

**FELINE HAEMOBARTONELLOSIS - LESSONS FROM RECLASSIFICATION
AND NEW METHODS OF DIAGNOSIS**
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INTRODUCTION

Haemobartonella felis infection of cats has been recognised worldwide and despite it being regarded as a common cause of anaemia, there is limited information available regarding its epidemiology and the disease it causes. One of the major factors limiting the investigation of this organism is that it has not yet been successfully cultivated outside the host. To date, attempts to culture the organism using *Mycoplasma* media and cat blood agar have failed.

RECLASSIFICATION

Until recently *H. felis* was classified as a rickettsial organism, primarily due to its uncultivated status, proposed transmission by arthropod vectors, small size and staining properties, and haemotropic character. However rickettsiae are known to possess cell walls and multiply within host cells, in contrast to *H. felis* which lacks a cell wall and multiplies on the surface of the host cell. Molecular studies have recently resulted in the reclassification of *Haemobartonella* spp., together with the related group of *Eperythrozoon* spp., within the genus *Mycoplasma*. This reclassification is based upon phylogenetic analysis of 16S ribosomal ribonucleic acid (rRNA) gene sequences from a number of American *H. felis* isolates which demonstrated their relatedness to *Mycoplasma* spp. (1-3). Certain phenotypic characteristics of *Haemobartonella* spp. are consistent with this new classification such as the lack of cell walls (which may contribute to the pleomorphism of these organisms) and fastidious growth requirements. *Mycoplasma pneumoniae*, which is closely related to *Haemobartonella*, can bind to erythrocytes *in vitro* and can induce cold agglutinins, features also seen with *Haemobartonella* spp.

Sequencing of 16S rRNA gene polymerase chain reaction (PCR) products from infected cats (1, 4-6) resulted in the recognition of different strains of *H. felis*. Under the new classification it has been proposed that the very closely related Ohio-Florida, Illinois and Oklahoma strains of *H. felis* (also called large forms) are now named '*Candidatus Mycoplasma haemofelis*' while the California strain (also referred to as the small form) is named '*Candidatus Mycoplasma haemominutum*'. Isolates with near identical 16S rRNA sequences to US strains have been reported in the UK, France, Israel and Australia (7, 8), suggesting worldwide distribution of these strains. The *Candidatus* status reflects that this classification is provisional because the uncultivated nature of these organisms means that there are few phenotypic characteristics with which to strengthen the classification. Phylogeny of the 16S rRNA gene of members of *Haemobartonella* and *Eperythrozoon* spp., collectively known as the haemoplasmas, reveals the presence of two subclusters. '*Candidatus M. haemofelis*' (CMhf) groups closely with '*Candidatus M. haemomuris*' (formerly *H. muris*) and *H. canis*, whilst '*Candidatus M. haemominutum*' (CMhm) groups with '*Candidatus M. haemosuis*' and '*Candidatus M. wenyonii*' (formerly *E. suis* and *E. wenyonii* respectively). The position of CMhf and CMhm within different subclusters suggests that they may represent distinct species. Additional data useful for classification may be obtained when genes other than 16S rRNA are sequenced and used for phylogenetic analysis.

The proposal for reclassification and renaming of *Haemobartonella* spp. was made in May 2001 (9, 10) whilst the existence of different strains was first reported in 1997 (1). Studies published before these dates do not make a distinction between strains so when work from such studies is discussed in this manuscript, the organism will be referred to as *H. felis*.

PATHOGENESIS

Clinical Signs and Haematological Features

In experimental infection studies performed to date, differences in pathogenicity appear to exist between CMhf and CMhm. Experimental infection of cats with two different isolates of CMhm (5, 11) resulted in minimal clinical signs and only minor or negligible haematological changes. Conversely experimental infection with CMhf often results in a severe haemolytic anaemia (4, 5, 11). Additionally, experimental inoculation of CMhf in cats already infected with CMhm appeared to result in more severe clinical disease and anaemia than that caused by CMhf alone (11). This finding may be because prior infection with one strain predisposes to immune-mediated disease when cats become infected with another strain. The nature of such superinfections and characterisation of the host's immune response to infection have not yet been investigated. At this time, it is impossible to make definitive statements concerning pathogenicity of CMhf and CMhm. Since organisms cannot be cultivated or accurately counted, it cannot be determined if experimentally inoculated cats received similar doses of organism which may have influenced clinical findings. Additionally, most of the experimental infections with defined strains to date have used intravenous inoculation (4, 6, 11). Natural routes of transmission of *H. felis* have not yet been defined and it is possible that route of transmission (including possible vectors) may affect pathogenicity.

