‘Candidatus Mycoplasma turicensis’ Infection: Reactivation, Tissue Distribution and Humoral Immune Response

Graduate School for Cellular and Biomedical Sciences
University of Bern
PhD Thesis

Submitted by

Marilisa Novacco
from Italy

Thesis advisor

Prof. Dr. Regina Hofmann-Lehmann
Clinical Laboratory
Vetsuisse Faculty
University of Zurich
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Bern, Dean of the Faculty of Medicine

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## 1. Abbreviations

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<td>Hemoplasma</td>
<td>Hemotropic mycoplasma</td>
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<td>‘<em>Candidatus M. turicensis</em>’</td>
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<tr>
<td>SPF</td>
<td>Specified pathogen free</td>
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<tr>
<td>PCR</td>
<td>Polymerase chain reaction</td>
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<tr>
<td>SEM</td>
<td>Scanning electron microscopy</td>
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<tr>
<td>TEM</td>
<td>Transmission electron microscopy</td>
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<tr>
<td>CR1, CD35</td>
<td>Complement receptor type I</td>
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<tr>
<td>ELISA</td>
<td>Enzyme-linked immunosorbent assay</td>
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<tr>
<td>FISH</td>
<td>Fluorescence in-situ hybridisation</td>
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<tr>
<td>FeLV</td>
<td>Feline Leukemia Virus</td>
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<td>FIV</td>
<td>Feline Immunodeficiency Virus</td>
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2. Abstract

Hemotropic mycoplasmas (aka hemoplasmas) are the causative agents of infectious anemia in numerous mammalian species. ‘Candidatus Mycoplasma turicensis’ (‘Candidatus M. turicensis’) is a hemoplasma species of felids. It has been first isolated in a cat with hemolytic anemia and it was subsequently described worldwide in domestic cats and wild animals. After bacteremia, cats may become chronic carrier of ‘Candidatus M. turicensis’. To date, it is assumed that infected animals do not completely clear the infection and a possible sequestration of feline hemoplasmas in tissues has been suspected. We hypothesized that ‘Candidatus M. turicensis’ chronically infected cats may be able to reactivate the infection under particular conditions, such as immunosuppression, and therefore represent a source of infection for other animals. Thus, the aims of the present study were to (1) assess a potential in vivo reactivation of ‘Candidatus M. turicensis’ bacteremia in chronically infected cats using high doses of methylprednisolone acetate, (2) identify potential sequestration sites prior to and during the attempted reactivation of the infection and (3) monitor the humoral immune response throughout the experiment.

Ten specified pathogen-free cats that had ostensibly recovered from experimental ‘Candidatus M. turicensis’ infection were used: five cats received high doses of methylprednisolone acetate (attempted immunosuppression) in order to assess a potential in vivo reactivation of ‘Candidatus M. turicensis’ (group 1), while five cats served as untreated controls (group 2). Besides weekly blood collection, tissue samples were collected from bone marrow, kidney, liver and salivary glands at selected time points. ‘Candidatus M. turicensis’ blood and tissue loads were monitored by real-time Taqman® PCR and the humoral immune response to ‘Candidatus M. turicensis’ was evaluated by DnaK enzyme-linked immunosorbent assay (ELISA). All cats tested ‘Candidatus M. turicensis’ PCR-negative in blood at the beginning of this study. One week after the administration of methylprednisolone acetate, cats from group 1 showed a significant decrease in lymphocyte counts and DnaK antibodies compared to cats from group 2. Additionally, one blood and three tissue samples from group 1 cats tested PCR-positive. Before the administration of methylprednisolone acetate, only one tissue sample tested PCR-positive. Remarkably, an increase of ‘Candidatus M. turicensis’ tissue loads was detected in the latter sample after the attempted immunosuppression. Four weeks after the methylprednisolone administration, blood and tissue samples of cats from groups 1 and 2 tested PCR-negative and DnaK antibodies returned to approximately pretreatment levels. This is the first study to report the presence of ‘Candidatus M.}
turicensis’ in tissues of chronically infected cats and the persistence of DnaK antibodies in the absence of detectable bacteremia. Methylprednisolone administration did not lead to a significant reactivation of ‘Candidatus M. turicensis’ under the chosen conditions. In conclusion, chronically ‘Candidatus M. turicensis’-infected cats do not appear to be a major source of infection for other cats at this stage of the infection. The results of the present study marked a step toward the understanding of the pathogenesis of ‘Candidatus M. turicensis’ infection and are clinically relevant to the prognosis of hemoplasma-infected cats.
3. Introduction

In the past few decades there has been growing attention on emerging diseases and infections with a zoonotic potential. In the current study, we focus on infectious anemia, which is induced by hemotropic mycoplasmas (also known as hemoplasmas). These bacterial agents are detected in an increasing number of mammalian species, including pet, production animals and wildlife, and they are recognized worldwide. Hemoplasmas have been suspected to be a potential source of infection for humans. Hemoplasmas have been recognized in humans, particularly when immunocompromised or those with close contact to infected animals (Archer et al., 1979; Congbin et al., 2010; dos Santos et al., 2008; Duarte et al., 1992; Kallick et al., 1972; Puntaric et al., 1986; Steer et al., 2011; Sykes et al., 2010; Yang et al., 2000; Yuan et al., 2009). The pathogenic potential of hemoplasmas in humans is still controversial and clinical signs of the infection are not clearly defined.

In this study we aim to better understand mechanisms exploited by hemoplasmas for evading the immune system of the host and establishing a chronic carrier stage. These efforts will hopefully be translated in new strategies to persistently eliminate the infection from the host.
3.1. Hemoplasmas

3.1.1. Reclassification as *Mycoplasma* spp

The species formerly known as *Haemobartonella* and *Eperythrozoon* are composed of small, uncultivable, wall-less bacteria that attach to the surface of erythrocytes of different mammalian species. *Haemobartonella* and *Eperythrozoon* spp were classified as members of the order Rickettsiales based on their biologic and phenotypic characteristics (obligate parasitism, small size, erythrocyte tropism and suspected arthropod transmission) (Kreier and Ristic, 1972, 1981; Ristic and Kreier, 1979). However, their lack of a cell wall, small size and genome, fastidious growth requirement, resistance to penicillin and susceptibility to tetracycline led to growing doubts on this classification and organisms were suspected to be more closely related to members of the class Mollicutes (family *Mycoplasmaceae*) (Tanaka et al., 1965). The phylogenetic analysis of the 16S ribosomal RNA gene of *Haemobartonella* and *Eperythrozoon* spp revealed little similarity with the other members of the Rickettsiaceae but supported a close phylogenetic relation with *Mycoplasma* spp. (Johansson et al., 1999; Neimark and Kocan, 1997; Rikihisa et al., 1997). Consequently, *Haemobartonella* and *Eperythrozoon* spp were reclassified within the genus *Mycoplasma* and a *Candidatus* designation was appended to those taxa that were newly and incompletely described (Murray and Schleifer, 1994; Murray and Stackebrandt, 1995; Neimark et al., 2001, 2002). Within the genus *Mycoplasma*, *Haemobartonella* and *Eperythrozoon* spp are most closely related to the members of the *pneumoniae* group but they represent a new phylogenetic cluster of bacteria defined as hemoplasmas for their peculiar tropism for red blood cells as well as for the relatively low sequence similarity of the 16S rRNA gene when compared to the closest related mucosal mycoplasma species. Subsequently, the rnpB gene was proposed for phylogenetic analysis of hemoplasma and other mycoplasma species (Peters et al., 2008a). This gene is universally present in bacterial species and encodes the RNA subunit of endoribonuclease P (Peters et al., 2008a). The rnpB gene is more suitable for phylogenetic discrimination of closely related taxa when compared with 16S rRNA sequences due to its higher rate of nucleotide variation (Tapp et al., 2003). The rnpB gene analysis confirmed the close phylogenetic relation of *Haemobartonella* and *Eperythrozoon* spp with *Mycoplasma* spp and all hemoplasma species were located within a single clade in both the rnpB and 16S rRNA gene phylogenetic trees.
Both phylogenies showed that hemoplasmas were most closely related to *M. fastidiosum*, followed by other members of the *M. pneumoniae* group (16S rRNA gene phylogeny, Fig. 1A) or *M. iowae* and *M. penetrans* (rnpB phylogeny, Figure Fig. 1B) (Peters et al., 2008a). The position of hemoplasma within the genus *Mycoplasma* remains, however, controversial (Uilenberg et al., 2004, 2006).
Figure 1 A and B: Phylogenetic analysis of the nearly complete 16S rRNA gene (A) and the RNase P RNA gene (rnpB) (B) for sequenced hemplasma isolates and other mycoplasma species. A phylogenetic tree was constructed by the neighbor-joining method. GenBank accession numbers are shown for all sequences (Peters et al., 2008a). Both phylogenetic trees were previously published by Peters and colleagues (Peters et al., 2008a).
3.1.2. **Ultrastructural morphology and mechanism of interaction**

Hemoplasmas are cell wall-less bacteria (rod-shaped, spherical, or ring shaped). They are found individually or in chains across the red blood cell surface (Small and Ristic, 1967) (Fig. 2). Hemoplasmas are enclosed by a single limiting membrane (Simpson et al., 1978). Although they have no nucleus, small granules and a few filamentous structures are found in the cytoplasm (Simpson et al., 1978).

