



## Haemotropic mycoplasmas (haemoplasmas): a review

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### ABSTRACT

Haemoplasmas contain *Eperythrozoon* and *Haemobartonella* species, which are widespread causes of animal and human infections. These obligatory cell parasites were previously known as *Hemobartonella*; now hemotropic mycoplasmas. These bacteria are identified by the lack of cell wall and their small genome. The usual diameter is between 0.3 and 1 μm. Mycoplasmas are polymorphic and seen in circular or bar shape, which aggregate and form pinion teeth on the surface of RBCs. Despite the small genome of mycoplasmas, the GC content is 23 to 40%. The genome contains a circular double stranded DNA. Mycoplasmas are notable to grow on culture media. Animals are usually infected with the hidden form of the disease and hence become carriers and sources for the spread of the infection. Since these bacteria are difficult to grow, the common methods of diagnosis are cytology and microscopic examinations. Today, the most reliable and definitive method for the detection of haemoplasmas polymerase chain reaction (PCR). The most important way for the transmission of bacteria is the insect bite including ticks. High density of hemotropic mycoplasmas causes RBC hemolysis and hence many symptoms including jaundice, lethargy, anemia, fever and acidosis. Mycoplasmas respond to antibiotic therapy. Effective antibiotics are available such as lincomycin, enrofloxacin, oxytetracycline and doxycycline. Hemoplasmas cause a wide range of diseases and play the role of a cofactor in many viral and neoplastic diseases. These diseases are common in many countries, including Iran.

**Keywords:** *Eperythrozoon*, *Haemobartonella*, *Mycoplasma*, mandatory parasites, RBC

### History

Blood parasites were observed in mice (*M. coccoides*) and dogs (*M. canis*) for the first time in Germany in 1928 (Schilling, 1928). In 1930, *Eperythrozoon* spp. was officially reported in pigs in United States. In 1941, Lotz et al. identified this bacterium in cattle in United States. Years later, Flint and Moss identified *M. haemofelis* in cats. In 1942, Clark reported *Eperythrozoon* infection in an anemic cat in South Africa and named it *E. felis*. In 1953, Flint and Moss reported a similar microorganism that caused infectious anemia in cats in United States. Although haemobartonellosis was defined for the first time in 1953 (Grindem et al., 1990), a few number of studies had been carried out related to incidence and prevalence

of the disease and its risk factors for decades. In 1955, Flint and Mckelvie suggested that this microorganism could be called *Haemobartonella felis*. The microorganism was known under this name until a decade ago. *Hemobartonella* and *Eperythrozoon* infections are widely spread in world countries, including Iran.

### Introduction

Mycoplasmas are one of the smallest bacteria dividing into two groups, haemotropic and non-haemotropic mycoplasmas. To date, several violent *Mycoplasma* species have been identified. For example, *M. penetrans* attacks human genital epithelial cells (McOrist, 2000) and *M. genitalium* infects human lung fibroblasts (Baseman, 1995). *M. gallisepticum* can also attack ancestral HeLa cells and chick embryo fibroblasts (Winner et al., 2000). Pathogenic bacteria attacks to eukaryote cells include benefits for the pathogen such as resistance to the immune system response, reduced effectiveness of antibiotics and nutritional benefits.

Haemotropic mycoplasmas were previously known as *Haemobartonella* and *Eperythrozoon*, but now are reclassified within the genus *Mycoplasma* according to 16S rRNA gene sequencing. Haemoplasmas are able to survive without oxygen. Since the cell wall does not really exist and because of the resistance to some antibiotics, identification of the bacteria is a great challenge. Haemoplasmas can infect a variety of mammals. In cats, three species of haemoplasmas have been identified, including *Candidatus Mycoplasma haemominutum*, *C. M. haemofelis* and *M. turicensis*. In dogs, two species have been characterized, including *M. haemocanis* and *C. M. haematoparvum*. *Eperythrozoon* spp. already identified include *M. (Eperythrozoon) suis* and *E. parvum* in pigs, *M. (Eperythrozoon) ovis* in sheep and goats, *M. (Eperythrozoon) Wenyonii*, *E. teganodes* and *E. tumoi* in cattle, *E. coccoides* in mice, *E. mariboi* in foxes and *C. M. haemolamae* in llamas and alpacas. In cats, haemoplasmas include Ohio strain or the spindle form of *M. felis* which is the cause of anemia in infected cats and the low virulence form, California strain. Furthermore, *M. canis* in dogs, *M. muris* in rats and *M. procyoni* in raccoons have been reported. *Mycoplasma synovia* (MS) is the bird *Mycoplasma* spp., causing subclinical diseases of the upper respiratory tract in most cases. MS can cause serious damages to the air sacs when combined with diseases such as Newcastle, bronchitis or both (Neimark et al., 2004).

Mycoplasmas cannot grow in culture media and are parasites of RBCs of mammals, including cats, dogs, cattle, sheep, goats, pigs and rodents (Hoelzle, 2008; Groebel et al., 2009). Symptoms are not specific, but generally include anemia, pallor mucosa, lethargy, anorexia, weight loss and depression. Constant fever, especially in acute stage of the disease, can be seen often. Splenomegaly and lymphadenopathy may be occur due to extra medullary hematopoiesis (EMH). Sometimes, jaundice caused by hemolysis is seen. The anemia is reversible, combined with reticulocytosis, anisocytosis, macrocytosis and polychromasia. Hematocrit may decrease to below 20%, depending on the severity of infection (Foley et al., 1998; VanSteenhouse et al., 1993). *M. haemofelis* is a common cause of normoblast presence in the blood (Hammer and Wellman, 1999). However, the anemia may be irreversible sometimes (VanSteenhouse et al., 1993). Symptoms such as anemia and jaundice are seen in pigs. Although incidence of the infection is low in pigs but includes a high mortality rate (Messick, 2004). A few reports have been published on the presence of the bacteria in horse blood. Infected horses show symptoms such as decreased stamina, weight loss, anemia, fever, lymphadenitis and impaired blood flow (Dieckmann et al., 2010). In cats, symptoms of the acute form of the disease include fever, anorexia, weight loss, jaundice, lymphadenopathy and splenomegaly (Ural et al., 2009). The chronic form of the disease is characterized by the symptoms such as anemia, weight loss, paralysis of the hind limbs, dehydration, depression and hypersensitivity (Akkan et al., 2005). A hidden form of the disease has been reported (Akkan et al.,

2005). Animals are usually infected by the hidden form and become carriers and sources of the infection (Messick, 2004). The bacteria are the cause of infectious anemia due to binding to erythrocytes, increasing cell permeability and thereby rupturing cells (Heinritzi and Plank, 1992).

