/ml) of stringently purified endotoxin-free recombinant WSP, obtained from the nematode *Dirofilaria immitis* (Brattig et al., 2004; Bazzocchi et al., 2000), were used to challenge the cells. Proteinase k-treated WSP (pkWSP) (Brattig et al., 2004; Bazzocchi et al., 2000) was used at a concentration of 5 μg/ml. Logarithmic phase cultures of *E. coli* and *Enterococcus faecalis* were washed three times in PBS and re-suspended in Hank-balanced salt solution (Sigma) at OD (A600 nm) of 0.4 prior to heat inactivation at 80 °C. For challenge, 30 μl of a 1:1 mixture of heat killed *E. coli* and *E. faecalis* were used per well. Logarithmic phase cultures of *E. coli* K12 TETr strain (NEB) were washed and re-suspended in PBS to a final OD (A600 nm) of 0.05. For challenge, 25 μl of the bacterial culture was added to 3h conditioned cell culture or 3h incubated Schneider medium (cell-free). Cell medium was collected at 3 and 9h post *E. coli* addition, plated in serial dilutions onto LB-TET agar plates and the next day the number of CFUs was determined.

8.3.3 RNA isolation, cDNA synthesis and real-time quantitative reverse transcription PCR (qRT-PCR)

Total RNA was isolated using TRIzol reagent (Invitrogen) and DNAseI (NEB) treated. First strand cDNA syntheses were performed in a 10 μl reaction volume with 1-1.5 μg of total RNA using the High Capacity RNA-to-cDNA kit (Applied Biosystems). Real-time quantitative reverse transcription PCR (qRT-PCR) amplifications were performed with Express SYBR GreenER PCR mastermix (Invitrogen) and analyzed using the Chromo4TM detection system (Bio-Rad) following manufacturer's instructions. Expression levels were calculated by the relative standard curve method, as described in Technical bulletin #2 of the ABI Prism 7700 Manual (Applied Biosystems), using as an endogenous reference ribosomal proteins S7 and L17 for *An. gambiae* and *Ae. albopictus* cell lines, respectively. pkWSP was used as the exogenous calibrator in all experiments. Primers were designed using GeneiousTM software (Biomatters Ltd) and sequences are listed in Table 1. Data from 4 independent biological repeats was analyzed with a Wilcoxon rank of sum test.

	Forward primer	Reverse primer
An gambiae		
APL1	ACCAGCCGCAGTTTGATAG	CAATCCCAGTCATTATGCGA
RpS7*, CEC1, DEF1 ref (Pinto et al., 2009) and GAMB, TEP1, FBN9 ref (Dong et al., 2006)		
Ae Albopictus[1]		
DEF (D) *	TTCGATGAACTACCGGAGGA	AGCACAAGCACTGTCACCAA
RpL17*	AGTGCGTTCCATTCCGTC	CTTCAGCGTTCTTCAACAGC
CEC (A1), TEP (Müller et al., 1999), PGRP (SP1) and CLIP (B37) ref (Blagrove et al., 2012)		

Table 1. Primers used in qRT-PCR. *RpS7 was used as the reference gene in *An. gambiae* analysis while RpL17 was the reference for *Ae. albopictus*. The *Ae. albopictus* immune gene primers have been determined via degeneracy against the corresponding *Ae. aegypti* orthologous genes shown in brackets.

8.4 RESULTS

8.4.1 WSP is a strong innate immune response elicitor in *An. gambiae* cells

In the *An. gambiae* cells, the antimicrobial peptideencoding genes Cecropin 1 (CEC1) and Gambicin (GAMB) showed elevated levels of transcription in the presence of WSP compared to negative controls (naïve and proteinase K-treated-pkWSP) (Brattig et al., 2004) and responded in a dosage dependent fashion, when different concentrations of WSP up to 5 μg/ml were used (Fig. 1A). Their mRNA levels were increased in the presence of WSP to similar degrees and statistically significant differences were observed for all WSP quantities used. In contrast, Defensin 1 (DEF1) which has been shown to be primarily active against Gram-positive bacteria (Vizioli et al., 2001), showed only a small degree of up-regulation that was not statistically significant. Increased concentrations of WSP also increased the transcription levels of complement-like gene TEP1, *Anopheles Plasmodium*-responsive Leucinerich repeat 1 (APL1) and Fibrinogen 9 (FBN9) (Fig. 1A). In comparison to the AMPs, TEP1 and APL1 showed a higher induction level with respectively 4 and 5-fold peaks. Significant up-regulation was also seen at a concentration of 5 μg/ml of WSP for all three genes ($p<0.05$). This data suggests that in this naturally *Wolbachia*- uninfected mosquito species, WSP is capable of inducing the transcription of innate immune factors such as AMPs,

complement-like proteins and fibrinogen genes, all of which are involved in anti-parasitic responses in *An. gambiae*.

8.4.2 WSP is a mild innate immune response elicitor in *Ae. albopictus* cells

We next examined whether WSP has the same capacity to elicit an immune response in a species that naturally harbours *Wolbachia*. Uninfected *Ae. albopictus* Aa23 cells (O'Neill et al., 1997) were challenged with WSP and transcription level of immunity genes monitored as for the *An. gambiae* cell line. All genes tested showed elevation in mRNA levels with increased WSP concentration up to 5 μg/ml (Fig. 1B), but these were less pronounced when compared to the 4a3A cell line. Statistically significant up-regulation was seen only for CEC and TEP when 5 μg/ml WSP was used ($p<0.05$, Fig. 1B).