**Figure 2 A to D:** SEM images of erythrocytes infected with ‘*Candidatus M. turicensis*’. Discoid-shaped organisms, about 0.3 mm in diameter, are attached to the surface of RBCs (indicated by an arrow in A). Bars represent 1 mm. Figure from Willi et al. (Willi et al., 2011).
The receptor that is used by hemoplasmas for adhesion on erythrocytes is not yet defined. An overexpression of the complement receptor type I (CR1, CD35) on erythrocytes was described in *Mycoplasma suis* (*M. suis*) infected human patients (Congbin et al., 2010). The CR1 is a multiple-modular protein that plays a role on erythrocyte immunity. The expression levels of CR1 were significantly higher in patients with severe hemoplasma infection compared with healthy patients. In addition, the expression levels of CR1 were lower after antibiotic treatment. The CR1 receptor is also used for adhesion and invasion of erythrocytes by other infectious agents, such as *Plasmodium falciparum*, *Streptococcus pneumonia*, *Porphyromonas gingivalis* (Awandare et al., 2011; Belstrom et al., 2011; Li et al., 2010). CR1 was never tested in samples from hemoplasma-infected animals and its role should be evaluated. The characterization of potential ligands between hemoplasma and erythrocytes would mark an important step toward the understanding of pathogenic mechanisms of hemoplasmas and it may be useful for the development of an effective vaccine.

The blood type of the host appeared to influence the susceptibility to hemoplasma infections. It was reported that two sibling cats of blood type B experimentally infected with ‘*Candidatus M. turicensis*’ showed a different kinetic of the infection compared to the other cats of blood type A (Museux et al., 2009). Indeed, one cat of blood type B developed lower blood loads than blood type A cats and its brother never showed detectable bacteremia, although the cat seroconverted subsequently. The blood type seems to play an important role also for other red blood cell parasites, such as *Plasmodium falciparum* (Fry et al., 2008).

Recently, a remarkable new finding changed our view of hemoplasma as bacteria with only epierythrocytic capabilities. Indeed, a virulent isolate of *M. suis* was reported within the erythrocytes (Groebel et al., 2009). The entry of *M. suis* seemed to begin with invagination of the erythrocyte membrane in an endocytosis-like process (Groebel et al., 2009). The depression in the erythrocyte membrane deepened and conformed to the shape of the bacterium, as shown by scanning and transmission electron microscopy (SEM, TEM) (Groebel et al., 2009). Upon entry, the erythrocyte membrane appeared normal and *M. suis* was found in intracellular vacuoles within the erythrocyte but it was also found floating freely in the cytoplasm of the infected cells (Groebel et al., 2009). A similar mechanism of erythrocyte invasion has been described also for other infections, *Plasmodium falciparum* and *Bartonella bacilliformis* (Benson et al., 1986; Haldar and Mohandas, 2007). Such colonization should protect the bacteria from the host’s immune response and hamper antibiotic treatment. An intracellular life cycle may explain the chronic nature of hemoplasma infections and should be
considered for the development of new strategy for treatment of the infection. Additionally, in the same study, *M. suis* was found in reticulocytes and normoblast (erythrocyte precursor) of infected pigs (Groebel et al., 2009). It was speculated that hemoplasmas infect erythrocyte precursors in the bone marrow. This would imply the possibility that certain hemoplasma isolates can propagate within the bone marrow to the erythrocyte precursors. Among the feline hemoplasma species, DNA of *M. haemofelis* and ‘*Candidatus* M. turicensis’ was detected by PCR in bone marrow of infected cats during the acute phase of the infection (Museux et al., 2009; Novacco et al., 2009; Tasker et al., 2009) but the exact localization of the organisms was not known. Successively, a study revealed no evidence of *M. haemofelis* in the erythrocyte precursors within the bone marrow by a fluorescence in-situ hybridisation assay (FISH) (Peters et al., 2011). However, the detection sensitivity of the assay was low and false negative results may have been detected (Peters et al., 2011).

### 3.1.3. The genomic basis

Hemoplasmas depend on the host cell for essential compounds they cannot produce. The genomic sizes of *M. suis* and *M. haemofelis* are comparable with the small size of the other members of the genus *Mycoplasma*. The cell wall and many biosynthetic systems of mycoplasmas have been probably lost during the process of reductive evolution from a branch of gram-negative walled bacteria (Woese, 1987). Mycoplasmas are thought to have retained only those genes that are essential for life.

Recently, an important step toward the understanding of the genomic basis of hemoplasmas was achieved. The whole genome sequence of *M. haemofelis* and *M. suis* were obtained (Barker et al., 2011; Guimaraes et al., 2011; Oehlerking et al., 2011; Santos et al., 2011). The genome of *M. haemofelis* consists of a single circular chromosome and the average gene length (1,147,259-1,152,484 bp) is shorter than that of other mycoplasmas (Barker et al., 2011; Santos et al., 2011). The genome of *M. haemofelis* encodes a large number of unique proteins. Probably this different set of genes is required to adapt to the blood environment (Santos et al., 2011). The analyses of the whole genome sequencing of *M. haemofelis* revealed that the metabolic pathways are reduced, requiring a supply of many nutrients and metabolites from the host for survival (Santos et al., 2011). Genes involved in carbohydrate metabolism were limited to enzymes of the glycolytic pathway, with glucose appearing to be the sole energy source (Barker et al., 2011). The pentose phosphate pathway genes appear to be incomplete or absent, suggesting alternative mechanisms as source of purine and pyrimidine bases (Santos et al.,
The latter may be obtained as scavenging from the host. Interestingly, two coding sequences of primary virulence genes were identified: endopeptidase o-sialoglycoprotein and superoxide dismutase (SOD) (Santos et al., 2011). The gene encoding for endopeptidase o-sialoglycoprotein has been found also in the *M. suis* genome (Guimaraes et al., 2011). This enzyme might be directly involved in erythrocyte lysis by cleavage of glycoproteins such as glycophorin A, which is an abundant component of the erythrocyte membrane (Santos et al., 2011). The superoxide dismutase is absent in the genome of *M. suis* and it is thought to detoxify reactive oxygen species, protecting *M. haemofelis* from oxidant damage (Santos et al., 2011). Additionally, it was shown that the core proteome of *M. haemofelis* contains an abundance of paralogous gene families (Santos et al., 2011). Paralogous repeats are strategically located in the genome of *M. haemofelis* and they might encode for putative surface expressed proteins of approximately 200 amino acids (Santos et al., 2011). It was speculated that these genes might code for phase variation in the surface protein expression of *M. haemofelis* (Santos et al., 2011). This pool of genes may represent a source of different antigenic epitopes that can be changed in order to evade the immune system and allow the establishment of a chronic carrier state (Santos et al., 2011).

The genome of *M. suis* (709,270-742,431 bp) consists of a single circular chromosome and includes 32 tRNAs and a single-copy 16S rRNA (Guimaraes et al., 2011; Oehlerking et al., 2011). It differs from other mycoplasma genomes in the high portion of predicted proteins without functional assignment (Oehlerking et al., 2011). *M. suis* generates ATP through glycolysis (Guimaraes et al., 2011; Oehlerking et al., 2011). The pentose-phosphate pathway, metabolism of co-factors and vitamins, pyruvate dehydrogenase and NAD+ kinase are missing (Guimaraes et al., 2011; Oehlerking et al., 2011). *M. suis* showed the presence of a large paralogous families, as was reported for *M. haemofelis* (Guimaraes et al., 2011). This pool of genes may be important for immune system evasion and antigenic variation (Guimaraes et al., 2011).

Overall, the genome sequence studies were able to predict metabolic pathways of hemoplasmas but gaps remain to be filled and enzyme activities to be experimentally characterized.
3.1.4. **Pathogenesis**

Hemoplasmas cause acute hemolytic anemia, either directly or by immune mediated destruction of red blood cells. The attachment of hemoplasma to the erythrocyte membrane leads to indentations and small depressions on the surface of the erythrocytes (Jain and Keeton, 1973). Contrast-free circular regions (around 0.3 µm in diameter) similar to holes were reported by SEM on the surface of the erythrocytes during ‘*Candidatus* M. haemominutum’ and ‘*Candidatus* M. turicensis’ infections (Willi et al., 2011). The attachment of the organisms to the membrane of the erythrocytes may cause a direct damage and this, in turn, may be responsible for the increased osmotic fragility of the erythrocytes and subsequent hemolysis observed during infection (Maede, 1975; Maede and Hata, 1975; Willi et al., 2005). The direct interaction between hemoplasmas and red blood cells include also the invasion of the erythrocytes by the bacteria, as it was observed for *M. suis*. The intracellular invasion of the virulent *M. suis* isolate within the erythrocytes resulted in cell damage and subsequent lysis of the cell (Groebel et al., 2009; Hoelzle et al., 2007b).