Clinical signs are non-specific but include those typically associated with anaemia such as pallor and lethargy, as well as anorexia, weight loss and depression. Intermittent pyrexia is often seen, particularly in the acute stages of the disease. Splenomegaly and lymphadenopathy may be seen, reflecting extramedullary haematopoiesis. Icterus, due to haemolysis, is occasionally seen. Typically a regenerative anaemia results with reticulocytosis, anisocytosis, macrocytosis and polychromasia. Normoblasts may also be visible and, although these can be a feature of diseases which are not associated with regenerative anaemias, *H. felis* was found to be the most common cause of normoblastaemia in a recent retrospective study (12). Non-regenerative anaemias due to haemobartonellosis have also been reported. This may arise due to concurrent retroviral infection but in some cases the reason for the absence of a regenerative response is not known.

Anaemia is induced by haemolysis and sequestration. The attachment of organisms to red blood cells (RBC) results in direct damage to the RBC membrane. This leads to an increase in osmotic fragility and a shortened RBC lifespan (13). RBC damage may expose hidden erythrocyte antigens or result in alteration of erythrocyte antigens inducing production of antierythrocytic antibodies. Antibodies may also be directed against the organism itself resulting in RBC destruction as an "innocent bystander" (14). Recently immunoblot studies (15) have documented the presence of antibodies to CMhf antigens as early as 14 days post-infection. The same study showed the presence of antibodies specific for RBC antigens in sera from cats immune to CMhf that were not present in pre-immune sera. Such antibodies may be involved in immune-mediated RBC-destruction. Although some intravascular haemolysis may occur by direct damage to the RBCs, the majority of haemolysis is extravascular in nature. Macrophage erythrophagocytosis occurs in the spleen, liver, lungs and bone marrow. Splenic macrophages can also remove *H. felis* organisms from the surface of RBCs (in a process called "pitting"), returning unparasitised cells into the circulation (16). This may explain the rapid increase in PCV sometimes seen following clearance of *H. felis* from the circulation.

Carrier Status

It is believed that cats which recover from infection remain chronically infected with *H. felis* for an undetermined period of time, which may be lifelong. A recent study detected chronic infection by PCR in cats up to 6 months after initial infection despite doxycycline treatment (4). Parasitaemia is not generally visible on blood smears during this period and cats appear clinically normal. These cats appear to be in a balanced state in which replication of organisms is balanced by phagocytosis and removal (14), although reactivation of infection can occur and may result in clinical disease (5).

DIAGNOSIS

Examination of Blood Smears

The most commonly used method to diagnose *H. felis* infection is demonstration of organisms on the surface of erythrocytes by microscopic examination of a good quality Romanowsky stained blood smear. The organism is typically found on the periphery of RBCs and may be found singly, in pairs, or in chains. Morphologically on Wright-Giemsa stained blood smears CMhf is said to be approximately twice as large and substantially darker than CMhm (5), although in the author's opinion the strains cannot be reliably distinguished by appearance alone.

False positive diagnoses arise when artifacts caused by improper drying or fixation and stain precipitation are confused with the organism. The use of fresh filtered stain solutions is imperative. Organisms must also be distinguished from feline erythrocyte inclusions such as Howell-Jolly bodies and Pappenheimer bodies. Acridine orange and direct fluorescent antibody staining methods are reported to be more sensitive than standard Romanowsky stains but both these techniques require a fluorescent microscope. False negative diagnoses are also a problem. While parasitaemia can be heavy during acute infection, the rapid clearance of parasites from the blood can result in negative cytology within a few hours (15). High concentrations (1.5%) of EDTA anticoagulant have been reported to detach organisms from the erythrocyte surface within a couple of hours (15) and this has been cited as a reason for false negative diagnoses on smears made from EDTA anticoagulated blood. However the concentration of EDTA used in routine blood collection tubes is lower (0.15%) and preliminary work in our laboratory suggests that this concentration does not elute organisms from erythrocytes.