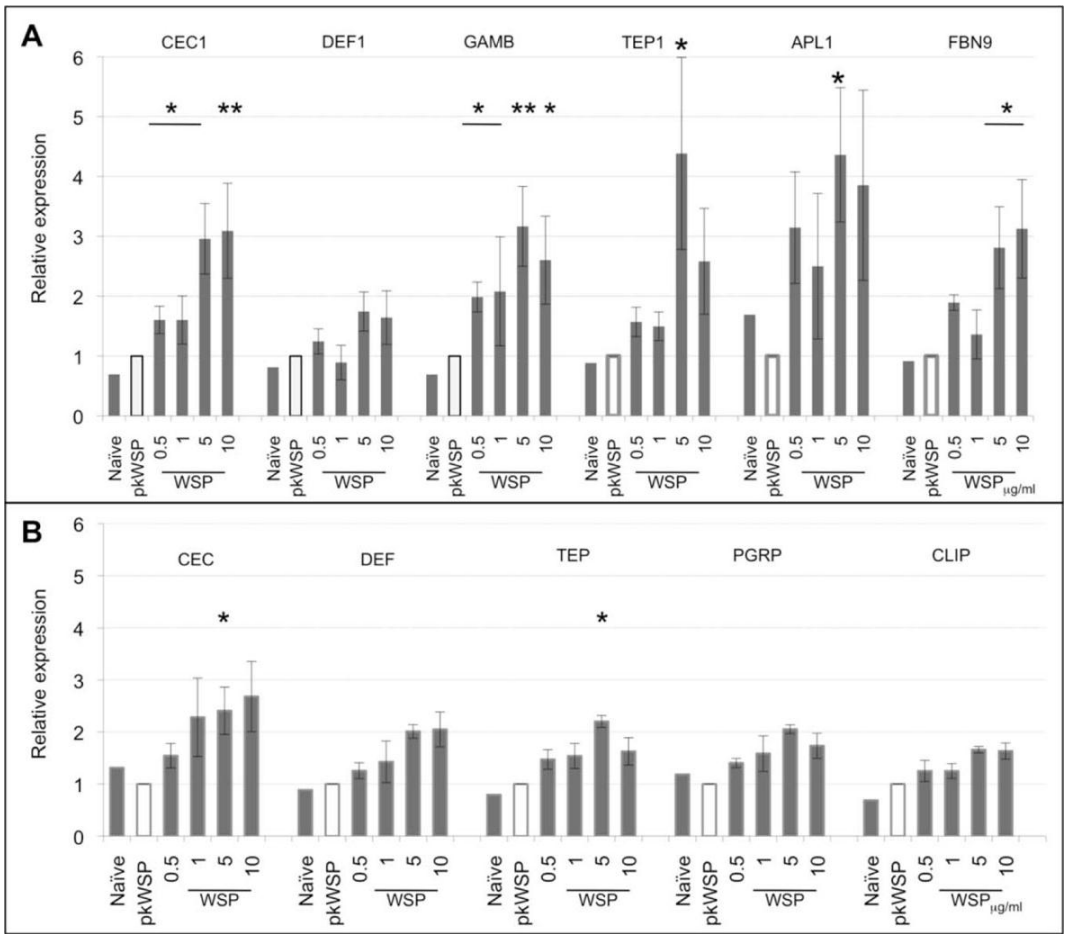


Figure 1. WSP challenge in mosquito cells. qRT-PCR analysis of AMPs and innate immune genes at 3h post-WSP challenge in 4a3A (A) and Aa23T (B). Increased expression dependent on WSP

quantities up to 5 μg/ml was detected in all genes tested. Relative expressions were calculated to pkWSP (WSP protein treated with proteinase K) challenged cells and represent the average of 4 biological repeats +/- SE. Statistical analysis where performed using a Wilcoxon rank sum test (*p<0.05, **p<0.01).

8.4.3 Only early phase induction is seen after WSP challenge in both cell lines

Innate immune response activation is commonly divided into early phase response (2-4 h post challenge) and late phase response (24 h post challenge), and so far we have shown that WSP can be a strong PAMP at this early phase response (3 h post challenge). To determine the dynamics of this immune response, both cell lines were stimulated with 5 μg/ml and monitored at 3, 9 and 24 h post challenge. In the 4a3A cell line all innate immune transcription is shut down at 9 h post infection. For only CEC1 and GAMB a mild induction (2-fold) at 24 h post challenge was detected, however this induction was not statistically significant (Fig. 2A). In the case of Aa23T cell line immune activation is decreased back to basal levels at 9 h post infection and no late phase induction was detected.

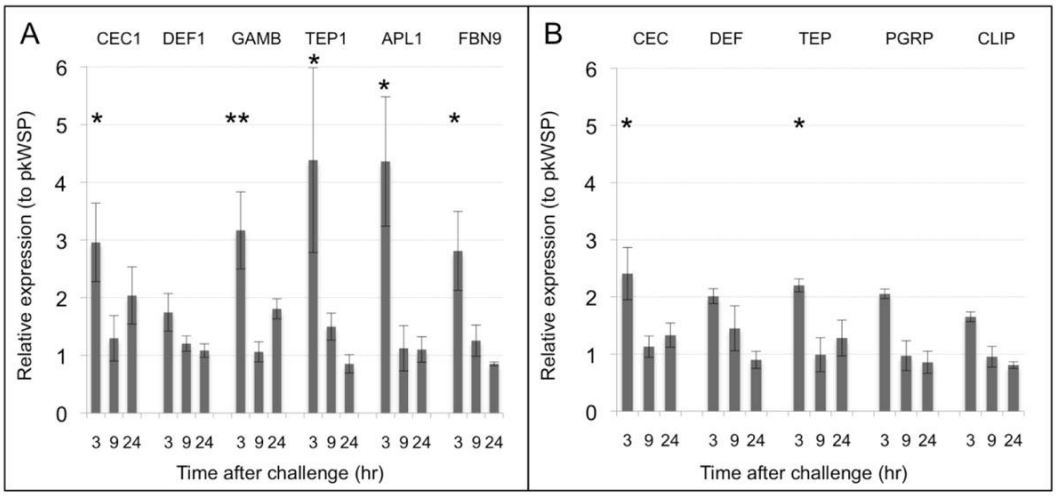


Figure 2. Dynamics of WSP challenge in mosquito cells. qRT-PCR analyses in 4a3A (A) and Aa23T (B) cell lines at 3, 9 and 24 h after WSP challenge detect significant upregulation for all tested genes at 3h post-challenge. With the exception of CEC1 and GAMB, mRNA levels return back to control levels at 24 h. Relative expressions were calculated to pkWSP-challenged cells and represent the average of 4 biological repeats +/- SE. Statistical analysis where performed using Wilcoxon Rank Sum Test (*p<0.05, **p<0.01).

8.4.4 The *Ae. albopictus* cells are capable of mounting a strong immune response

To exclude the possibility that the differences observed between these cell lines may be due to an impaired immune response in the particular *Ae. albopictus* line used, the responses of both cell lines to bacterial challenge and their capacity to clear a live bacterial infection was tested. Both cell lines were challenged with a mixture of heat-killed *E. coli* and *E. faecalis*, and relative transcription monitored from 3-24 h as above. In the 4a3A cell line peak immune induction of both DEF1 and TEP1 was seen at 6 h rather than 3 h, which for DEFD and TEP in Aa23T line already showed strong transcription levels. When looking at the peak levels of up-regulation, in Aa23T cell line DEFD and TEP levels reach 4.5 and 3-fold respectively, while DEF1 and TEP1 show 3-3.5-fold levels in the 4a3A cell line (Fig. 3A). To test for the capacity of each cell line to clear an *E. coli* infection, live *E. coli*- TETr was added to 3 h conditioned cell culture. Cell medium was collected at 3 and 9 h post *E. coli* addition, diluted in LB-TET medium and plated on LB-TET plates. Colony forming units (CFU) where counted for several dilutions for each condition. The Aa23T cells at 3 h post-*E coli* addition had cleared 99 % of bacteria from the culture medium in comparison with only 14 % of bacteria cleared in 4a3A cell culture when compared to the same amount of bacteria incubated in cell-free (CF) medium.