Alternatively, immunological mechanisms are suggested to be involved in the destruction of the erythrocytes. The up-regulation of autoreactive antibodies was reported during the acute hemolytic anemia in hemoplasma infected animals (Maede and Hata, 1975; Tasker et al., 2010; Willi et al., 2005). Autoreactive antibodies may target either the host actin or unspecified glycoproteins on the red blood cell surface (Felder et al., 2010). Alternatively, antibodies may be directed against hemoplasmas, which are attached to the surface of the erythrocytes. Removal of red blood cells had been observed by phagocytosis (Felder et al., 2010).

Recently, a new mechanism was proposed for the development of hemolytic anemia: hemoplasmas may trigger the programmed cell death of the erythrocyte (eryptosis) (Felder et al., 2011). Eryptosis is characterized by cell shrinkage, microvesiculation and phosphatidylserine exposure on the outer membrane of the erythrocytes. The phenomenon was observed in pigs experimentally infected using different *M. suis* isolates. All isolates induced eryptosis but with different characteristics. It is not yet clear which mechanisms are responsible for eryptosis. It was shown that soluble mycoplasma substances or stress signals were present in the blood of diseased animals (Felder et al., 2011). Alternatively, hemoplasma might act as competitor for nutrient shortening the life span of the erythrocyte. The depletion of energy and oxidative stress may be a trigger to eryptosis.
3.2.  Feline hemoplasmas

3.2.1. Introduction

Feline hemoplasmas are the causative agent of infectious anemia in felids. They were first identified in 1942 (Clark, 1942). Later on, feline hemoplasma were identified all over the word and recognized as *Haemobartonella felis* (Flint and Moss, 1953; Flint et al., 1958; Harbutt, 1969; Small and Ristic, 1971). In 2001, *Haemobartonella felis* was reclassified within the genus *Mycoplasma* and renamed as *Mycoplasma haemofelis* (Neimark et al., 2001). Currently, three hemoplasma species have been recognized in cats: *Mycoplasma haemofelis* (*M. haemofelis*), ‘*Candidatus* Mycoplasma haemominutum’ (‘*Candidatus* M. haemominutum’) and ‘*Candidatus* Mycoplasma turicensis’ (‘*Candidatus* M. turicensis’) (Foley et al., 1998; Foley and Pedersen, 2001; Willi et al., 2005). Feline hemoplasmas are found worldwide, although prevalence varies geographically (Willi et al., 2010). Hemoplasma infection has been reported also in nine different captive and free ranging wild felid species originating from Europe, Africa and South America (Willi et al., 2007c). The pathogenic potential of feline hemoplasma in these species is not completely understood (Willi et al., 2007c). Wild felids may have a role as reservoirs and asymptomatic carriers of hemoplasma infections. This possibility is not yet clarified.

3.2.2. Pathogenicity and risk factors

The hemoplasma species that are currently recognized vary in their pathogenicity, with some isolates inducing hemolytic anemia whereas others result in few clinical signs. *M. haemofelis* is the most pathogenic of the three feline hemoplasma species and experimental infection often results in hemolytic anemia. However, some prevalence studies failed to demonstrate an association between *M. haemofelis* infection and anemia in naturally infected cats, and blood loads were not always inversely correlated with packed cell volume (PCV) values (Tasker et al., 2003a; Tasker et al., 2004a; Willi et al., 2006a). It was reported that a cat with high *M. haemofelis* blood loads (> 10^6 copies/ml of blood) and transient Feline Leukemia Virus (FeLV) antigenemia remained clinically healthy throughout a 124 days follow-up period (Willi et al., 2006a). The lack of clinical signs even in the presence of high *M. haemofelis* blood loads could be due to a less virulent *M. haemofelis* isolate. Alternatively, a different susceptibility of the cat to hemoplasma infections may have played a role.
Prevalence studies revealed that ‘Candidatus M. haemominutum’ is the most prevalent hemoplasma species. The pathogenic potential of ‘Candidatus M. haemominutum’ is still controversial. Generally, naturally infected cats did not show an association between anemia and ‘Candidatus M. haemominutum’ blood loads but exceptions are also reported (Hornok et al., 2008; Reynolds and Lappin, 2007). The pathogenic potential of ‘Candidatus M. haemominutum’ seems to depend on co-factors, such as immunosuppression or pre-existing retroviral infections. Indeed, it was shown that ‘Candidatus M. haemominutum’ resulted in severe anemia in cats co-infected with FeLV (George et al., 2002). Interestingly, it was argued that ‘Candidatus M. haemominutum’ may play a role in inducing bone marrow disorders (George et al., 2002) and in reducing the regenerative potential of the bone marrow (Sykes et al., 2007). It was suspected that ‘Candidatus M. haemominutum’ may replicate on bone marrow cells, decreasing the regenerative response to the infection.

‘Candidatus M. turicensis’ induced mild to marked anemia in experimentally infected cats depending on the inoculation route (Museux et al., 2009; Willi et al., 2005). The pathogenic potential of ‘Candidatus M. turicensis’ seems to depend on co-factors, such as immunosuppression or co-infection with other hemoplasmas. Indeed, it was reported that a remarkable number of ‘Candidatus M. turicensis’ naturally infected cats had a concurrent disease, such as neoplasia or Feline Immunodeficiency Virus (FIV) infection (Willi et al., 2006a).

Overall, the pathogenic potential of feline hemoplasmas is difficult to assess. It may vary greatly depending on the hemoplasma isolate, route of the infection, cat signlament and history. Concurrent infections probably play an important role on the outcome of hemoplasma infections. Additionally, some risk factors related to the host have to be considered. Old male non-pedigree cats are believed to be at increased risk of hemoplasma infection, although young cats are possibly more likely to show clinical signs of the infection. Cat bite abscess, retroviral infection and outdoor access have been also reported as risk factors in cats (Grindem et al., 1990; Luria et al., 2004; Tasker et al., 2003a; Willi et al., 2006a). The association of feline hemoplasma with male gender, abscess and outdoor access seem to suggest a possible transmission of the infection either by aggressive contact or by exposure to blood-sucking arthropods. The influence of many of these factors has not yet been completely clarified. For this reason generalized predictor of hemoplasma infection outcome cannot be foreseen.
3.2.3. **Stages of the infection**

A clear definition of the acute and chronic phase of hemoplasma infections is missing. Commonly, the acute phase of infection is identified with the appearance of bacteremia and consequent clinical signs. Clinical signs of hemoplasma infections are pallor, lethargy, anorexia, weight loss, depression, dehydration and pyrexia. However, this definition of the acute phase is problematic considering the heterogeneity of the outcome of hemoplasma infections. The acute infection is not always associated with clinical signs of the infection, particularly in ‘*Candidatus M. haemominutum*’ and ‘*Candidatus M. turicensis*’ infection. For this reason, we used to define the acute phase of ‘*Candidatus M. turicensis*’ infection as period of time when the bacteremia takes place. Infections with *M. haemofelis* more often result in a severe hemolytic anemia. Moreover, episodes of *M. haemofelis* blood load fluctuations have been observed. After primary infection, cats may become clinically healthy carriers of hemoplasmas. Carriers may represent a source of infection particularly upon reactivation of the infection. The investigation of the chronic phase of hemoplasma infection was the subject of the present thesis.

3.2.4. **Blood load variations**

During experimental *M. haemofelis* infections rapid and simultaneous disappearance of the organisms from the peripheral blood had been demonstrated. *M. haemofelis* causes an acute hemolytic anemia in infected cats and reaches the peak load (10^8 - 10^9 copies/ml of blood) 14 - 15 days post infection with marked fluctuation in copy numbers over time (Tasker et al., 2006; Tasker et al., 2003b; Willi et al., 2006a). Fluctuation in blood loads can be as large as a 4 log difference over 2 or 3 days, or a 7 log difference over 12 days (Tasker et al., 2006; Tasker et al., 2003b; Willi et al., 2006a). The duration of a cycle is typically between a few days and a few weeks. Different mechanisms were proposed to explain the changes from a high level of bacteremia to undetectable organism loads reported for *M. haemofelis*. Messick and colleagues proposed that an antigenic variation may conceal behind the cyclic bacteremic episodes of *M. haemofelis* (Messick, 2004). The host immune system may clear some of the organisms from the blood (nadir of the cycle) and *M. haemofelis* may change certain immunodominant antigens, not yet identified by the immune system, and be able to restart the replication in peripheral blood. The antigenic variation is a mechanism that other mycoplasma species use for escaping the immune recognition and developing a chronic carrier state (Razin et al., 1998). It was indeed shown that mycoplasmas possess a surface architecture that is antigenically and functionally extremely versatile.
This remarkable antigenic variability was documented for *Mycoplasma gallisepticum, Mycoplasma bovis, Mycoplasma hyorhinis* and *Mycoplasma pulmonis* (Razin et al., 1998). This theory is supported by the finding of paralogs as well as tandem repeats in the genome of *M. haemofelis* (Santos et al., 2011). This pool of genes is thought to be critical for antigenic variation (Santos et al., 2011).