Various strains of *H. felis* have been detected by PCR. These strains include Ohio (large) and California (small) strains (Berent *et al.*, 1998). Since these bacteria are difficult to grow; the common method of detection is limited to cytology and microscopic examination. Several factors can cause false positive or false negative results using this method; therefore, it is not considered as a gold standard for the definitive diagnosis of the infection. Today, the most definitive and reliable method for the detection of haemoplasmas PCR (Tasker *et al.*, 2001). Furthermore, staining with acridine orange and electron microscopy are used to identify these pathogens. Although the transmission of the bacteria has not completely been understood, the most important way is likely insect bites such as ticks. Other ways have been described including intrauterine transmission, direct transfer of bacteria during the animal fight and transfer to newborns by milk. In some cases, the infected animals are infected with FIV or FeLV, simultaneously (Berent *et al.*, 1998). Various antibiotics are effective on these bacteria, including lincomycin, enrofloxacin, oxytetracycline, doxycycline and tiamidolium (Baneth *et al.*, 1998; Winter, 1993; Ojeda and Skewest, 1987). The bacteria are resistant to azithromycin.

### Ultra structure morphology

Several characteristics of *Mycoplasma* species, including mycoplasmas in rats and mice (*H. muris*, *E. coccoides*) and *M. polmonis*, have been compared with each other via electron microscopy. Despite the divergence in classification, the ultra structural details of *H. muris* and *E. coccoides* were similar. Both bacteria were spherical, 350 to 700 nm in diameter and free of cell wall. The proliferation style of these bacteria is binary division, but no proven evidence is available on the cell cycle. In contrast, multi forming is considerable in *M. polmonis*. However, the remaining structure is similar to that of the two other species. The host is important for the differentiation of these three species, which is different in *M. polmonis*. Morphological structures of other mycoplasmas such as *H. felis*, *H. canis*, *E. ovis*, *E. wenyonii* and *E. suis* and other blood parasites are reviewed many times in the last 20 years. More recently, mycoplasmas have been found in Camelidae family using optical microscopy. Generally, similar findings in erythrocyte parasitic species have been identified. These microorganisms are round to rod shaped, 0.3 to 3 μm in diameter and usually surrounded by a membrane. The bacteria have no nucleus but contain granules. They attach to surface of RBCs but cannot penetrate; in fact, they reside in 15-nm holes and thereby seem attached to RBCs.

*M. haemofelis* includes membrane with polar spots, which facilitates connection to the host. These spots consist of a protein network; therefore, the bacteria can bind to eukaryotic cells. Mycoplasmas are dependent to host cells due to their small genome size. The membrane structure of *M. haemofelis* is special, meaning that the plasma membrane contains sterols which are made from the host cholesterol. Furthermore, *M. haemofelis* receive their biosynthetic precursors from the host. Therefore, it can be easily understood why *M. haemofelis in vitro* growth is difficult. Although these bacteria are cell surface parasites, but they do not penetrate the cell overall.

### Genomic structure

For more than half a century, researchers were much confused about the actual structure of *Eperythrozoon* and *Haemobartonella*. In 1993, the bacteria were categorized in Anaplasmataceae family based on their phenotypic characteristics. *M. haemofelis* possesses a circular chromosome which is about 1.199 kb in length. Like other mycoplasmas, GC content in *M. haemofelis* is low and about 38.5% (Berent

*et al.*, 2003). One of the characteristics of these bacteria is replacing UGA codon, tryptophan encoder gene, instead of stop codon (Baseman *et al.*, 1997). *M. haemofelis* is unique, compared to other mycoplasmas. A reason is the presence of superoxide dismutase enzyme gene.

### Pathogenicity

Like other mycoplasmas, haemoplasmas are not freely available in nature but are able to survive as parasites in their host. These bacteria used to have cell wall but have lost their cell wall and biosynthetic systems during evolution and only a part of their genome which is important for survival has been conserved. This small genome provides features of transcription, translation and protein synthesis. The bacteria includes a double-stranded circular DNA molecule containing necessary information for synthesis of essential proteins. *M. haemofelis* consists specific genes, which can protect this species against oxidative stress and increase biosynthesis of amino acids in a microorganism. Various mechanisms have been suggested for the pathogenicity of these bacteria. For example, the bacteria produce free radicals to damage the host cell membrane (Somerson *et al.*, 1965). Furthermore, nutrient depletion or biosynthetic precursors are released from damaging cell membrane caused by the bacteria (Russel, 1966). The bacteria sometimes trigger auto antibodies, which cause disturbances in the immune system of the host (Denman, 1991). Another mechanism in human *M. penetrans* includes increasing the cell permeability that results in cell death. Chronic illnesses are seen in intracellular infections; in cases, who do not respond to immune or antibiotic treatment. Furthermore, some genes code super antigens that bind directly to major histocompatibility complex (MHC) molecules and stimulate large numbers of lymphocytes. Production of inflammatory cytokines and host immune response (activating lymphocytes) leads to symptoms such as chronic arthritis (Cole and Alkins, 1991). Evidences suggest that the host immunity plays an important role in severity of symptoms and duration of disease (Biberfeld, 1971). A majority of pathogenic mycoplasmas include flask shaped accessories containing specific organelles that bind to the target cell (Razin *et al.*, 1998). This structure is essential for the attachment of the bacteria. There are also proteins, which help bacteria to bind to the target cell membrane (Baseman, 1993).