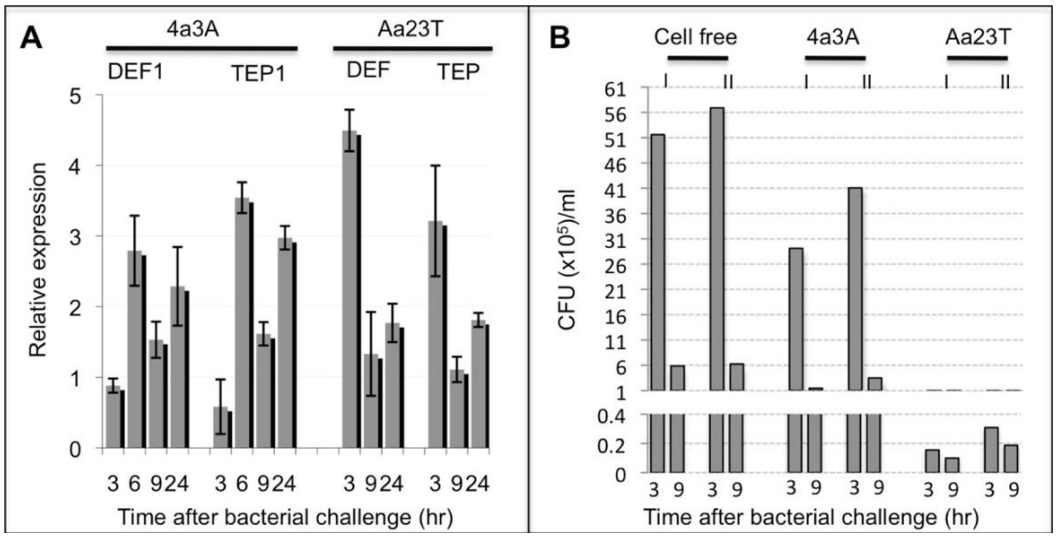


Figure 3. 4a3A and Aa23T immune response to bacterial challenges. (A) qRT-PCR analysis at 3, 6, 9 and 24 h after cell challenge with a mixture of heat-killed *E. col*i and *E. faecalis* show both

early and late phase induction of DEF and TEP in both mosquito species. The time of early phase induction varies between species. Up-regulation levels for each gene are similar between the two cell lines. Relative expressions were calculated to PBS-challenged cells and represent the average of 3 biological repeats +/- SE (B) 99% of *E. coli* is rapidly cleared by Aa23T cell line at 3 h post-infection while for 4a3A only about 14% have been killed when compared to the same amount of bacteria incubated in cell-free (CF) medium. The starting amount used in each case was 25 µl per well of culture with an OD600 reading of 0.05, which represents approximately 15-18M CFU/ml. I -Set I; II -Set II.

8.5 DISCUSSION

Obtaining a better understanding of *Wolbachia*-host immune interactions in insects is particularly important at the current time given the recently described effects of *Wolbachia* in inhibiting the development or dissemination of several very important mosquito-borne human pathogens. This study shows that, as previously observed using mammalian cells, the *Wolbachia* WSP protein is a potent innate immune elicitor in insects. The responses between the two mosquito cell lines to WSP challenge are mechanistically similar: 1) they are dosage dependent, increasing with increasing amounts of WSP up to 5 µg/ml; 2) peak induction is seen at 5 µg/ml, while higher concentrations sometimes reduce the mRNA levels; and 3) the immune gene transcription was at a maximum at 3 h post challenge (early phase induction) and do not show late phase induction. The major difference is the level of up-regulation between the two species: detected peak induction of 3 to 5-fold in the naturally *Wolbachia*-uninfected cell line compared to just 2-fold induction in the naturally infected one. Tolerance effects due to previous natural *Wolbachia* exposure have been described (Turner et al., 2006) and seem likely to be contributing to the differences observed between these cell lines in their response to WSP. The control experiments also show that Aa23T can show strong induction of immune gene transcription and can effectively clear a bacterial infection. Thus the differences seen between WSP-associated immune induction between these cell lines are not due to impaired immune responses in Aa23T.

In this experimental set-up the WSP protein will be extracellular, and although *Wolbachia* itself is mostly located within intracellular vacuoles in insects, bacterial protein will be released into the hemocele, for example through regular

cellular turnover / apoptosis. This mirrors the situation in humans where WSP elicits antibody responses in lymphatic filariasis patients despite *Wolbachia* itself being located inside vacuoles within the filarial nematodes (Bazzocchi et al., 2000). In the insect hemocele WSP has the potential to elicit innate immune responses from hemocyte immune cells, and the same applies in these cell lines.

Further studies of insect immune responses to WSP may include the examination of levels of immune response to intracellular WSP, using transformation / transfection studies (although these will not exactly replicate the intravacuole localization of *Wolbachia* itself). Furthermore, the possibility of different levels of immune response to WSP derived from various insect *Wolbachia* strains can be examined, particularly in the case of the *Ae. albopictus* cells which are derived from a naturally *Wolbachia* infected species and could thus show varying degrees of tolerance to different WSP molecules. These basic biology questions are also relevant to the important applied aim of identifying potent PAMPs that might be incorporated in transgenic strategies to 'prime' the mosquito immune system, and thus impair pathogen transmission. The *Dirofilaria Wolbachia*-derived WSP used here appears to hold potential in this respect, since it induces the up-regulation of genes (particularly TEP1 and APL1) that are directly involved in *Plasmodium* killing in *Anopheles* mosquitoes.

8.6 CONCLUSIONS

Similarly to mammals, the major surface protein of the endosymbiotic bacteria *Wolbachia* (WSP) can induce strong innate immune responses in insects at the transcriptomic level. Antimicrobial peptides as well as important immune effector genes are up-regulated when recombinant WSP is used to challenge mosquito cell lines. Interestingly the response between a naturally-uninfected mosquito and a naturally -infected mosquito is qualitatively similar but quantitatively distinct. The *Wolbachia* naïve host is capable of mounting a very strong up-regulation to WSP as opposed to the *Wolbachia* cleared host suggesting that tolerance effects due to previous *Wolbachia* exposure may be contributing to this particular phenotype.