Alternatively, a tissue sequestration may explain the copy number variation seen during *M. haemofelis* infection. Many years ago a temporary tissue sequestration had been demonstrated in cats acutely infected with *M. haemofelis* (Maede, 1979). More recently, the same hypothesis was tested by Tasker and colleagues (Tasker et al., 2009). Tissue samples from *M. haemofelis*-infected cats were collected at different time points of the infection and the ratio between hemoplasma blood and tissue loads was calculated. Tissue collected during the nadir of *M. haemofelis* copy number cycling contained no detectable *M. haemofelis* copies or only very low copies (Tasker et al., 2009). For this reason, the authors conclude that no significant *M. haemofelis* sequestration was found and they hypothesized that a better explanation for the copy number cycling may be related to the impressive capability of *M. haemofelis* to rapidly replicate in blood of infected cats (Tasker et al., 2009). In the same study, it was shown that PCR-negative samples, if tested in replicates, actually contained very low *M. haemofelis* copy numbers. This observation points out that *M. haemofelis* may be present in the blood although the cat is considered negative (Tasker et al., 2009).

Recently, a possible tissue sequestration was reported in ‘*Candidatus* M. turicensis’ chronically infected cats (Novacco et al., 2011). DNA of ‘*Candidatus* M. turicensis’ was detected in tissue samples more than one year after exposure. Results of the study are extensively discussed in section 3.6.

Fascinating hypotheses are possible concerning the reason of the cycling variation of hemoplasmas but enormous difficulties in their proof are present. Moreover, it is important to mention that not all the *M. haemofelis* isolate showed the same mechanism of copy number variation. And also using the same *M. haemofelis* isolate is possible to have differences in blood fluctuation with some animal showing high variation of *M. haemofelis* copy numbers and others not. The lack of an in vitro culture method hampers the possibility to obtain a standard inoculum to use in experimental studies. Moreover, the characteristic of the cats (age, sex, blood type) as well as genetic factors of each cat may influence the outcome of the infection.
3.2.5. Prevalences

Currently, the prevalence of hemoplasma infections is mostly based on PCR investigations. Feline hemoplasmas have been shown to exhibit worldwide geographical distribution and isolates from three different continents have shown near sequence identities (Clark et al., 2002; Tasker et al., 2003c; Watanabe et al., 2003; Willi et al., 2006b). Prevalence of feline hemoplasmas in Europe are shown in Table 1. Geographic variations in the prevalence of hemoplasma infections are reported. This phenomenon is thought to be related to the presence of vectors of the infection. Different climate conditions may influence the permanent presence of distinct blood sucking arthropods species. For instance, it was reported that hemoplasma infections were more prevalent in the west and in the south part of Switzerland compared to the rest of the country where the mean annual temperature are lower and less arthropod species are present (Willi et al., 2006a). The same phenomenon was also reported for canine hemoplasma, where a high prevalence was detected in countries with Mediterranean climate, where the tick *Rhipicephalus sanguineus* is highly prevalent (Novacco et al., 2009). In future it would be interesting to assess the sero-prevalence of hemoplasmas in order to understand the number of animals that were exposed to the infections. Probably, a larger prevalence would be reported in comparison to the PCR results, as it was described in a cat population from UK (Barker et al., 2010).
**Table 1:** Percentage of PCR-positive cats for feline hemoplasma in Europe.

<table>
<thead>
<tr>
<th>Feline hemoplasmas</th>
<th>Switzerland</th>
<th>Germany</th>
<th>UK</th>
<th>Spain</th>
<th>Italy</th>
</tr>
</thead>
<tbody>
<tr>
<td>N° of cats</td>
<td>615</td>
<td>397</td>
<td>2011</td>
<td>191</td>
<td>307</td>
</tr>
<tr>
<td><em>M. haemofelis</em></td>
<td>0.5%</td>
<td>1.4-7.4%</td>
<td>1.6-2.8%</td>
<td>2.1%</td>
<td>5.9%</td>
</tr>
<tr>
<td>‘Candidatus M. haemominutum’*</td>
<td>8.5%</td>
<td>8.9-23.3%</td>
<td>11.2-17.1%</td>
<td>7.9%</td>
<td>17.3%</td>
</tr>
<tr>
<td>‘Candidatus M. turicensis’*</td>
<td>1%</td>
<td>0.3-2.2%</td>
<td>1.7-2.3%</td>
<td>nd</td>
<td>1.3%</td>
</tr>
<tr>
<td>Co-infection</td>
<td>1%</td>
<td>0.8-3%</td>
<td>1.6-1.9%</td>
<td>2.1%</td>
<td>5.5%</td>
</tr>
</tbody>
</table>

Reference (Willi et al., 2006a) (Bauer et al., 2008; Just and Pfister, 2007; Laberke et al., 2010) (Peters et al., 2008b; Tasker et al., 2003a; Willi et al., 2006b) (Roura et al., 2010) (Gentilini et al., 2009)

nd= not detected. *Including co-infected animals
3.2.6. **Immunological protection**

Co-infections with different feline hemoplasma species are often reported in prevalence studies (Tasker et al., 2003a; Willi et al., 2006a; Willi et al., 2010). The presence of co-infection may suggest the lack of an immunological cross-protection between different hemoplasma species. To rule out this possibility, cats chronically infected with *Candidatus* *M. turicensis* were exposed to *M. haemofelis*. The chronically infected cats were not protected from the challenge and a boost effect in *M. haemofelis* DnaK antibodies was noted (Bauman et al., 2011).

An immunological protection within the same hemoplasma species has been recently observed (Novacco et al., in preparation). *Candidatus* *M. turicensis* chronically infected cats appeared to be protected from a second challenge (Novacco et al., in preparation). No boost effect in *M. haemofelis* DnaK antibodies was noted and one cat that became ELISA-negative was anyway protected from the repeated challenge, suggesting that antibodies against DnaK play only a marginal role in the protection. Mechanisms involved in the immunological protection are not yet clarified.

3.2.7. **Transmission**

The natural route of transmission of feline hemoplasma infections has not been confirmed; however, blood sucking arthropods are likely to play a role. Indeed, DNA of feline hemoplasmas has been found in fleas collected from cats (Barrs et al., 2010; Shaw et al., 2004; Willi et al., 2007a) and a transient transmission of *M. haemofelis* via the hematophagous activity of the cat flea *Ctenocephalides felis* was demonstrated (Woods et al., 2005).

DNA of *Candidatus* *M. haemominutum* and *Candidatus* *M. turicensis* were isolated in saliva and salivary glands of infected cats (Dean et al., 2008; Willi et al., 2007a). The presence of *Candidatus* *M. turicensis* in saliva of infected cats suggested the possibility of having a direct transmission of *Candidatus* *M. turicensis* between cats. To investigate this possibility, an experimental transmission study was performed. Cats had been exposed sequentially to *Candidatus* *M. turicensis* PCR-positive saliva and blood either oronasally or subcutaneously (Museux et al., 2009). None of the cats exposed to saliva developed bacteremia (Museux et al., 2009); however, some cats seroconverted as shown by Western Blot (Museux et al., 2009) and further confirmed by ELISA (Novacco et al., submitted). In contrast, the subcutaneous inoculation of a very little amount of infectious blood (10 µl of blood with 1 x 10³ copies of *Candidatus* *M. turicensis*) resulted in bacteremia. This indicated that social contact,
such as the sharing of food dishes or grooming, with ‘Candidatus M. turicensis’-infected cats is unlikely to pose a risk of infection for non-infected animals. However, aggressive interaction with exchanging of blood or transmission by arthropod vectors may play a role in the transmission. The mechanism by which ‘Candidatus M. turicensis’ arrives to the blood through the skin is unknown. However, it can be speculated that ‘Candidatus M. turicensis’ is drained by the lymphatic vessels from the skin to the lymph nodes and from there it may reach the thoracic duct and return into the blood stream by the superior vena cava. Only experimental transmission studies evaluating tissue sequestration of ‘Candidatus M. turicensis’ at different time points after inoculation may validate this hypothesis. The subcutaneous route may simulate the natural route of transmission between cats and be helpful for the comparison to natural infections. Indeed, less severe clinical signs are often reported in hemoplasma naturally infected cats compared to experimentally infected cats. This difference may be related to the inoculation route and the dose of hemoplasma infection used in experimental studies. This hypothesis was proven for ‘Candidatus M. turicensis’: cats subcutaneously infected developed bacteremia later than cats inoculated intravenously or intraperitoneally and the bacterial loads were lower (Museux et al., 2009; Willi et al., 2005). Because hemoplasma are not cultivable in vitro and our knowledge on these organisms is mainly based on in vivo experimental transmission studies, it is important to be aware of these differences and take them in account, particularly when comparing natural and experimental infections.