In 1973, researchers found that the most important vector of *M. haemocanis* in dogs was *Rhipicephalussanguineus* tick. Berkenkamp *et al.* (1988) found that *Polyplaxserrata* and *P. spinulosa* fleas were vectors of *M. coccoides* in mice. Prullage *et al.* (1993) identified *Stomoxyscalcitrans* and *Aedesaegypti* as the vector of *M. suis* in pigs. Willi *et al.* (2007) studied the *M. haemofelis* transmission by ticks, mice, cats and infected cat saliva. Results showed that saliva and feces could be transmission ways, but might lose their ability of transmission over the time.

Various virulence factors exist in mycoplasmas. Virulence factors result in more severe infections in the host. Some of these virulence factors include toxins and cytolysins. Two genes have been investigated to be associated with virulence factors in *M. haemofelis*. The first one, existing in *M. suis*, is called O-sialoglycoproteinase dopeptidase gene (Guimaraes *et al.*, 2011). This enzyme is involved in lysis of erythrocytes. The second enzyme, existing in *M. haemofelis*, but not in *M. suis*, is superoxide dismutase (SOD) gene (Berent and Messick, 2003). SOD protects *M. haemofelis* against damages caused by oxidants. Lipoprotein membrane of mycoplasmas is recognized by the host immune system (Chambaud *et al.*, 1999). Some genomic regions within the mycoplasmas respond to environmental changes. This genomic regions present as repetitive units on the genome (Van Belkum *et al.*, 1998). There are variable numbers of these repetitive units in *M. haemofelis* and *M. suis* (Santos *et al.*, 2011).

### Metabolism

Most research on metabolism in haemotropic *Mycoplasma* has been carried out in two species, *M. haemofelis* and *M. suis*.

### ***Energy metabolism***

*M. haemofelis* uses Embden-Meyerhof-Parnas pathway (EMP) for its energy metabolism. In *M. haemofelis*, protein-encoding specific sequences (CDSs) on the genome produce enzymes, which play a role in phospho transferase transition system and lead to change glucose into pyruvate molecules. Non-phosphorylated NADPH dependent glyceraldehyde-3-phosphate dehydrogenase (GAPN) causes a subsidiary pathway in glycolysis by changing glyceraldehyde-3-phosphate to 3-phosphoglycerate. This enzyme, which exists in some *Mycoplasma* spp. and a few species of other bacteria, causes resistance to damaging effects of oxidation and reduction by decreasing the conversion rate of NADP to NADPH (Iddar et al., 2002, 2005; Boyd et al., 1995). Other cell wall-free bacteria (Mollicutes) use F0F1 ATP synthase complex to produce energy. No orthologues exist for the pyruvate dehydrogenase complex and acetate kinase and no coenzyme A metabolic enzyme is present to support the lack of pyruvate dehydrogenase complex (Santos et al., 2011).

### ***Nicotinate/nicotinamide metabolism***

The end products of the metabolism of niacin (nicotinate and nicotinic amide) include  $\text{NAD}^+$  and  $\text{NADP}^+$ . Since the lack of these two key enzymes, nicotinate phosphoribosyl transferase and  $\text{NAD}^+$  synthetase, in metabolism of nicotinate, *M. haemofelis* uses nicotinamide as precursor of  $\text{NAD}^+$  and  $\text{NADP}^+$ . The pathway uses purine nucleoside phosphorylase, which is involved in purine metabolism. Furthermore, ribosylnicotinamide kinase and subsequently nicotinate-nucleotide adenylate transferase are involved in this pathway. Study of metabolic pathways among *Mycoplasma* species suggests that *M. suis* uses these enzymes but lacks the  $\text{NAD}^+$  kinase to produce  $\text{NAD}^+$  (Guimaraes et al., 2011). This enzyme is involved in transferring  $\text{NAD}^+$  and  $\text{NADP}^+$ ; therefore, plays an important role in maintaining the intracellular NADH/NADPH balance in the bacteria. Based on the previous studies, it can be concluded that only nicotinamide is required for the production of  $\text{NAD}^+$  and  $\text{NADP}^+$  in *M. haemofelis*, while  $\text{NADP}^+$  is further required in *M. suis* (Santos et al., 2011).

### ***Lipid metabolism***

The major cell membrane lipids of mycoplasmas include phospholipids, glycolipid and sterols. Mycoplasmas are not able to produce fatty acids from acetyl coenzyme A due to lack of specific genes (Pollack, 1978). However, *M. haemofelis* can produce phospholipids from glycerol because this species includes genes required for the synthesis of glycerol kinase and hence produces glycerol-3-phosphate (Yus et al., 2009). Glycerol-3-phosphate also plays a role in synthesis of the simplest membrane phospholipid, phosphatidate. Phosphatidate is changed to cardiolipin by an enzyme called phosphatidyl glycerophosphate synthase. Since the bacteria lacks this enzyme, another enzyme—cardiolipin synthetase—converts cytidine 5'-diphosphate diacylglycerol directly to cardiolipin. In general, mycoplasmas need sterols in culture media (Santos et al., 2011).

### ***Vitamin metabolism***

Vitamins for the proper development of mycoplasmas include nicotinate spermine, thiamine, pyridoxal, nicotinic acid, riboflavin, choline, folic acid and coenzyme A/pantothenate. None of these vitamins are



made by the bacteria (Yus et al., 2009). Only one enzyme is involved in folate metabolism; called serine hydroxy methyl transferase and seen in *M. haemofelis*. Based on this fact, it can be concluded that *M. suis* possibly converts L-serine to glycine, but requires folate derivatives. The derivatives are obtained from the bacterial environment, which plays an important role in providing the necessary and vital vitamins. It is remarkable that serine hydroxyl methyl transferase does not exist in *M. suis*; therefore, it absolutely depends on the environment (Guimaraes et al., 2011).

### ***Pentose-phosphate pathway***

Pentose phosphate pathway does not exist in *M. suis* and *M. haemofelis*; therefore, these species use other ways (e.g. environment) to produce ribose and restore NADPH (Yus et al., 2009).