9. DEVELOPMENT OF A BROAD-RANGE 23S rDNA REAL-TIME PCR ASSAY FOR THE DETECTION AND QUANTIFICATION OF PATHOGENIC BACTERIA IN HUMAN WHOLE BLOOD

9.1 INTRODUCTION

Blood culture (BC) is the most widely used method for the diagnosis of blood stream bacterial infections (BSIs) (Mylotte and Tayara, 2000). Among the major limitations of culture techniques one must consider the intrinsic poor cultivability (or non-cultivability) of some bacteria, and the inhibitory effects of concurrent antibiotic therapy. In addition, the turn-around time of BC ranges from 24 to 72 h, which implies that results might become available too late to be of clinical utility (Paolucci et al., 2010). In recent years, molecular methods have been proposed as additional diagnostic tools for BSIs (Gaibani et al., 2009; Paolucci et al., 2010). Several studies reported the development and clinical assessment of broad-range real-time PCR protocols, capable of rapid detection and identification of a vast proportion of cultivable and uncultivable bacteria, from different types of biological samples (Anthony et al., 2000; Rosey et al., 2007; Zucol et al., 2006; Jiang et al., 2009; Matsuda et al., 2007; Rampini et al., 2011; Yang et al., 2002; Zapater et al., 2008; Clarridge, 2004). The majority of the broad-range real-time PCR protocols are based on universal bacterial primers (pan-bacterial primers), that are targeted on highly conserved regions of the gene coding for the 16S ribosomal RNA (16S rDNA) (Clarridge, 2004; Bacchetti De Gregoris et al., 2011; Cherkaoui et al., 2009; Ferri et al., 2010; Gentili et al., 2011; Zemanick et al., 2010). A major pitfalls of 16S-based pan-bacterial primers is their cross reactivity with human ribosomal RNA genes; to overcome the problem, Kommedal and co-workers proposed a 16S rDNA-based dual-priming protocol (Kommedal et al., 2012). In addition to 16S rDNA, the gene coding for the large subunit ribosomal RNA (23S rDNA) has also been exploited for the development of PCR methods for bacterial detection, but only a limited number of studies evaluated the utility of

23S-based pan-bacterial primers (Anthony et al., 2000; Hunt et al., 2006), and no studies have so far exploited this target for BSI monitoring.

9.2 PURPOSE

The aim of this study was to develop a novel 23S rDNA-targeted real-time pan-bacterial PCR method, suitable for the detection of a wide range of bacterial species, for the monitoring of BSIs. The use of a real time PCR assay capable to specifically detect with elevated sensitivity a wide range of human pathogens could be a useful tools that microbiology laboratories could use for the identification of infecting bacteria in patients suffering for invasive microbial disease, and in particular in the case of blood stream infections.

9.3 MATERIALS AND METHODS

9.3.1 Design of the 23S rDNA universal primers

Complete 23S rDNA sequences from 50 bacterial species, spanning the eubacterial diversity, were retrieved from the NCBI database (http://www.ncbi.nlm.nih.gov/). Alignment of the sequences was performed using the MUSCLE software (Edgar, 2004) and manually checked. 23S rDNA sequences from *Caenorhabditis elegans*, *Candida albicans*, *Drosophila melanogaster* and *Homo sapiens* were also included in the alignment, in order to evaluate the specificity for the bacterial DNA of the designed primers. Primers were manually designed on the obtained alignment, and then evaluated using mfold (http://mfold.rna.albany.edu/?q=mfold) and the Operon oligo analysis tool (http://www.operon.com/tools/oligo-analysis-tool.aspx). The sequences of the 23S rDNA-targeted pan-bacterial primers are: (PAN23S-F) 5'-TCGCTCAACGGATAAAAG-3' and (PAN23S-R) 5'-GATGAnCCGACATCGAGGTGC-3' and the amplified fragment size is 97 base pairs. The designed primers were then compared to the non redundant nucleotide

eukaryotic database using the Blast software (http://blast.ncbi.nlm.nih.gov/Blast.cgi), to highlight possible unwanted matches.

9.3.2 Bacterial isolates and DNA extraction

A panel of 47 different bacterial isolates, 20 Gram-positive and 27 Gram-negative, from a total of 43 species plus four further taxa at the species/subspecies rank, were included in the study (Table 1). These strains were either obtained from routine cultures, and the identification performed at the Unit of Clinical Microbiology, St. Orsola Malpighi Hospital, or obtained from the bacterial collection at the same Institution (BACSO). A cell suspension containing 10^8 CFU/ml was obtained from each bacterial isolate, and the DNA was extracted using QIAmp DNA blood mini kit (Qiagen) following the manufacturer's instructions and eluted in 50 µl of sterile water. In addition, five eukaryotic species from the genus *Candida* (*C. albicans*, *C. glabrata*, *C. tropicalis*, *Candida parapsilosis* and *C. guillermondii*) were included in the study.

Microorganisms species	Origin of the isolate
Acinetobacter baumannii	URINE
Acinetobacter lwoffii	URINE
Alcaligenes xylosoxydans	URINE
Bacteroides fragilis	CEREBROSPINAL FLUID
Campylobacter jejuni	FECES
Citrobacter brakii	ABDOMINAL DRAINAGE
Citrobacter freundii	BLOOD
Citrobacter koserii	URINE
Corynebacterium jeikeium	BLOOD
Corynebacterium minutissimum	BLOOD
Corynebacterium striatum	BLOOD
Corynebacterium urealyticum	BLOOD
Enterobacter cloacae	URINE
Enterobacter erogene	URINE
Enterococcus casseliflavus	BLOOD
Enterococcus faecalis	BACSO/ATCC 29212
Enterococcus faecium	BLOOD
Enterococcus gallina rum	BLOOD
Escherichia coli	BACSO/ATCC 25922
Haemophilus influenzae	BACSO/ATCC 49247
Haemophilus influenzae	BACSO/ATCC 49766
Hafnia alvei	BILE
Klebsiella oxytoca	BLOOD

Klebsiella pneumoniae	URINE
Morganella morganii	URINE
Nocardia spp.	BRONCHIAL ASPIRATE
Proteus mirabilis	URINE
Proteus vulgaris	BRONCHIAL ASPIRATE
Providencia stuartii	URINE
Pseudomonas aeruginosa	BACSO/ATCC 27853
Pseudomonas luteola	BRONCHIAL ASPIRATE
Salmonella spp. Group B	FECES
Salmonella spp. Group C	FECES
Salmonella spp. Group D	FECES
Serratia marcescens	URINE
Staphylococcus aureus	BACSOATCC 29213
Staphylococcus epidermidis	BLOOD
Staphylococcus haemomlyticus	BLOOD
Staphylococcus hominis	BLOOD
Staphylococcus warnerii	BLOOD
Stenotrophomonas maltophilia	FARINGEAL SWAB
Streptococcus agalactiae	URETRAL SWAB
Streptococcus anginosus	BLOOD
Streptococcus mitis	BLOOD
Streptococcus parasanguinis	BLOOD
Streptococcus piogene	FARINGEAL SWAB
Streptococcus pneumoniae	BACSO/ATCC 49619

Table 1. Bacterial strains utilized in this study. The strains were either obtained from the bacterial Collection of the S.Orsola Hospital (BACSO) or derived from routine workflow. In this last case the procedure for identification are the standard and reported in the Qualty Assurance files of the Laboratory.

9.3.3 23S rDNA real-time PCR

PCR reactions were effected in a final volume of 25 µl, containing 12.5 µl of SYBR Green PCR Master Mix Reagent (Applied ByoSystems), 250 nM of each primer, and 5 µl of the extracted DNA solution. PCR was performed in an IQ5 thermocycler (Biorad–Hercules) with an initial step of 5 min at 95 °C, followed by 40 cycles of 15 s at 95 °C and 30 s at 58 °C. After PCR amplification, the melting curve was established by increasing the temperature from 55 °C to 95 °C.