The vertical transmission of hemoplasmas from the queen to kittens during pregnancy (transplacental passage), at birth or via lactation has been supposed to occur (Harvey and Gaskin, 1977). Early studies reported hemoplasma infections in young kittens born from chronically infected queens (Fischer et al., 1983; Gretillat, 1984). Further studies in this field using specific PCR are required to prove this option. Blood transfusions have been reported as a source of *M. haemofelis* and ‘Candidatus M. haemominutum’ infections (Gary et al., 2006; Willi et al., 2006a). The risk of transmission depends on the viability of hemoplasmas in stored blood. The viability of *M. haemofelis* in blood collected in heparin or EDTA anticoagulants is very short (less than one hour), as inoculation with blood stored for longer periods have failed to produce bacteremia (Tasker, 2010). The viability of ‘Candidatus M. haemominutum’ in EDTA blood appeared to be different compared to *M. haemofelis*. Indeed, Geret and colleagues reported a successful transmission of ‘Candidatus M. haemominutum’ after 24 hours storage of the infectious blood at room temperature (Geret et al., 2011).
Because of the close phylogenetic relationship of feline, canine and rodent hemoplasmas, a cross-species transmission has been suspected (Willi et al., 2007a). It was shown that the inoculation of feline hemoplasmas into splenectomized dogs did not result in detectable bacteremia, and cats inoculated with blood from hemoplasma infected dogs remained asymptomatic (Lumb, 2001). However, dogs re-inoculated with the infectious blood from the latter cats developed bacteremia, suggesting that cats might act as carriers of canine hemoplasmas (Lumb, 2001). A potential interspecies transmission of ‘Candidatus M. turicensis’ from rodents to cats was also suspected (Willi et al., 2007a). For this reason, a large number of free-living Swiss rodents was tested for the presence of feline hemoplasmas. All samples tested PCR-negative for feline hemoplasmas, although the rodent hemoplasma Mycoplasma coccoides was detected (Willi et al., 2007a). The results obtained from this study seem to indicate that an interspecies transmission of ‘Candidatus M. turicensis’ between rodents and cats is unlikely to occur.

3.2.8. Diagnosis

Cytological examination: Formerly the diagnosis of hemoplasma infection was based on cytological examination of Romanosky-stained blood smears (Bobade and Nash, 1987). The organisms stain blue to purple with Weight-Giemsa stain (Messick, 2004). Acridine orange staining may also reveal the presence of these organisms (Bobade and Nash, 1987). Hemoplasmas appear as rounded bodies singly, in pairs, or occasionally in chains on the surface of erythrocytes as shown in Fig. 3. Diff-Quick or filtered Giemsa stains can be used. A diagnostic sensitivity of less than 20% has been reported for cytological examination (Tasker et al., 2003a) and the diagnostic specificity is often hampered by confusing the organisms with stain precipitates, Howell-Jolly bodies or artifacts (Tasker and Lappin, 2002). In addition, cytology cannot differentiate between hemoplasma species. For all these reasons, blood smear examination alone is not a reliable method to diagnose hemoplasma infections.

Polymerase chain reaction: Nowadays, PCR is thought to be the gold standard for the diagnosis of hemoplasma infection. Most feline hemoplasma PCR assays are based on the amplification of segments of the hemoplasma 16S rRNA gene. Conventional PCR assays detect and distinguish both M. haemofelis and ‘Candidatus M. haemominutum’, but not ‘Candidatus M. turicensis’ (Berent et al., 1998; Criado-Fornelio et al., 2003; Jensen et al., 2001). Real-time quantitative PCR assays have a higher specificity and can differentiate the three feline hemoplasma species. In addition, real-time PCR allow quantification of bacteria copy numbers. This is an important feature when considering the infection
status or monitoring the response to the treatment. Usually, peripheral blood of the cat is used for the
diagnosis of hemoplasma infections. Real-time PCR assays were also used to quantify bacterial loads in
tissues samples (Novacco et al., 2011; Tasker et al., 2009). Because many cats have been reported to be
infected with feline hemoplasmas but lack any clinical signs of hemoplasmosis, a positive PCR result
should be always interpreted together with clinical and laboratory findings (Willi et al., 2007b).
Serology: To date, no routine serological assay for the diagnosis of feline hemoplasma infections is
available. Experimental serological assays have been described using hemoplasma antigen either on
blood smears or purified from large volumes of blood from infected cats (Alleman et al., 1999; Foley et
al., 1998; Peters et al., 2010). These methods are not a convenient source of antigens, and preparations
of whole-cell or membrane antigens are difficult to standardize. The first recombinant hemoplasma
antigen, *M. suis* HspA1, was produced in 2007 and used to analyze samples collected during an
experimental *M. suis* study (Hoelzle et al., 2007a). The antigen belongs to the heat shock protein 70
(HSP70) family and was found to be DnaK-like (Hoelzle et al., 2007a). Subsequently, a truncated *M.
haemofelis* DnaK form was produced for feline hemoplasma (Museux et al., 2009). This agent was
applied in Western Blot analyses for the detection of the humoral immune response in ‘*Candidatus M.
turicensis*’-infected cats under experimental conditions (Museux et al., 2009). Recently, the complete
DnaK gene of *M. haemofelis* was identified by our group (Wolf-Jackel et al., 2010). The protein was
recombinantly produced and applied in an enzyme-linked immunosorbent assay (ELISA) (Wolf-Jackel
et al., 2010). This assay is able not only to detect but also to quantify the humoral immune response to
all three feline hemoplasma species (Novacco et al., 2011; Novacco et al., submitted; Wolf-Jackel et al.,
2010). The DnaK antigen was recognized and recombinantly produced also by another research group
(Barker et al., 2010). The cross-reactivity of the *M. haemofelis* DnaK antigens is not yet established. The
developed ELISAs may be affected by problems of specificity and they should be used only under
experimental conditions.
Different antigens were described for *M. haemofelis* (Alleman et al., 1999; Foley et al., 1998; Peters et
al., 2010). A combination of more antigens may help to better understand the kinetic of the humoral
immune response during hemoplasma infections. Overall, the combination of the ELISA and PCR is
desirable for the complete diagnostic profiling of hemoplasma infections.
3.2.9. Antibiotic treatment

Different antibiotic protocols are suggested for the treatment of hemoplasma infection (Table 2). Tetracycline and quinolones are effective in reducing hemoplasma blood loads, although elimination of the infection has been never achieved (Dowers et al., 2009; Ishak et al., 2008; Tasker et al., 2004b). The antibiotic treatment is often used for solving the hematological abnormalities related to hemoplasma infection and the clinical signs of infected animals improve after treatments. No significant differences in the efficacy were noted among tetracycline and fluoroquinolones treatments and the best treatment regime is not yet validated. The response to an antibiotic treatment may vary greatly among the different hemoplasma species and in relation to the different isolates considered. In addition, the route of
administration of antibiotics may play a role in the efficacy of the treatment. Currently, all the antibiotics are suggested to be administered orally (Table 2). This may affect their efficacy, although a clinical response to antibiotics had been proven. Different administration routes should be investigated in the future. Moreover, antibiotics were administered in monotherapy protocols and this may have been inadequate. Probably, the choice of a combination of antibiotics may be recommended in clinically refractory cases. Interestingly, in the genome of *M. haemofelis* one gene was identified related to antimicrobial resistance, a ribosomal RNA adenine dimethylase family protein (MHF_1613) (Santos et al., 2011). The product of this gene is a methylase, responsible for modification of the 16S rRNA. Lack of methylation by this enzyme may modify the ribosomal binding site for the aminoglycoside, leading to resistance (Santos et al., 2011). Whether or not this enzyme plays a role in antimicrobial resistance for *M. haemofelis* has to be experimentally confirmed.