### ***Purine metabolism***

Cell wall free bacteria cannot synthesis purine and pyrimidine nucleotides; hence, media containing these compounds are needed (Williams and Pollack, 1985; McElwain et al., 1988; Pachkov et al., 2007). Encoding genes are also needed to produce required proteins and enzymes of purine nucleotides in *M. haemofelis* (Yus et al., 2009). Hypoxanthine is the end product of RBC metabolism; therefore, this feature provides a greater compatibility for *M. haemofelis* in blood environment. *M. haemofelis* includes hypoxanthine phospho ribosyle transferase enzyme that changes hypoxanthine to inosyne 5'-monophosphate and guanine to guanine 5'-monophosphate (GMP). However, hypoxanthine can be regarded as a precursor of purine nucleotides. *M. haemofelis* can produce GTP and dGTP from guanosine and ATP and dATP from adenine. The latter reaction is catalyzed by an enzyme existing only in *M. haemofelis*; called adenosine kinase. If the first and the second steps in both directions are reversible, adenine and guanine can be used as purine nucleotides precursors. Moreover, it can be concluded that hypoxanthine, adenine and guanine can be used as precursors of purine nucleotides ATP/GTP (RNA) and dATP/dGTP (DNA) in *M. haemofelis* (Santos et al., 2011).

### ***Pyrimidine metabolism***

Mycoplasmas do not have orotate gene required for pyrimidine synthesis (Sasaki et al., 2002). In *M. haemofelis*, uracil can be used as precursor of cytosine in synthesis of RNA (Yus et al., 2009). In DNA synthesis, thymidine is a precursor of dTTP synthesis because this species lacks thymidine phosphorylase enzyme. There are differences between various *Mycoplasma* spp.; for example, *M. haemofelis* includes cytidylate kinase converting cytidine 5'-monophosphate (CMP) to cytidine 5'-diphosphate (CDP). This enzyme is absent in *M. Suis* (Guimaraes et al., 2011).

### ***Amino acid metabolism***

Mycoplasmas lack the ability to synthesize amino acids. Amino acid synthesis genes only exist in *M. haemofelis* genome; called permease ABC transporter. This amino acid is necessary for the proper growth of the bacteria (Yus et al., 2009).

### ***Protein homeostasis***

The loci of chaperons and proteases have been well saved on the genome of *Mycoplasma*. The most important roles of these genes include stabilization of the protein formation as well as reduction of

harmful proteins to the cell (Lund, 2001). In most bacteria, two main chaperon systems exist: GroE and DnaK. In mycoplasmas, no GroE system is reported including GroEL and GroES in *M. haemofelis* and *M. suis* and several other species of mycoplasmas (Guimaraes et al., 2011; Minion et al., 2004). Another chaperon, existing in *M. haemofelis* but not in *M. suis*, is called trigger factor (Tig). The most important differences between the Tig system and the previous systems include the Tig system, unlike the previous systems, is not ATP-dependent and is involved in cold shock (Wong and Houry, 2004). The most important roles of proteases include reduction and elimination of dense and harmful proteins (Dougan et al., 2002). Proteases in *M. haemofelis* include protease Lon, which is a heat shock ATP-dependent and a membrane-bound protease called FtsH. This *Mycoplasma* sp. lacks specific proteases. Proteolysis seems to be involved in protein homeostasis more than protein folding due to lack of specific proteases and GroEL and GroES chaperon systems (Wong and Houry, 2004). This can be effective in pathogenicity and viability of the bacteria (Sinha and Bhatnagar, 2010).

## Diagnostic techniques

### *Blood smears*

Microscopic study of the blood smears is the most common way to detect mycoplasmas. Blood smears are dyed with Romanowsky stains including Giemsa, Wright and Wright-Giemsa. Microscopic observation demonstrates bacteria in single, pairs or chain on the surface of erythrocytes, but rarely free in the plasma. However, this method includes a high rate of false-positive results. For example, dye sediment caused by inappropriate staining or fixation may be confused with the bacteria. Other mistakes include water droplets and Howell-Julie and Heimer-Papen bodies. Acridine orange and fluorescent antibody staining methods need fluorescent microscope to detect bacteria; however, both of these methods are more sensitive in detecting bacteria than Romanowsky dyes (Bobade and Nash., 1987; Small and Ristic., 1967). However, Howell-Julie bodies may be confused with the bacteria and cause false-positive results (Harvey and Gaskin., 1977). Although the bacteria exist in the blood in acute phases, it can be cleared from the blood within a few hours. As a result, the blood sample shows false negative result (Alleman et al., 1999; Harvey and Gaskin, 1977). Use of anticoagulants in blood samples and resampling reduce false-negative results. If highly concentrated EDTA is used, bacteria may be detached from the erythrocytes. Therefore, it is better to prepare smears immediately after blood sampling or use other anticoagulants such as EDTA (Alleman et al., 1999).

### *Serology*

Western immune blotting method can be used to detect *M. haemofelis* after 14 days of infection (Alleman et al., 1999). In 1998, Foley et al. infected cats experimentally with *M. haemofelis*. After 21 days of infection, the bacteria were detected in blood by indirect immune fluorescent antibodies. This method also can be used up to six months after the infection. The antibodies are quite strain specific and used to detect acute and subacute infections (Foley et al., 1998). Serological methods are not routine in detection of haemotropic mycoplasmas.

### *PCR*

PCR is a highly sensitive diagnostic method, amplifying certain fragments of the DNA to identify the microorganisms. Studies have shown that diagnostic rates of cytopathology and PCR are nearly 37.5 and 100%, respectively. *M. haemofelis* can be detected by PCR after eight days of infection until no

antibiotics are used. PCR shows reliable results after completion of three to 35 days of antibiotic therapy (Berent et al., 1998; Foley et al., 1998). Usually, PCR may remain positive for a long time in asymptomatic animals. Positive results do not always reflect the occurrence of clinical symptoms, but can show previous infections (Tasker and Lappin, 2002).

### **LAMP**

In loop-mediated isothermal amplification(LAMP) method,only a tube is used to amplify DNA (Notomi et al., 2000). This method is an alternative to PCR for the diagnosis of diseases, especially in developing countries. LAMP is a novel approach for nucleic acid amplification using constant-rate incubation instead of using thermal cycles. Turbidity caused by pyrophosphate magnesium can be detected in the reaction by photometer to detect amplified products (Mori, 2001). In 2012, a report was released on detection of *M. wenyonii* in 330 cattle using LAMP in China. LAMP was found more sensitive than PCR. Seventy one samples were reported positive using LAMP, while 62 samples were reported positive using PCR. Furthermore, 26 lice, 30 flies and 26 mosquitoes by LAMP and 18 lice, 20 flies and 21 mosquitoes by PCR were reported positive, when detecting carriers. Based on the results of the study, LAMP is a simple diagnostic tool to identify *M. wenyonii* as well as epidemiologic studies.