9.3.4 PCR sensitivity test

An external standard for absolute quantification (i.e. the target 23S rDNA gene fragment, cloned into a plasmid vector) was prepared. PCR was effected on *Staphilococcus aureus* DNA using the above described primers PAN23S-F and

PAN23S-R according to standard PCR conditions. The band resolved on a 2% agarose gel was excised and the PCR product was then purified, quantified, and cloned using the pGEM T-easy vector (Qiagen) according to manufacturer instructions. Ten randomly selected clones were sequenced with ABI technology. A plasmid containing the 23S rDNA insert was purified from one of the clones, using the QIAprep Spin Miniprep Kit (Qiagen). After quantification, a serial dilution of the plasmid was used to assess the sensitivity of the above PCR assay, with plasmid at concentrations ranging from 10^8 to 10^1 copies per reactions, to generate the standard curve. The pGEM T-easy vector was also used to clone the PCR products obtained from *S. aureus* cultures, blood from healthy donors and blood from healthy donors spiked with *S. aureus* DNA; cloned PCR products were then sequenced to verify whether just bacterial DNA had been amplified and cloned.

9.3.5 Quantification of the 23S rDNA present in extracted DNA samples

The developed real time PCR assay was tested on the DNA extracted from the 47 bacterial isolates (Table 1). Each extracted DNA were tested by real-time PCR, and the *CT* values were applied to the standard curve generated in the same experiment to obtain the corresponding copy number of bacterial gene targets in each reaction. Additionally, real time PCR was performed on DNA extracted from blood from healthy donors, used as negative control.

9.4 RESULTS

The novel 23S rDNA real time PCR assay showed a high efficiency under the experimental conditions outlined above. Primer alignment against the 47 bacterial species included shows an almost complete identity, with no mismatches at the 3' end, while a high number of mismatches are present in the alignment with the eukaryotic organisms, such as *H. sapiens*, *C. elegans*, *C. albicans* and *D. melanogaster*, as shown in Figure 1.

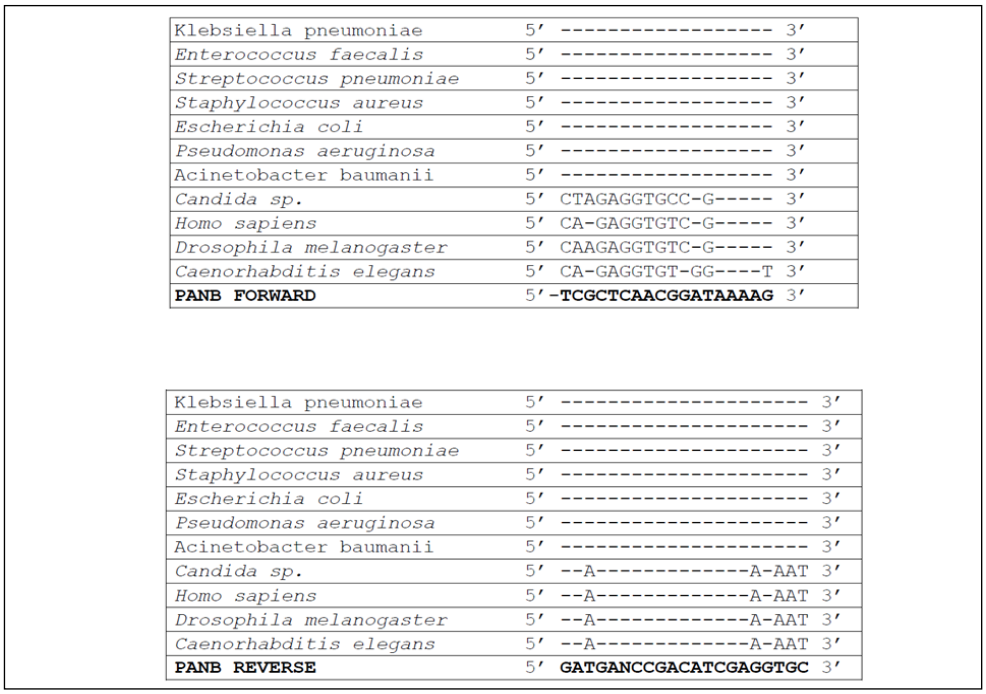


Figure 1. Analysis of PANB-forward and PANB-reverse primers homology sequences against the 23S rDNA of the most common pathogenic bacteria species, *H.sapiens, C. elegans, C. albicans* and *D. melanogaster* in their binding areas.

PCR sensitivity was evaluated on a serial dilution of the plasmid containing the 23S rDNA fragment, at concentrations ranging from 10^8 to 10^1 copies per reactions (Fig. 2) to generate a standard curve (R value: 0.97; slope value: -2,527). Each dilution was tested in triplicate. Since amplifications from the 10^1 dilution could not be distinguished from those from the negative controls (Fig. 2), PCR products from this dilution and from negative control were resolved on agarose gel, purified and cloned; ten clones for each sample were sequenced using the amplification PAN23S-F and PAN23S-R primers. All of the sequences obtained from the 10^1 dilution of the plasmid were the 23S rDNA of *S. aureus*, while the sequences from the negative controls were identified as derived from contaminating bacteria. Based on these results, we can conclude that the developed PCR procedure is capable to detect up to 10 bacteria.