Table 2: Different protocols of antibiotics for feline hemoplasma infections

<table>
<thead>
<tr>
<th>Antibiotics</th>
<th>Dosage (mg/Kg)</th>
<th>Administration</th>
<th>Route</th>
<th>Time (days)</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td>Oxytetracycline</td>
<td>22</td>
<td>q8h</td>
<td>PO</td>
<td>21</td>
<td>(Tasker and Lappin, 2002)</td>
</tr>
<tr>
<td>Doxycycline</td>
<td>5-10</td>
<td>q12-24h</td>
<td>PO</td>
<td>21</td>
<td>(Braddock et al., 2004; Tasker et al., 2004b)</td>
</tr>
<tr>
<td>Enrofloxacin</td>
<td>2.5-10</td>
<td>q24h</td>
<td>PO</td>
<td>12-14</td>
<td>(Dowers et al., 2002; Tasker et al., 2004b)</td>
</tr>
<tr>
<td>Marbofloxacin</td>
<td>2.75</td>
<td>q24h</td>
<td>PO</td>
<td>14</td>
<td>(Ishak et al., 2008)</td>
</tr>
<tr>
<td>Pradofloxacin</td>
<td>5-10</td>
<td>q24h</td>
<td>PO</td>
<td>14</td>
<td>(Dowers et al., 2009)</td>
</tr>
</tbody>
</table>

PO= per os (oral administration).
The response to the antibiotic treatment should be measured by quantitative PCR. Infected cats may turn PCR-positive after the end of the antibiotic treatment and monitoring of the hemoplasma blood loads may be useful during the follow-up. If the therapy is not considered optimally effective, it is suggested to continue the administration of the antibiotic up to 8 weeks (Tasker, 2010). The proof of a PCR-negative result for hemoplasma at the end of the treatment would be desirable. PCR-positive cats may be a source of infection for other cats. Recently, our group has highlighted the possibility of using an ELISA assay for monitoring the effectiveness of the antibiotic treatment (Novacco et al., submitted). The ELISA was used in combination to the PCR for evaluating the kinetics of the humoral response together with the presence of the organism in blood. It was shown that doxycycline treatment resulted in a decrease of ‘Candidatus M. turicensis’ blood load and antibody levels in experimental ‘Candidatus M. turicensis’-infected animals (Novacco et al., submitted). However, the complete clearance of the infection after antibiotic treatment has not been proven in this study. Overall, the ELISA assay would be a less expensive alternative for monitoring the efficacy of the treatment over time for veterinarian clinicians than PCR.
3.3. ‘Candidatus Mycoplasma turicensis’

‘Candidatus M. turicensis’ is a hemoplama species that was described worldwide in wild and domestic felids (Willi et al., 2007c). It has been isolated for the first time in a Swiss cat with hemolytic anemia (Willi et al., 2005). Phylogenetic analyses on the 16S rRNA gene showed that ‘Candidatus M. turicensis’ is closely related to the two rodent hemotropic mycoplasma species, Mycoplasma coccoides and Mycoplasma haemomuris (Willi et al., 2005).

The kinetic of the acute phase of ‘Candidatus M. turicensis’ infection was monitored by real-time PCR (Museux et al., 2009; Willi et al., 2005). Experimental transmission studies showed that cats developed bacteremia within 14 - 45 days post infection with a peak load ranging from \(10^3 - 10^5\) copies/mL and the cats stayed PCR-positive for 10 - 21 weeks after ‘Candidatus M. turicensis’ inoculation (Museux et al., 2009). No fluctuation of blood loads were detected during the early phase of the infection (Museux et al., 2009). In immunocompetent cats, clinical signs of the infection are limited (Museux et al., 2009; Willi et al., 2005). The peak bacteremia is accompanied with an increase in the osmotic fragility of red blood cells and a decrease of the PCV (Museux et al., 2009; Willi et al., 2005). The decrease of the PCV is usually proportional to the bacterial blood loads developed during the infection. Differently, the pre-administration of methylprednisolone acetate in “amplificatory” cats led to clinical signs of hemolytic anemia and a drop in packed cell volume (Museux et al., 2009; Willi et al., 2005). The cats used to amplify ‘Candidatus M. turicensis’ reached peak loads of \(10^6 - 10^7\) copies/mL of blood, 4 - 11 days after intraperitoneal administration (Museux et al., 2009; Willi et al., 2005). The cats became anemic and hemoplasma loads were inversely correlated with the PCV value (Museux et al., 2009). Mild clinical signs of pallor and lethargy were observed (Willi et al., 2005).

The humoral immune response to the acute phase of experimental ‘Candidatus M. turicensis’ infection was monitored by Western Blot (Museux et al., 2009). The serological assay was based on a truncated \(M. haemofelis\) DnaK form (see also section 3.2.8). Experimentally infected cats seroconverted 3 to 4 weeks after ‘Candidatus M. turicensis’ exposure. Cats remained seropositive until the end of the study. Interestingly, cats treated with antibiotics become seronegative after the treatment. The intensity of the Western Blot band appeared to decrease and/or vanish over time (Museux et al., 2009).

At the beginning of this study little was known about the chronic phase of ‘Candidatus M. turicensis’ infection. Important questions on possible tissue sequestration of the organisms and their subsequent reactivation and release in peripheral blood were not answered. In the meantime, the complete DnaK
gene of *M. haemofelis* was identified and a new serological assay for the detection and quantification of the humoral immune response to hemoplasma infections was developed by our group (Wolf-Jackel et al., 2010). The application of the ELISA for monitoring the humoral immune response in cats chronically infected with *Candidatus M. turicensis* was demanded.

4. **Aims of the study**

The aims of the present study were to:

1. Assess a potential *in vivo* reactivation of *Candidatus M. turicensis* bacteremia in chronically infected cats using high doses of methylprednisolone acetate;
2. Identify potential sequestration sites prior to and during the attempted reactivation of *Candidatus M. turicensis*;
3. Apply a serological assay for *Candidatus M. turicensis* to monitor the humoral immune response during chronic stage and reactivation.
5. Accepted publication

Chronic “Candidatus Mycoplasma turicensis” infection

Marilisa Novacco1*, Felicitas S Boretti2, Godelind A Wolf-Jäckel1, Barbara Riond1, Marina L Meli1, Barbara Willi2, Hans Lutz1 and Regina Hofmann-Lehmann1

1Clinical Laboratory, Vetsuisse Faculty, University of Zurich, Winterthurerstrasse 260, 8057 Zurich, Switzerland
2Clinical for Small Animal Internal Medicine, University of Zurich, Winterthurerstrasse 260, 8057 Zurich, Switzerland

*Corresponding author: Marilisa Novacco
Clinical Laboratory
Vetsuisse Faculty, University of Zurich
Winterthurerstrasse 260
8057 Zurich, Switzerland
mnovacco@vetclinics.uzh.ch
6. Discussion and perspective

Tissue sequestration of hemoplasmas has been proposed as mechanism to explain the copy number variation seen during *M. haemofelis* acute infection. However, tissue collected during the nadir of *M. haemofelis* copy number cycling contained no detectable *M. haemofelis* copies or only very low copies (Tasker et al., 2009). Moreover, *M. haemofelis* was reported only on the surface of red blood cells during the potential sequestration within tissues at this stage of the infection (Peters et al., 2011).

In the present study, we hypothesized that hemoplasmas may be sequestered within tissues during the chronic phase of the infection. The tissue sequestration may protect the bacteria from the host’s immune response and may explain the chronic nature of hemoplasma infections. An intracellular life cycle was previously reported for *M. suis* and other mycoplasma species (Groebel et al., 2009; Razin et al., 1998; Simpson and Love, 1970). We decided to investigate this hypothesis using ‘*Candidatus* Mycoplasma turicensis’ in specified pathogen free cats.

After ‘*Candidatus* M. turicensis’ bacteremia, cats became chronic carriers. During this stage, ‘*Candidatus* M. turicensis’ blood loads were below the detection limit of real-time PCR but antibodies remained at high levels. A tissue sequestration of the organisms was suspected to occur. Tissue samples were collected from chronic carrier cats and analyzed for the presence of ‘*Candidatus* M. turicensis’. DNA of the bacteria was detected, although at low levels, within some tissues more than one year after exposure. No significant contribution of the ‘*Candidatus* M. turicensis’ blood loads to the tissue loads was expected because all the cats resulted PCR-negative in blood at the beginning of the study. The viability of the organisms in tissues could not be proven but it is generally believed that the DNA of a dead organism would be quickly removed from the body (Dowers et al., 2009). Therefore, we assume that ‘*Candidatus* M. turicensis’ is able to remain quiescent in tissue niches for a long time after exposure. ‘*Candidatus* M. turicensis’ may reach the tissues through the blood supply, as reported for *M. haemofelis* (Tasker et al., 2009). Because tissue cells are not the primary target for hemoplasmas, the growth of the bacteria is probably hampered by the lack of nutrient. Hence, ‘*Candidatus* M. turicensis’ most likely needs to decrease its metabolism to survive. A similar mechanism was observed in *M. suis* after cultivation in a classical mycoplasma medium (Schreiner et al., in preparation). The medium used for cultivation appeared to induce a transformation of *M. suis* into nanoparticles (nanotransformation).
(Schreiner et al., in preparation). The same phenomenon was reported also in other mycoplasmas (Chernov et al., 2007; Demina et al., 2010). ‘*Candidatus* M. turicensis’ may use this mechanism to survive for many months or years within the host cells. The exact localization of ‘*Candidatus* M. turicensis’ within tissues is not known. A fluorescence in-situ hybridisation (FISH) technique was used for studying tissues and cell types important in survival and persistence of feline hemoplasma species (Peters et al., 2011). ‘*Candidatus* M. turicensis’ was detected by PCR but not by FISH in tissues of acutely infected cats. The low copy numbers of ‘*Candidatus* M. turicensis’ hampered the possibility to clearly localize the organisms and establish the type of cells involved in its survival (Peters et al., 2011). The tissue copy numbers reported by PCR in ‘*Candidatus* M. turicensis’ acutely infected cats tended to be lower than copies reported for *M. haemofelis* with the exception of the tonsil, mesenteric lymph node, mid-jejunum as well as colon and colonic lymph node (Peters et al., 2011). The FISH technique could not detect any signal for hemoplasmas in tissues of chronically infected cats due to the low sensitivity of the assay (Peters et al., 2011). Thus, tissue cells involved in the sequestration of hemoplasma during the chronic phase of the infection are still unknown.