### **Treatment**

However haemotropic *Mycoplasma* species are sensitive to tetracyclines, including tetracycline and oxytetracycline, side effects may occur such as fever (Wilkinson, 1968). Another disadvantage of these antibiotics includes dosage. Since the antibiotics must be prescribed orally several times a day, they are difficult to use. However, due to good responses to these antibiotics, they are the first choice drugs. Therefore, other derivatives of this family, including doxycycline, are used. Benefits of these antibiotics include less side effects and lower doses. Doxycycline is prescribed 5–10 mg/kg orally once a day for 14–21 days. The Point is that although antibiotics can eradicate symptoms such as anemia, they cannot completely clear organs from the bacteria (Berent et al., 1998; Foley et al., 1998). Other effective drugs are available such as enrofloxacin, 10mg/kg once a day for 14 days (Winter, 1993) and fluoroquinolones or macrolides such as azithromycin 15 mg/kg twice a day (Westfall et al., 2001). Glucocorticoids such as prednisolone 2 mg/kg orally once a day for three weeks are also effective (VanSteenhouse et al., 1993). This can be prescribed along with antibiotics. Supportive therapies such as blood transfusion may be needed. Complete blood transfusion is necessary in severe anemia and rapidly decreased hematocrit conditions to less than 12%. However, it must ensure that animal blood is not contaminated with the bacteria (Tasker and Lappin, 2002).

### **Recent research**

More recently in Brazil, Dubravka et al.(2010) diagnosed rare zoonosis disease (haemotropic mycoplasmas) in a systemic lupus erythematosus patient caused by *Nocardia asteroides* pneumonia. Systemic lupus erythematosus is an autoimmune diseases that caused by suppression of immune system and exposure to opportunistic infections. This report describes a 21-year-old woman with zoonosis and a rare disease, haemotropic *Mycoplasma*, in infancy lupus deals. Treatment of *Nocardia* spp. was started with glucocorticoids and cyclophosphamide and resulted in a complete recovery of the patient. According to the literature review, the only case of the *Eperythrozoon* disease in humans was reported by Croatian authors a couple of decades ago. A recent report is presented based on haemotropic mycoplasmas in humans with symptoms of fever and hemolytic anemia (Steer et al., 2011). A 62-year-old English white woman with three-week history of fever, abdominal pain, joint pain, weight loss and night sweats had



traveled to Australia. She fed kangaroos in Australia and swam in a warm-water pool. After carrying out biochemical and serological tests, treatment was started with doxycycline. This is the first report on human haemoplasma infection. In pigs, more symptoms including loss of appetite, poor growth rate and reduced fertility have been observed. Recent studies by Woods *et al.* demonstrated that *Ctenocephalides felis* mites transfer *M. haemofelis* and *C. M. haemominutum* in cats. Tasker *et al.* researched to find a relationship between *M. haemofelis* and FIV in cats. Data in United States have shown that there is a possibility of existing *M. haemofelis* in cats with FIV. However, studies failed to demonstrate any relationships between these two agents. *Mycoplasma* infection has been reported in people with HIV, recently. These studies are not complete and show that mycoplasmas are possibly causes of zoonotic diseases. Since *Mycoplasmas* have not been cultivated *in vitro* yet; therefore, the load of infection has not been measured accurately. This explains why outcomes of research are contradiction.

## Epidemiology

### *Haemotropic mycoplasmas in cats (feline infectious anemia)*

*Mycoplasmas* cause anemia in cats. The anemia is so called haemoplasmosis. Feline infectious anemia or reversible anemia is caused by *M. haemofelis* (Mhf). The disease is a global epidemics. Mhf is one of the haemotropic mycoplasmas that usually infects mammals. The organism was previously known as *Haemobartonella* and the relative disease was named haemobartonellosis. Changes in taxonomy of *Haemobartonella* made by PCR and DNA analysis in 2001 have changed the classification to *Mycoplasma* (Niemark *et al.*, 2001). Two other species of *Mycoplasma*, *M. haemominutum* and *M. turicensis*, are weaker than Mhf in pathogenicity. The *M. haemofelis* genome consists of a circular chromosome. The interesting fact about the *Mycoplasma* genome is that despite a small genome, the GC content is 23 to 40% in *M. haemofelis*. Unlike other mycoplasmas, *M. haemofelis* tends to attach to the host erythrocytes and is more persistent in various blood conditions because of its different genome (Santos *et al.*, 2011). The shortest genome among mycoplasmas belongs to *M. haemofelis* (Guimaraes *et al.*, 2011). However, despite a small bacterial genome, it contains a high percentage of prologue genes (Pushker *et al.*, 2004). These prologues result in survive of parasite on the erythrocytes (Santos *et al.*, 2011). Epidemiologic studies on the prevalence of mycoplasmas have identified *M. haemofelis* in Iranian cats using molecular techniques such as PCR. Vahedi *et al.* reported this species in nearly 6.25% of healthy and ill cats but no *C. M. haemominutum* or *C. M. turicensis* was detected. Phylogenetic analysis of the Iranian cat isolates showed that these isolates were more similar to isolates from China and Thailand than those from other countries. In this study, male gender and low PCV had a direct correlation with *Mycoplasma* infection (unpublished data).