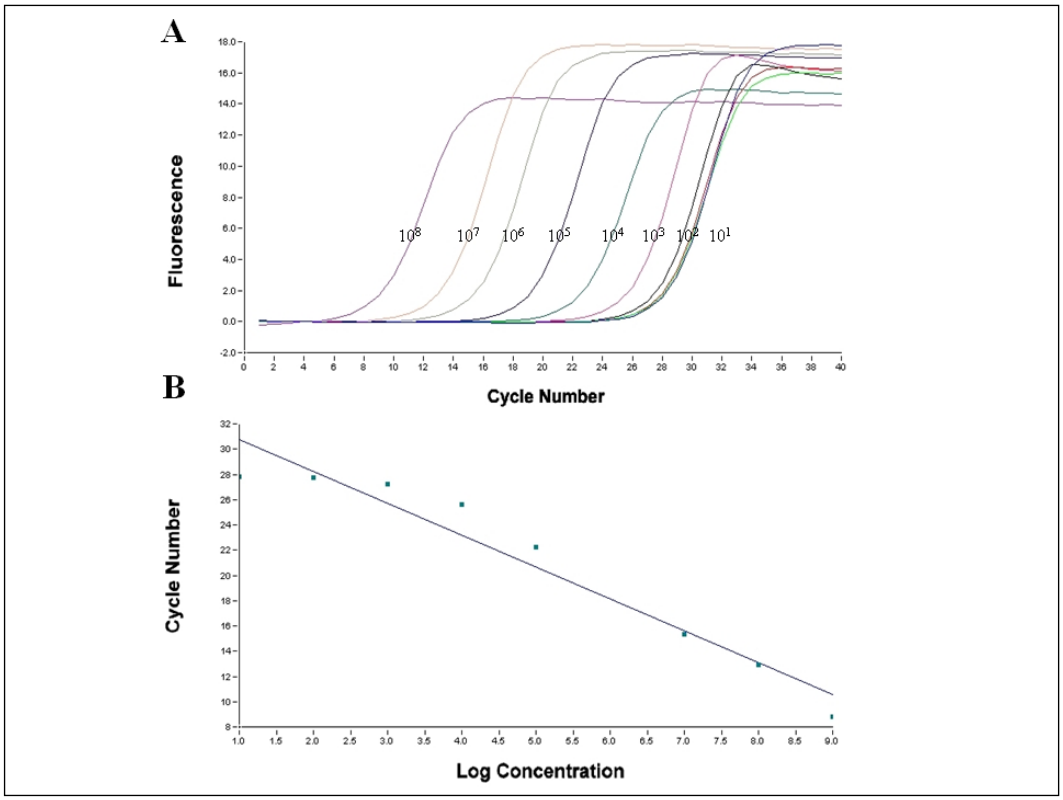


Figure 2. Standard curve amplification of cloned bacterial 23S rDNA plasmid real-time PCR ranging from 10^8 to 10^1 copies per reactions. Panel A showed the amplification curve constructed by PCR assay. The fluorescence and the corresponding cycle numbers were showed in the vertical and orizontal axis, respectively. Panel B showed the relative standard curve ranging from 10^1 to 10^8 copies per reactions.

In order to verify the specificity of the amplifications, PCR products from a *S. aureus* culture, from blood samples of three healthy donors, and from blood samples of three healthy donors spiked with *S. aureus* DNA were resolved on agarose gel, purified, cloned and sequenced. All of the obtained sequences were bacterial, indicating that the novel primers PAN23S-F and PAN23S-R did not amplify from human DNA. The specificity of the novel 23S rDNA-targeted primers was also evaluated on a total of 20 Gram-positive and 27 Gram-negative bacterial species/taxa (see Table 1 for details) and on five eukaryotic species, from the genus *Candida* (*C. albicans*, *C. glabrata*, *C. tropicalis*, *C. parapsilosis* and *C. guillermondii*). PCR amplifications were obtained from the 47 bacterial DNAs (results not shown), while all the specimens containing *Candida*-derived DNA led to amplification curves comparable to those obtained from negative controls (i.e. sterile PBS solution).

9.5 DISCUSSION

In this study, we developed and assessed the analytical performance of a novel real time PCR method targeted a conserved region of the 23S ribosomal DNA gene, for the detection and quantification of a wide range of human pathogenic bacteria in human blood. Up to today, the 16S rDNA was the most widely used sequence for tests aimed at the phylogenetic analysis of different bacteria and for methods intended to detect the presence of a broad range of pathogenic germs in human derived materials. One possible concern was raised about the specificity of this approach, given the likely cross-reactivity with human DNA, but this issue was not largely investigated (Kommedal et al., 2012; Harris and Hartley, 2003; Rantakokko-Jalava et al., 2000; Vandercam et al., 2008). In fact, the possible cross-reactions can result in aspecific amplification of human DNA that consequently results in false positive microbiological results (Handschur et al., 2009). Given this aspect of the 16S rDNA targeted assays, the choice of the 23S rDNA region as target for a broad range PCR was recognized as a possible way to overcome the cross-reactivity with human derived DNA while still being able to detect a broad range of bacterial species. Several theoretical advantages are foreseen for using this alternative sequence as PCR target, such as the following: i) the high content of characteristic sequence stretches due to a greater length, ii) the unique insertions and/or deletions, iii) possibly a better phylogenetic resolution because of a higher sequence variation. Most of these characteristics are presently available thanks to the accessibility to a wide range of the complete genomic sequences for an high number of bacterial species (Hunt et al., 2006; Ludwig and Schleifer, 1994; Pei et al., 2009).

The novel real time PCR assay described in this study was able to detect and amplify specifically all the bacteria species included in a study panel that encompassed more than 90 % of the reported causes of blood stream infections (Paolucci et al., 2010), and no false positive reactions were observed when eukaryotic DNA of diverse origin was tested. The presence of bacterial products, including nucleic acids, in the blood is nowadays a well recognized phenomenon that frequently occurs when pathological condition allow translocation from

highly colonized sites, such as the bowel (Gomez-Hurtado et al., 2011; Kramski et al., 2011).

10. BACTERIAL SCREENING IN *HYALOMMA MARGINATUM* (IXODIDA, IXODIDAE) FROM SOCOTRA ISLAND (YEMEN), DETECTION OF NEW ISOLATED *FRANCISELLA*-LIKE TICK ENDOSYMBIONT.

10.1 INTRODUCTION

The ticks are blood-sucking ectoparasites able to parasite a multitude of terrestrial vertebrates as mammals, birds, reptiles and amphibians (Sonenshin, 1991 and 1993). Nowadays ticks are considered the group of arthropods that can transmit the wider variety of pathogenic agents to humans and animals (Jongejan and Uilenberg, 2004). Microorganisms such as bacteria (e.g. *Rickettsia* spp., *B. burgdorferi*, *Erlichia* spp. and *Francisella* spp.), protozoa and viruses (like Crimean-Congo hemorragic fever, Tick Borne Encephalitis) can be transmitted to host as a result of a tick bite (Sonenshine, 1991; Jongejan and Uilenberg, 2004). Ticks play also an important role as reservoirs for population of these bacteria in nature (Parola and Raoult, 2001). Recently, the intra mitochondrial bacterium *M. mitochondrii*, originally discovered in the tick *I. ricinus*, was found to be widespread in many tick genera (Lo et al., 2006; Epis et al., 2008).

Approximately 870 species of ticks are described (Nava et al., 2009), subdivided into three families: Argasidae, Ixodidae and Nuttalliellidae (Horak et al., 2003; Nava et al., 2009). The 26 species belonging to the genus *Hyalomma* Koch, 1844 are widespread in Palearctic and Afrotropical biogeographic regions (Horak et al., 2003; Apanaskevich and Horak, 2008; Estrada-Peña et al., 2012). In details *Hyalomma marginatum* Koch, 1844 is widespread in Central and Southern Europe, Northern Africa and in Asia east to Iran (Manilla, 1998; Apanaskevich and Horak, 2008). *H. marginatum* is a two-host species showing a low host specificity, in fact the adults feed on different species of large mammals (ungulates and livestock), while the immature stage feed on birds or small mammals (Manilla, 1998) increasing its ability to spread. This species can transmit a variety of pathogens for human and animal (Hoogstraal, 1956) and it is

considered one of the most important tick species involved in the transmission of the virus of the Crimean-Congo haemorrhagic fever (Hoogstraal, 1979; Estrada-Peña et al., 2012); furthermore it is known to transmit bacteria of the genus *Rickettsia* (e.g. *R. conori* the causative agent of the Mediterranean spotted fever) and *Coxiella burnetii*, the causative agent of Q-fever (Hoogstraal, 1956). Recently Ivanov and colleagues (2011) isolates *Francisella*-like endosymbionts (FLEs) in *H. marginatum* collected from Bulgaria.