The persistence of ‘*Candidatus* M. turicensis’ within tissues may also explain the high level of antibodies detected more than one year after exposure. The constant presence of the antigen may continuously stimulate the immune system, which in turn keeps the bacterial copy numbers at very low levels in tissues. In order to down-regulate the immune system and provoke a potential reactivation of ‘*Candidatus* M. turicensis’, chronically infected cats were administered with high doses of methylprednisolone acetate. After the attempted immunosuppression, cats showed a significant drop in lymphocyte counts and a significant transient decrease of the measured antibodies (*M. haemofelis* DnaK antibodies). Additionally, one blood and three tissue samples tested PCR-positive for ‘*Candidatus* M. turicensis’ and increased tissue loads were detected in one cat. The decrease of antibodies and lymphocyte values appeared to be associated with the increase in ‘*Candidatus* M. turicensis’ tissue loads but overall the reactivation of ‘*Candidatus* M. turicensis’ was limited. The reactivation might have been more successful at an earlier time point after infection. Indeed, ‘*Candidatus* M. turicensis’ tissue loads appeared to decrease over time. ‘*Candidatus* M. turicensis’ tissue loads were higher immediately after bacteremia compared to the tissue loads reported six months after exposure (Novacco et al., 2011; Peters et al., 2011). Alternatively, the protocol used for the attempted immunosuppression might be not successful to
reactivate ‘Candidatus M. turicensis’ infection. However, the methylprednisolone dose used in the present study has been reported to successfully reactivate a latent FeLV infection in cats (Rojko et al., 1982). We cannot exclude that in naturally infected cats the reactivation of the infection may occur under different immunosuppressive conditions, such as a concurrent disease or neoplasia. In addition, the impossibility to significantly reactivate ‘Candidatus M. turicensis’ should be also considered. ‘Candidatus M. turicensis’ showed a different kinetic of the infection compared to M. haemofelis and ‘Candidatus M. haemominutum’. The bacteremia reported for M. haemofelis and ‘Candidatus M. haemominutum’ lasted for longer period of time compared to ‘Candidatus M. turicensis’. Additionally, no evident blood load variations were detected in ‘Candidatus M. turicensis’ acutely infected cats. Thus, ‘Candidatus M. turicensis’ may be soon recruited within tissues and organisms may remain sequestered in tissue niches even for months without the possibility of being successfully reactivated. After methylprednisolone administration, a significant decrease in the antibody levels was detected. The level of antibodies returned to approximately pretreatment levels in four weeks, suggesting the persistence of ‘Candidatus M. turicensis’ within tissues. A complete clearance of ‘Candidatus M. turicensis’ from tissues appeared unlikely to occur, although all cats tested PCR-negative in the analyzed tissues at the end of the experiment. Probably, ‘Candidatus M. turicensis’ organisms were below the detection limit of real-time PCR or localize in other organs. We collected tissue samples only from organs easily to be localized by manual palpation; analyses of a larger number of organs might have been more successful in ‘Candidatus M. turicensis’ detection. We hypothesized that the high levels of antibodies reported in chronically infected cats might be important to sustain an effective anti-hemoplasma immune response. For this reason, the ‘Candidatus M. turicensis’ chronically infected cats reported within this study were re-exposed to ‘Candidatus M. turicensis’. Cats were protected from reinfection (Novacco et al., in preparation). Mechanisms involved in the protection are not yet clarified. All the cats showed a decrease of M. haemofelis DnaK antibodies after reinfection (Novacco et al., in preparation). Moreover, a chronically infected cat that became seronegative before reinfection was nevertheless protected from the second challenge. This result suggested that antibodies against M. haemofelis DnaK play only a marginal role in the immunological protection. Probably, an interaction between the humoral and cellular immune response is required.
Although PCR is the gold standard for the detection and differentiation of hemoplasmas during the acute phase of the infection, the ELISA is a precious help for monitoring the chronic phase of ‘Candidatus M. turicensis’. Indeed, the detection of hemoplasmas in chronically infected cats is problematic because the bacterial loads are at, or below, the detection limit of real-time PCR. Therefore, a PCR-negative result cannot rule out a low-level infection. By monitoring the kinetics of antibodies we may have indirect information of the presence of ‘Candidatus M. turicensis’. The combination of the ELISA and PCR is desirable for the complete diagnostic profiling of hemoplasma infections.

In conclusion, the results of the present study contribute to the understanding of the pathogenesis of ‘Candidatus M. turicensis’ infection. Infected cats that had recovered from bacteremia without antibiotic treatment remained chronic carriers. ‘Candidatus M. turicensis’ remained detectable by PCR in tissues at low levels for more than one year after exposure. Additionally, high levels of DnaK antibodies were detected in the majority of the cats until the end of the study. The reactivation of ‘Candidatus M. turicensis’ seemed to be unlikely to occur under the chosen conditions. For this reason, chronically infected cats appeared not be a major source of infection for other cats.

6.1.1. Significance in veterinary medicine

We demonstrated that cats that ostensibly recovered from the acute ‘Candidatus M. turicensis’ infection conceal ‘Candidatus M. turicensis’ within some tissues. However, no significant reactivation of ‘Candidatus M. turicensis’ could be provoked at this stage of the infection. For this reason, ‘Candidatus M. turicensis’ chronically infected cats seem not to be a major source of infection for other cats. However, we cannot exclude a successful reactivation under different conditions or for other feline hemoplasma species. In future, a potential reactivation of M. haemofelis in chronically infected cats should be tested. Moreover, high levels of DnaK antibodies were measured during the study, probably due to the persistent low levels of ‘Candidatus M. turicensis’ within tissues. Chronically infected cats appeared to remain carrier of ‘Candidatus M. turicensis’ but they are protected from reinfection. Immunological mechanisms involved in the protection need further investigation.
The prevalence of hemoplasma infections in cats depends on different factors but it varies from 1 to 23% in Europe. Because hemoplasma may cause potential life-threatening hemolytic anemia, it is important to develop assays for a precocious and reliable diagnosis. The combination of the ELISA and PCR is required for the complete diagnostic profiling of hemoplasma infections. The ELISA could be particularly useful for the identification of chronically infected cats and for monitoring the antibiotic treatment. For this reason, further studies are required to develop easy and fast serological assay for the detection of antibodies in naturally infected cats. Probably, a combination of different hemoplasma antigens should be considered.

6.1.2. Significance in human medicine

The significance of hemoplasma infections in humans is still under investigation. Hemoplasmas have been reported in humans with close contact with animals, i.e. pet owners, farmers and veterinary doctors (Archer et al., 1979; Congbin et al., 2010; dos Santos et al., 2008; Duarte et al., 1992; Kallick et al., 1972; Puntaric et al., 1986; Steer et al., 2011; Sykes et al., 2010; Yang et al., 2000; Yuan et al., 2009). Transmission routes are not yet clarified. The zoonotic potential of hemoplasma is still controversial but the close phylogenetic relation of the human isolates with the hemoplasma species from animals suggest a possible interspecies transmissibility. Indirect transmissions are also supposed to occur (Yang et al., 2000; Yuan et al., 2009). Vertical transmission of hemoplasmas (from the mother to the fetus) was also reported (Yang et al., 2000). The majority of infected humans are asymptomatic carrier of hemoplasma infections (Congbin et al., 2010; Yang et al., 2000; Yuan et al., 2009). However, cases with evident clinical signs (pyrexia, lymphoadenopathy and hemolytic anemia) are reported (Steer et al., 2011; Yang et al., 2000). Pregnant women and newborns appeared to have more severe clinical signs of hemoplasma infections (Yang et al., 2000). Often hemoplasma infections are reported in immunocompromised humans (Archer et al., 1979; dos Santos et al., 2008; Duarte et al., 1992; Kallick et al., 1972). In these cases hemoplasma infections are resistant and long treatments are required to eliminate the infection from the sick patient. Understanding mechanisms behind the ability of hemoplasmas to establish a chronic carrier state is a key point for the development of treatment against hemoplasma infections. We hope to be able to better understand these mechanisms in infected animals and transfer this information in human medicine, when required.
7. References