Latency of *M. haemofelis* varies from two to 30 days. This period lasts three to four weeks in acute form. In this step, changes in hematocrit can be observed. In cats, hematocrit may be normal, whereas the disease can reoccur in patients with chronic illnesses. Most cats with anemia are also FIA positive. Clinical symptoms are associated with the speed of anemia. Clinical findings indicate weakness, paleness of mucous membranes, tachycardia and collapse. Cats that are in final stages show increased body temperature. Physical examination shows organ disorders including heart murmur, enlarged spleen and jaundice. In chronic cases, weight loss and depression are significant. Laboratory findings demonstrate increased nucleated red blood cells (NRBC), polychromasia, anisocytosis, Howell-Julie bodies and increased reticulocytes. The first characteristic of the infection found in CBC is hyperchromic macrocytic anemia. *Mycoplasma* infection can almost be suspected by blood smears; however, blood smears have only a sensitivity of 50% (Hagiwara, 2009). Since the immune response of animals may take a short time

to occur, the bacteria is not possibly observed in blood smears a few days from the disease onset. Studies show that blood smears must be examined at least every four days (Ettinger, 2005). The Coombs test is positive nearly two weeks after the observation of the bacteria in the blood smear; however, it may be negative in chronic cases. Laboratory confirmation is carried out by electron microscopy; however, the sensitivity is lower than 50%. Thus, most researchers believe that PCR is the best method of diagnosis. Pathogenicity of *M. haemofelis* is more prominent than that of two other species. Conventional PCR uses presence or absence of genes after the amplification process, whereas real-time PCR uses fluorescent substances to mark the DNA during the gene amplification.

Although many studies have been carried out on mycoplasmas transmission, many facts have remained unidentified. Several routes have been suggested for the bacterial transfer including transfer via fights that has not been proven yet, transfer via mites especially in areas with high occurrence and transfer via other arthropods such as ticks. Furthermore, geographical diversity plays an important role in the bacterial transfer. Due to the ability of bacteria to survive outside the host body for seven days, the risk of infection heightens. The rate of infection is higher in young male cats which directly contact stray cats. The risk of anemia has been shown to be higher in cats with Mhf and FIV than cats with Mhf alone (1997). Pathogenicity of Mhf is not fully understood yet. The presence of parasites on RBCs is thought to stimulate the production of antibodies that results in extracellular hemolysis (Hagiwara, 2009). In fact, the immune response in cats results in the bacteria destruction in red blood cells. Therefore, healthy RBCs and subsequently hematocrit are increased. However, the hypothesis that healed cats remain as carriers is still accepted. The infection recurrence is possible in stress or pregnancy. Infected cats show anorexia and lethargy. Clinical findings are nonspecific and include pale mucosa, tachycardia, fever, jaundice and enlarged spleen. Fever is often frequent and reaches the highest level when the blood parasite proliferates. The anemia can be fatal in some cases.

Several other causes of anemia exist which must be differentially diagnosed. For example, infectious microorganisms such as *Babesia felis* and *Cytauxzoon felis* must be differentiated from mycoplasmas. Another cause, neoplasia, must be differentiated. Chronic inflammation, diabetes mellitus, feline immunodeficiency virus (FIV) and bone marrow diseases are other reasons for the reversible anemia. If the infection is not treated, one third of the infected cats will die. For an effective treatment, supportive therapies such as oxygen therapy or blood transfusion must be considered. A total of 10 mg/kg oral doxycycline for two weeks is an appropriate treatment. An alternative drug is 5mg/kg enrofloxacin administered orally. Glucocorticoid administration is still controversial. Doxycycline is the choice antibiotic for *M. haemofelis*. Enrofloxacin also can be a good option but is usually the second choice due to damages to the retina.

### ***Haemotropic mycoplasmas in dogs***

*M. haemocanis* causes mycoplasmas is, which damages RBCs. Similar to other mycoplasmas, *M. haemocanis* cannot grow *in vitro*. Incidence of *M. haemocanis* varies from 0.5 to 40%. The *Mycoplasma* genome has fully been sequenced to better understand the molecular and biological aspects. DNA of this species has been extracted from bacteremic dogs. Dogs do not show anemia symptoms until they suffer from other diseases such as cancer and congenital immune deficiency or had splenectomy. The disease includes mild symptoms except splenectomized patients which show loss of appetite, pale gums and infertility. Mycoplasmas are transmitted mainly by ticks. However the infection spreads through the fight between dogs, the vertical transmission has not been reported. Diagnosis in dogs may be possible by veterinarians through the case history, biochemical and serological tests and blood smears. Furthermore,

PCR can help to prove the diagnosis. The number of bacteria in blood smears can fluctuate dramatically. Although several observations occur in one smear, nothing may be seen after two hours. The infection can be treated with antibiotics such as tetracycline, oxytetracycline and doxycycline for three weeks. Based on the patient's condition, one or more antibiotics are prescribed. Mite control is the best way for the prevention of infection and the best pesticide can beprmetrine, which contains K9 ADVANTIX. Collars containing amitrazare used in some countries.

### ***Haemotropic mycoplasmas in mice***

*C. M. haemomurisis* detected in the blood of infected mice. In fact, this bacteria can cause infection in albino rats, albino mice, wild mice and hamsters. The routine detection methods of this bacterial species are similar to those of other haemotropic mycoplasmas and include staining blood smears with Romanowsky dyes. In microscopic views, the bacterium is seen in cocci shape with various lengths from 0.7 to 0.3  $\mu\text{m}$ . Sometimes, intra cytoplasmic dense granules can be seen in the blood parasite (Tanaka et al., 1965). The infection can result in death of the animal or makes the animal a carrier and causes the spread of infection in populations. The predisposing causes of the infection in animals are not fully understood, but it seems that the risk of infection is higher in splenectomizedmice than animals with spleen and also in young animals than adult ones. Tetracycline and oxytetracycline are effective in acute and hidden forms of the infection. The bacterial species is spread around the world. The infectious vector is usually a louse, which is called *Polyplaxspinulosa* (Rikihisa et al., 1997).