10.2 PURPOSE

The aim of this work was to study the ticks (Acarina, Ixodida) collected from Socotra Island (this Indian Ocean archipelago has a peculiar fauna since it has been isolated 35–41 Million Years ago; Girdler and Styles, 1974) and to screen the bacterial community associated with these ticks in this area.

10.3 MATERIAL AND METHODS

10.3.1 Sample collection, morphological identification and images acquisition

A total of 34 adult ticks specimens were collected in Socotra Island (Yemen) directly from livestock (sheep and goats) during field research (December 2010). All the collected specimens were immediately stored in absolute ethanol for further DNA extraction. Genomic DNA was extracted from all specimens individually following a procedure that allow to preserve the morphology for further analyses. Specimens manipulation were completed using the stereo microscope Leica MS5. All ticks were identified using standard taxonomic keys (Starkoff, 1958; Manilla, 1998; Apanaskevich and Horak, 2008). Male and female images were acquired by a machinery made and coptimized in order to scan the sample at different focus layers that were mounted with Zerene Stacker 1.0 64 bit (Student Edition).

10.3.2 DNA extraction and polymerase chain reaction (PCR)

Total genomic DNA was extracted and purified individually using Qiagen DNeasy Blood & Tissue Kit (Qiagen). All the tick preserved in ethanol were washed with distilled water and dried before DNA extraction. Afterwards, ticks were cut with a scalpel along the idiosome and left for 12 h at 56 °C into 180 µl of ATL lysis buffer (Qiagen) with 200 ng/ml proteinase K (Sigma Aldrich). The following extraction steps were performed according to the manufacturer's instructions. Extracted DNAs were quantified with Nanodrop 1000 (Thermo Scientific). In order to confirm the morphological identification of the ticks, a fragment of the mitochondrial ribosomal small subunit 12S rRNA gene was amplified (Beati and Keirans, 2001) and sequenced for all the samples. The extracted DNAs were examined, for the presence of *Francisella* spp., *Rickettsia* spp., *C. burnetii*, *B. burgdorferi* and *M. mitochondrii*, using specific PCR protocols. The primers used for the screening of bacterial species are reported in table 1. PCR amplification were performed in 25 µl reaction mix containing 1 µl of each primers (1 µM), 5 µl of GoTaq reaction Buffer (1x) with Mg^{2+}(1.5 mM $MgCl_2$), 0.5 µl of dNTPs (0.2 mM each dNTP), 0.2 µl of GoTaq DNA Plymerase (1.25 U). Successful amplification was determined by gel electrophoresis. Positive and unambiguous PCR products were directly sequenced in both strand by ABI technology (Applied Biosystems). The obtained sequences were manually corrected using Geneious Pro 5.3 and deposited in the EMBL data library (Accession numbers HE819515 for *H. marginatum* partial 12S rRNA gene and HE819516 for *Francisella*-like endosymbiont partial 16S rRNA gene).

Organism	Target gene	Primer sets	References
B. burgdorferi	16S rRNA	5'-ATGCACACTTGGTGTTAACTA-3' 5'-GACTTATCACCGGCAGTCTTA-3'	Marconi and Garon, 1992
C.burnetii	transposon-like repetitive region	5'-TATTGTATCCACCGTAGCCAGTC-3' 5'-CCCAACAACACCTCCTTATTC-3'	Willems et al., 1994; Berri et al., 2000
Francisella spp.	16S rRNA	5'-CAAGGTTAATAGCCTTGGGGGA-3' 5'-GCCTTGTCAGCGGCAGTCTTA-3'	Forsman et al., 1994
M. mitochondrii	16S rRNA	5'-GTACATGGGAATCTACCTTGC-3' 5'-CAGGTCGCCCTATTGCTTCTTT-3'	Epis et al., 2008

Organism	Target gene	Primer sets	References
Rickettsia spp.	citrate synthase *gltA*	5'-GCAAGTATCGGTGAGGATGTAAT-3' 5'-GCTTCCTTAAAATTCAATAAATCAGGAT-3'	Labruna et al., 2004

Table 1. Primers used in this study and relative reference.

10.3.3 Bioinformatic and phylogenetic analyses

The tick mitochondrial 12S rRNA and the bacterial 16S rRNA consensus sequences obtained by sequencing were subjected to BLAST analysis (http://www. ncbi.nlm.nih.gov/blast) and compared to the sequences available in GeneBank (http://www.ncbi.nlm.nih.gov/genbank/). A 16S rRNA sequences of *Francisella* spp. was retrieved from GeneBank in order to perform phylogenetic analyses. Sequences belonging to the four subspecies of *Francisella tularensis*, *Francisella*-like endosymbiont (FLEs) of tick and other *Francisella* spp. were included in the dataset. The obtained 18 sequences were aligned using MUSCLE (Edgar, 2004) then trimmed with Gblocks (Castresana, 2000) and analyzed with jModelTest 0.1.1 (Posada, 2008) to choose the most suitable model of nucleotide evolution. Phylogenetic reconstructions were performed with Bayesian inferences using MrBayes 3.1.2 (Huelsenbeck and Ronquist, 2001). Bayesian analyses were performed using GTR (Lanave et al., 1984) as model of evolution + I+ G; two parallel analyses, each composed of one cold and three incrementally heated chains were run for 2.5 million generations. Trees were sampled every 100 generations and burn-in fraction was calculated as 25 % of total sampled trees, according to the lnL stationary analyses. The majority rule consensus tree was rooted with the branch leading to *F. philomiragia* and *F. noatunensis*, node with values of Bayesian Posterior Probability (BPP) less than 0.5 were collapsed.

10.4 RESULTS

All the collected ticks, 11 males and 23 semi-engorged females, were morfologically identified as *Hyalomma marginatum*. Male and female images in dorsal view were reported in Figure 1 (respectively B and A). The DNA extracted

from the 34 specimens, quantified by Nanodrop, result in concentration ranging from 40 to 110 ng/µl. All tick samples were positive for 12S rRNA PCR; the PCR products were sequenced and morphological identification were confirmed by BLAST analysis (100% identity with *H. marginatum*, accession number AF150034).

All the specimens resulted negative in PCR for the presence of *Rickettsia* spp., *C. burnetii*, *B. burdorferi* and *M. mitochondrii*; while PCR amplification for *Francisella* spp. was positive in three specimens (2 females and 1 male, 8.8% of prevalence). No nucleotide differences were recovered between the three consensus sequences after a pairwise comparison. BLAST analyses confirm the positivity of the three specimens to bacteria of the genus *Francisella* and highlight that are 99% identity to reported sequences of FLEs of ticks.