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8. List of Publications

2011  


2010  


2009  
9. Curriculum vitae

Dr. med. vet. Marilisa Novacco

Address: Clinical Laboratory, Vetsuisse Faculty
University of Zurich, Zurich (Switzerland)
Winterhurerstrasse 260, CH-8057 Zurich
Website: http://www.vetlabor.uzh.ch
E-mail: mnovacco@vetclinics.uzh.ch
Phone: + 41 44 635 82 79
Fax: + 41 44 635 89 23

Education

2009- Present Clinical Laboratory, Vetsuisse Faculty
University of Zurich, Zurich (Switzerland)

PhD student in Cellular and Biomedical Sciences,
Graduate School of Bern (Switzerland)

2007-2008 Clinical Laboratory, Vetsuisse Faculty
University of Zurich, Zurich (Switzerland)
Completion of the doctoral thesis on feline and canine hemotropic mycoplasmas

2001-2007 Veterinary University of Bologna (Italy)

Dr. med. vet in Veterinary Medicine

Dissertation: Clinical Pathology Service, University of Bologna (Italy)
“Use of combined conventional and real-time PCR to determine the epidemiology of feline hemoplasma infection in northern Italy”

1995-2000 High School in Portogruaro (VE), Italy
Awards and Honors

2010  IOM Student Travel Award for attending the 18th Congress of the International organization for Mycoplasmology (IOM), Chianciano Terme (Italy)

2009  Forschungskredit (grant for research project as PhD) at the University of Zurich

2007  Recipient of a post-graduate grant to stay at a research institution abroad. Founding was obtained from the Veterinary University of Bologna, Italy. The period was spent at the Clinical Laboratory, Vetsuisse Faculty, University of Zurich, Switzerland.

Work Experience

2009  PhD at Clinical Laboratory, Vetsuisse Faculty, University of Zurich, Switzerland. Project: ‘Candidatus Mycoplasma turicensis’ Infection: Reactivation, Tissue Distribution and Humoral Immune Response

2008  Clinical laboratory, Vetsuisse Faculty, University of Zurich, Switzerland. Completion of the Doctoral thesis on feline and canine hemoplasmas prevalence

2007  Post graduate training period in the Veterinary Clinical Department, Veterinary University of Bologna (Italy)

2005-2007  Full time student volunteer involved in the daily clinical activity at small animal practice in the Teaching Hospital and in the Veterinary Clinical Pathology Service

Languages

Italian: maternal, English: very good knowledge, German: basic knowledge
**Expertise**

In addition to the education as veterinarian:

- Polymerase Chain Reaction (including quantitative real-time PCR, semiquantitative and conventional PCR)
- DNA sequencing (Applied Biosystems ABI310 sequencer)
- Cloning
- DNA extraction from cells and tissues
- Enzyme-linked immuno-sorbent assays (ELISA)
- Flow Cytometry (BD FACS Calibur, Gallios/Navios Beckman-Coulter)
- Bioinformatics (DNA sequence analysis including trace file editing, NCBI BLAST, multiple sequence alignments, annotation and GenBank submission)
- Laboratory animal management (domestic cats) under specified pathogen free (SPF) conditions.

**Continuing Education**

2011  
Introduction to epidemiology and biostatistics. University of Bern (CH) (20.06.11-01.07.11)

Training in flow cytometry, Gallios (Beckman Coulter International S.A.) at the Vetsuisse University, University of Zurich (CH) (19.09.11 - 21.09.11)

2010  
Foundation English grammar B1-B2.1. University of Zurich (CH) (25.02.10 - 3.06.10)

Scientific writing in the sciences or medicine. University of Zurich (CH) (20.4.10 - 29.4.10)

Effective scientific presentation for PhD students in the sciences or medicine. University of Zurich (CH) (11.5.10 - 3.6.10)

69th Annual Assembly of the Swiss Society for Microbiology. ETH Zurich (CH) (24.6.10)

Immunofluorescent staining, confocal microscopy and image analysis. University of Bern (CH) (5.10.10 - 6.10.10)
2009
Bioinformatics. University of Bern (CH) (19.02.09- 28.5.09)
Cellular and Molecular Immunology. University of Bern (CH) (17.09.09 - 10.12.09)
LTK module 1E: Introductory course in Laboratory Animal Science. University of Zurich (CH) (4.11.09 - 12.11.09)

2008
Real-Time PCR and Sequencing. Seminar Roche, Basel (CH) (25.9.08)

Oral and Poster Presentation

2011
“Protection from ‘Candidatus Mycoplasma turicensis’ reinfection”. Oral presentation at Mycoplasma Symposium, Göttingen (D) (4-6.07.11)

2010
“‘Candidatus Mycoplasma turicensis’ Infection: Attempted Reactivation and Tissue Distribution in Chronic Carrier Cats”. Oral presentation at 18th IOM Congress, Chianciano Terme (IT) (11-16.07.10)
“Cat Facility Management”. Oral presentation at LTK Module 20E at the Vetsuisse University, University of Zurich (CH) (30.03.10)
“Potenziale riattivazione e siti di sequestro di ‘Candidatus Mycoplasma turicensis’ in gatti con infezione cronica”. Oral presentation at 65° International Congress SCIVAC, Rimini (IT) (28.05.10)
“‘Candidatus Mycoplasma turicensis’ Infection: Attempted Reactivation and Tissue Loads in Chronic Carrier Cats”. Poster presentation at 4th Graduate School Student’s Symposium, University of Bern (CH) (27.1.10)

2009
“Housing, identification, conditioning, experimental design with cats”. Oral presentation at LTK Module 20E, Zurich (CH) (07.04.09)
“Importance of canine hemotropic mycoplasma infections with emphasis on their geographical distribution”. Oral presentation at Mycoplasma Symposium, Morschach (CH) (25-27.06.09)
10. Acknowledgements

I would like to thank all of my family for their love and constant support. A special thanks to my mum who always encouraged me to give my best and believe in my possibilities and to Paolo for taking care of me with love and patience, even when it was really hard. Moreover, I want to thank Zoe, my faithful dog, for her devoted love. I was always comforted by her presence around me.

My sincere thanks go to my supervisor Prof Regina Hofmann-Lehmann for her guidance and support during my PhD. I learn to be strong, independent and to NEVER EVER GIVE UP! Working with her has helped me to develop into a more rounded scientist and my stay in her group significantly improved my education. It was a privilege and honor to work in her group. I want to thank Prof Hans Lutz for his precious help and support. Many thanks also to my mentor, Prof. Thomas Lutz, for his support throughout my PhD. Moreover, I deeply thank my co-referee Prof Ludwig Hoelzle for our inspiring discussions on hemoplasmas. I was delighted to share my enthusiasm and doubts with him.

I would like to thank also to my colleagues of the Clinical Laboratory. Thanks to Marina M. for being always helpful and competent, for the time we spent together in laughing and for cheering me up when I was sad or lost. Above all, I want to thank you, my dear Marina, for your friendship. Thanks to Valentino C. who always helped me as friend and colleague. You love to grumble but we all know you have a heart of gold. Thanks also to Barbara R. for her excellent help with the cats and for the great time spent together in Milan and Dublin. Thanks to Kristina M. and Godelind W. for the nice time we spent together out of the lab and for our discussion on hemoplasmas. I want also to thank Vera R. and Andrea W. for their excellent support with the cats during all the blood collections. Thanks also to Celine R. and Mirjam L. for being helpful colleagues and great friends; I will never forget our red faces after Kondi! Moreover, I want to thank Julia B. for the lovely care she had for our cats and big help during our projects together. My sincere thanks go also to Marychelo R. and Daniela B.: they were friend and excellent animal caretaker. I am aware that without you all our research could not be done. Your help was essential. I want to thank also Eniko G, Theres M. and Beatrice W. for their excellent support in the lab. In particular, I want to thank Eniko for being the MAMA of the lab and Theres for her sense of humor. I want to thank all my friends and in particular: Marco, Vera, Catch, Bingo, Clod, Pelo, Dario and Step. Without your friendship and support anything would have been possible.

This thesis is dedicated to my grandparents, Pelio and Luisa.
11. Declaration of originality

Last name, first name: Novacco Marilisa
Matriculation number: 08-751-158

I hereby declare that this thesis represents my original work and that I have used no other sources except as noted by citations.

All data, tables, figures and text citations which have been reproduced from any other source, including the internet, have been explicitly acknowledged as such.

I am aware that in case of non-compliance, the Senate is entitled to divest me of the doctorate degree awarded to me on the basis of the present thesis, in accordance with the "Statut der Universität Bern (Universitätsstatut; UniSt)", Art. 20, of 17 December 1997.

Place, date

Zurich, 31.01.2012

Signature