### ***Haemotropic mycoplasmas in poultry***

*M. gallisepticum* and *M. synoviae* are two notable species in poultry. Symptoms caused by *M. synoviae* include impotence, breast blisters, enlarge knee joints, splenomegaly, drooping feathers, paralysis, pallor crown, problematic legs and hepatomegaly. *Mycoplasma* colonies are visible such as satellite plates. *M. synoviae* sometimes exists without any symptoms and mortality is less than 10%. The infection is diagnosed by specific lesionsand serological tests. The incubation period is almost long and generally varies from 11 to 21 days after contamination. Treatment includes antibiotics such as chlortetracycline, oxytetracycline and tylosin. Bacterial transfer through feces is one of the important ways. Immunofluorescent assay is one of the rapid methods to detect *Mycoplasma* colonies. The colony size of *M. synoviae* varies 1–3mm. *M. synoviae*is sensitive to temperatures over 39°C. The bacteria can survive at -70°C for a long timein broth media. The bacteria has been identified using laboratory assessment techniques including serum agglutination on the plate (SPA), tube agglutination test (TA), haemagglutination, agar gel precipitation (AGP) and enzyme-linked immunosorbent assay (ELISA). One of the major proteins of the bacteria, p41, has been well studied using spot ELISA technique. Another technique, PCR, is a rapid, simple and highly sensitive diagnostic technique; by which, *Mycoplasma* DNA can be identified in tissues and culture media.

### ***Haemotropic mycoplasmas in cattle***

Like other mycoplasmas, *M. wenyonii*is seen freely or bound to erythrocyte surfaces in the blood plasma (Messick, 2004). From the microscopic view in Romanowsky staining, the bacterium is observed in circular, rod or cocci form. The parasite often exists in immunodeficient or splenectomized cattle. No treatment is needed based on the clinical signs. Symptoms include anemia, lymphadenopathy, fever, growth retardation, depression, diarrhea, decreased milk production, infertility, hind limb and scrotal edema, swelled teats, weight loss and reproduction inefficiency (Radostits et al., 2007; Montes et al.,

1994; Smith et al., 1990). If treatment is needed, tetracycline will be appropriate. Transmission of the bacteria is not completely known but findings often indicate that it is mechanically transmitted via arthropods, direct transfer and transfer by individuals (iatrogenic) (Radostits et al., 2007).

### *Haemotropic mycoplasmas in horses*

Not much information is available about haemotropic mycoplasmas in horses, but a report of a 30-year-old infected horse in Nigeria possibly confirms the existence of these species. Symptoms include fever, lymphadenitis, blood circulatory disorders and mucosal pallor. Bacteria found in infected erythrocytes sized approximately 0.3  $\mu$  min diameter (Gretillat, 1987). In a study, presence of the bacteria was proven using SYBR Green real-time PCR. In this study, primers included 16S\_HAEMOforwGGCCCATATTCCT (AG)CGGGAAG and 16S\_HAEMOrevAC(AG)GGATTACTAGTGATTCCA (Hoelzle et al., 2011). Temperature and heart rate of infected horses were normal, while hematocrit decreased to 28–30% (normally 31–45%). Hematologic parameters were normal and bacterial culture was negative. However, foreign bodies were found on the surface of erythrocytes with approximate size of 0.4  $\mu$  min acrydine orange stained blood smears. Sequencing genetically showed 97.8% similarity between the samples (GenBank accession nos. FN421445 and FN421443). Phylogeny of the sequences was discussed by Dieckmann et al. (2010).

### *Haemotropic mycoplasmas in pigs*

In pigs, anemia and jaundice are caused by *C. M. haemosuis* (Splitter, 1950). This species is the largest species among haemotropic mycoplasmas and is common in United States more than other areas (Kreier and Ristic, 1984). In pigs, the bacteria may be addressed incorrectly as *Eperythrozoon parvum* (a nonpathogenic parasites), but this *Mycoplasma* species seems longer in blood smears. Sequence deposits of 16S rRNA gene are available in Gene Bank with accession nos. U88565 and AF029394 (Messick et al., 1999; Rikihisa et al., 1997). Detection techniques include Giemsa stained blood smears, PCR and electron microscopy. Yuan et al. (2009) carried out PCR partially on 16S rRNA gene of *C. M. haemosuis* using 5'-CACGCCGTAACGATGGGTAT-3' (upper primer) and 5'-CAGCCCAAGGCATAAGGGG-3' (lower primer). Data of this study have been deposited in Gene Bank with accession no. EU371555 (Yuan et al., 2009). Transmission of the bacteria in pigs include mechanical transmission by stable flies (*Stomoxys calcitrans*) and mosquitoes (*Aedes aegypti*) (Prullage et al., 1993). Lack of proper hygiene in farms causes the growth of mosquitoes (*Aedes* spp.), which are the main factors of the infection in pigs (Prullage et al., 1993). Other routes of infection transmission include foods contaminated with the blood of infected pigs. Contaminated needle is another important way of infection transmission. A report of human infection by *M. suis* has been published (Puntaric et al., 1986). In humans, symptoms include fever, hemolytic anemia and jaundice (the Yang et al., 2000). Pigs are discussed as source of human infections. People who are in direct contact with infected pigs (e.g. farm workers) are exposed to *M. suis* more than other people. The disease is usually subclinical and the symptoms can be wide ranging. The most common symptoms in infected swine include mild fever and subcutaneous hemorrhage. Subcutaneous bleeding seems to occur due to infected erythrocyte sedimentation because the mass of erythrocytes triggers immune complex formation causing vascular damages and hemorrhage (Yuan et al., 2009). In pigs, symptoms of the acute infection include anemia and jaundice. Despite a low epidemic morbidity, the mortality is very high (Messick, 2004). Chronic infections decrease fertility, reduce the animal growth and increase the incidence of other diseases in the animal (Messick, 2004; Heinritzi, 1989). In pigs, haemotropic *Mycoplasma* spp. are sensitive to tetracyclines (Rikihisa et al., 1997).