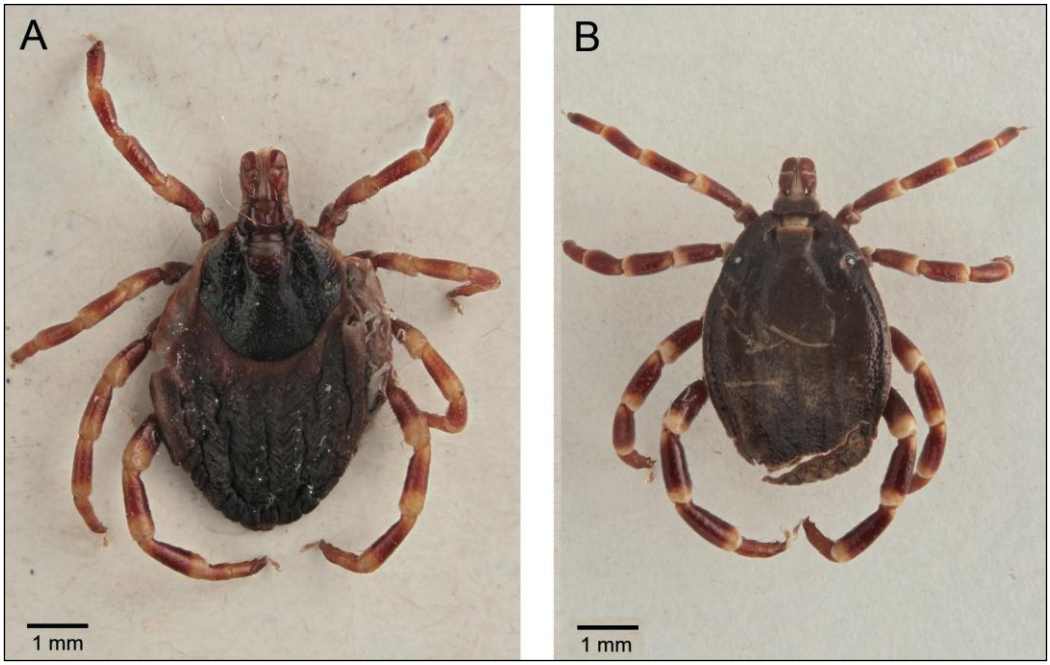


Figure 1. *Hyalomma marginatum* in dorsal view; A) female, B) male.

Phylogenetic analyses were performed on a dataset of a 660 bp of the bacterial 16S rRNA composed of eighteen taxa belonging to *Francisella* spp. from different origin (e.g. pure culture, soil samples, seawater, tick endosymbionts) in

order to understand the relationships of the newly sequenced bacterial strains. Bayesian analysis (Fig. 2) confirm that *Francisella* spp. harboured by *H. marginatum* collected from livestock in Socotra Island cluster within the group of tick FLEs. In detail the new sequence clusters with a BPP of 1 within a well supported group formed by two FLEs previously isolated from *H. marginatum* and *R. sanguineus* collected from Bulgaria. Not all the FLEs of ticks appear monophyletic.

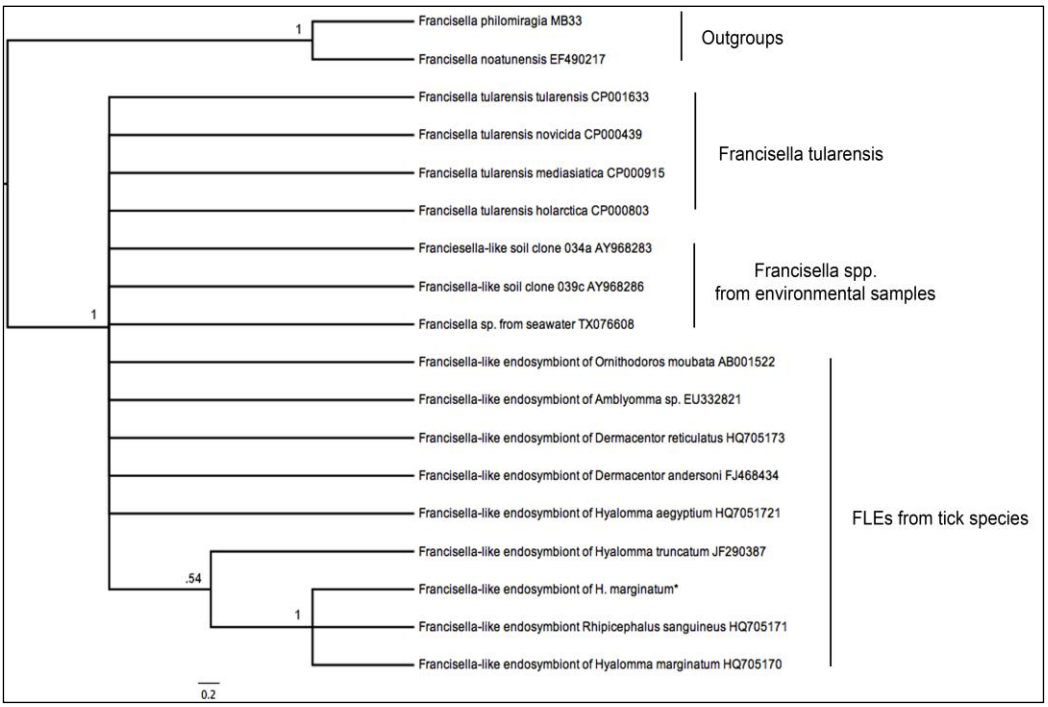


Figure 2. Bayesian consensus tree of *Francisella* spp. 16S rDNA gene. Bayesian trees were calculated according to GTR+I+Gamma model; above each node were reported Bayesian Posterior Probability (BPP) value, branches with BPP values less than 0.5 were collapsed; * identifies the sequence obtained in the present study.

10.5 DISCUSSION

This study is the first detection of bacteria harboured in tick collected from livestock in Socotra Island. Gram-negative bacteria belonging the genus *Francisella* are known to be distributed mainly in Northern Hemisphere (Foley and Nieto, 2010). Within this group there are bacteria of medical and veterinary

importance as the etiologic agent of Tularemia, *F. tularensis* and the FLEs of tick. At present FLEs have been identified in both soft (genus *Ornithodoros*) and hard ticks (*Amblyomma*, *Dermacentor*, *Rhipicephalus*, *Hyalomma*), furthermore their pathogenic rule is unknown even if genes implicated in the pathogenicity of *F. tularensis* have been detected (Machado-Ferreira et al., 2009). As resulted by our analysis, the monophyly of FLEs of ticks is at least doubtful, leading to the hypothesis that *Francisella*-like organisms could be widespread into the host by the mean of horizontal transmission. Our analysis is far from be exhaustive in the understanding of relationships within FLEs but in order to shed light in FLEs relationships a multi locus sequence analysis is required.

Considering the importance to human health of bacteria of the genus *Francisella* our results need to be taken in consideration for the control of new emerging diseases in Socotra Island.

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