Like other mycoplasmas, *M. suis* attaches to erythrocyte surface and does not penetrate the cell. Interestingly, only marginal bacteria are seen in acridyne orange stained smears in acute phases, while many bacteria have been found by PCR. These findings indicate that *M. suis* is capable of invading erythrocytes. Using fluorescent labels, laser confocal microscopy and electron microscopy, *M. suis* has been found to be an intracellular microorganism. This microorganism invades erythrocytes by endocytosis process and initially is surrounded by two layers. Then, the bacteria float freely in the cytoplasm. The bacterial colonization causes resistance to immune response and antibiotics. The intracellular release of *M. suis* provides iron in RBCs, which can be visible in the heminform in erythrocytes. Hemin supports the growth of invading bacteria, which is typically seen in *Bartonella quintana* (Seubert et al., 2002). However, this has not been established yet. The fact that haemotropic mycoplasmas cause chronic diseases without symptoms is very interesting (Messick et al., 2004). Since *M. suis* and other mycoplasmas invade the host cell cytoplasm, their infection can be very long lasting. To establish the hypothesis that *M. suis* attacks RBCs, pigs with no spleen were experimentally infected with *Mycoplasma* spp. Clinical symptoms were evaluated and blood samples were collected. Furthermore, confocal microscopy (CLSM) with fluorescent labeling and electron microscopy were used. In this study, enter of *M. suis* was associated with the cell membrane indentations and when the pathogen's attack increased, the pathogen's shape also changed. The bacteria can be found in vacuoles of infected erythrocytes as a consequence of the infection. A similar mechanism of endocytosis and invasion of erythrocytes can be seen in malaria caused by *Plasmodium falciparum*.

Haemotropic mycoplasmas cause life-threatening anemia, infertility and suppressed immune system in animals. Microorganisms with similar morphology have been identified in human blood. *M. suis* causes anemia, jaundice and fever in pigs (IAP), which is associated with the increase of bacteria in blood; approved by PCR. Symptoms can be treated by tetracycline. Infected pigs (even once) are considered as carriers and play an important role in epidemiology of the bacteria. Consequences of the acute form infection include impairment of fertility in matures, growth retardation in young animals and increased risk of respiratory infections in infants. The chronic form of the infection is important economically, especially in developing countries. Despite clinical findings about IAP which indicate low mortality but a high infection rate, recent observations have shown that the fatality rate has increased even when antibiotics are used. There is a big difference between the results of examinations and blood smear studies and those of real-time PCR, as more bacteria have been shown by PCR.

## Discussion and conclusion

In recent years, research have been carried out on haemotropic mycoplasmas in Iran. In 2013, Torkan et al. evaluated the prevalence and risk of *M. haemofelis* in 90 cats (45 healthy and 45 sick cats) using blood smears and PCR. Furthermore, age, sex, breed, sterility and living area were recorded. Overall, 72.2% of the patients were identified to be infected. Results revealed differences between the two groups. However, no significant differences were detected for gender, breed and sterility. Results also showed that the interactions between various risk factors could reduce the effectiveness of specific effects of each factor. Ghazi Saedi and colleagues (2009) investigated the molecular types of haemotropic mycoplasmas and assessed the rate of haemobartonellosis in cats in Tehran. Comparison of PCR and blood smear methods was another aim of the study. Blood samples were collected randomly from 100 mix bred cats (50 females and 50 males) with symptoms such as anemia, referred to Small Animal Teaching Hospital, Faculty of Veterinary Medicine, University of Tehran. Blood cells were counted and Giemsa stained smears were prepared. Furthermore, PCR was carried out for all samples using universal and special primers to detect haemoplasma infection. Cats were divided into two groups of infected and uninfected



animals (positive and negative). Twenty two percent of the cats were reported PCR positive. The prevalence of haemobartonellosis was reported 14–30% with 95% assurance. The rate of infected male cats was higher than that of female cats. Sensitivity and specificity were calculated 27 and 74.89%, respectively. The value of monocytes, eosinophils, hematocrit, MCV and platelets in infected cats was less than that in negative group, but MCHC, MCH, WBC and band neutrophils were higher. Based on PCR results, 63.63% of cats were infected with *M.haemofelis* (1309 bp), 54.54% with *C.M. haemominutum* (1354 bp) and 18.18% with *C.M.turicensis* (1317 bp). Furthermore, 18.18% of the patients were infected with *C.M.haemominutum* and *M.haemofelis*, 54.4% with *M.haemofelis* and *C.M.turicensis*, 54.4% with *C.M.turicensis* and *C.M.haemominutum* and 54.4% with the three species. First phylogenetic analysis of *C. M. haemofelis* was carried out by Vahedi *et al.* in Iran (unpublished data). In this study, 60 cat blood samples were collected from veterinary clinics in Tehran from 2011 to 2012. Giemsa stained blood smears have been examined using light microscopes and the positive samples were used for DNA extraction and PCR. Positive PCR samples were sequenced for the differentiation of bacterial species and phylogenetic analysis. Thirty-two samples were positive in direct examination; from which two samples were identified as *M.haemofelis* by the PCR. No positive samples of *C. M. haemominutum* or *C. M. turicensis* were found in PCR. Phylogenetic analysis of the isolates showed that these isolates were more similar to the isolates from China and Thailand than those from other countries. Based on the high sequence similarity between Iran, China and Thailand isolates, it can be concluded that these bacteria possibly had the same origin.

Haemotropic mycoplasmas are prevalent in other parts of world as well. The prevalence of mycoplasmas in anemic cats is about 25% in United States and the infection has been reported often with *M.haemofelis*. However, nonanemic cats have been infected with *haemominutum* species. Hematology and parasitology findings for FIA and *Mycoplasma* detection are similar. However, low hematocrit is a sign for both infections. Techniques such as PCR can help to final diagnosis. *Haemoplasma* can cause acute hemolytic anemia and chronic illnesses in a variety of vertebrate hosts. These bacteria, depending on host susceptibility, cause a wide range of clinically asymptomatic infections to fatal diseases. Older animals may be more susceptible to infection. Clinical characteristics of acute disease have been studied over a wide range of animals.

Infection with haemotropic *Mycoplasma* could be seen in a wide range of mammals. A few infected humans have been reported. These observations indicate the necessity of further research on this bacterium. Phylogenetic analysis supports strongly changed *Haemobartonella* and *Eperythrozoon* classification to haemotropic *Mycoplasmas*. Nowadays, PCR and real-time PCR are available for the diagnosis of the infection. LAMP, as a novel and reliable approach, is available as well. *Mycoplasma* infections vary from severe and fatal anemia to chronic blood diseases and even infertility. Furthermore, *Mycoplasma* spp. possibly play a role in some viral diseases as cofactor, which requires further extended research. Treatment approaches are still controversial. *Haemoplasma* infection is common in most parts of world and Iran is no exception.

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