Conventional PCR

PCR is a powerful and sensitive molecular technique whereby DNA template is amplified exponentially to detectable levels. The specificity of the PCR assay is dependent on primer design and reaction conditions. Primers are short strands of DNA which act as a starting point for DNA synthesis when annealed to complementary sequences in template DNA. They should therefore be specific for the template being amplified i.e. CMhf and/or CMhm DNA sequences. Temperature cycling in the PCR machine allows for repeated denaturation of DNA, primer annealing and synthesis of DNA, allowing many copies of DNA to be made from a single copy of template. The PCR product is then detected by loading onto an ethidium bromide (EtBr) stained agarose gel and performing electrophoresis. Visualisation under UV illumination allows detection of product since EtBr binds to double stranded DNA.

PCR is known to be more sensitive than examination of blood smears for the detection of CMhf and CMhm. Westfall et al. (11) found that only 37.5% of samples were positive on cytopathology after experimental infection compared to 100% positivity with PCR. Recently, Jensen and colleagues (17) applied a PCR assay that is able to detect and distinguish both CMhf and CMhm to blood samples from 220 American cats. The overall prevalence of infection was 19.5%, with 4.5% of cats CMhf positive, 12.7% CMhm positive and 2.3% dual infected. Anaemic cats were more likely to be infected with either CMhf or both CMhf and CMhm, than non-anaemic cats. These findings in naturally infected cats support the experimental findings of differing pathogenicity with the different strains. A recent study using PCR on 426 UK cats (7) reported an similar prevalence of infection (18%) but very few cats were infected with CMhf (1.4%) or dual infected (0.2%), precluding evaluation of any association between CMhf and anaemia. CMhm infection, however, was common (16.9% of cats) and was not associated with the presence of anaemia. Further studies are required to determine whether geographical variation in the prevalence of CMhf and CMhm infection exists. Experimentally, cats become PCR positive as early as day 8 post-infection. They may then become PCR negative during antibiotic treatment, but usually become PCR positive again 3 to 35 days after completion of antibiotics (4, 5). Blood samples for *H. felis* PCR should not therefore be submitted during antibiotic treatment, unless the cat is not responding to treatment, since a negative result during antibiotic treatment does not rule out the possibility of the cat becoming of carrier status. 10% of healthy cats in a UK study (7) were positive for *H. felis* by PCR.

Thus, it is apparent that a positive PCR result does not equate with clinical disease, making interpretation of results problematic. A positive PCR result should be interpreted in line with the strain identified, the haematological findings and clinical signs reported.

Real time PCR

Real time PCR synthesises DNA exponentially, in the same way as conventional PCR, but the PCR product can be detected as it is being made. A fluorescent labelled probe is used and this also confers additional specificity to the assay. The probe anneals specifically to the PCR product made, and is then cleaved by the enzyme synthesising DNA in the PCR, resulting in the release of the fluorescent reporter from a nearby quencher. The amount of fluorescence (measured at each cycle in the real time machine) is therefore proportional to the amount of PCR product made. Real time PCR is advantageous over conventional PCR because the reaction is carried out in sealed tubes with no need to run gels. This reduces the risk of PCR amplicon carry over and makes the assay very rapid. Importantly, reliable quantification of starting template is possible since the cycle number at which a threshold level of fluorescence is reached (due to PCR product synthesis) will be proportional to the amount of starting DNA template present in the sample i.e. the greater the amount of starting template, the sooner (a lower cycle number) the threshold level will be reached. The development of a real time PCR assay in our laboratory has allowed quantification of CMhf and CMhm DNA in blood samples collected from naturally and experimentally infected cats. The assay has a sensitivity of 3 and 8 starting copies of template DNA of CMhm, and CMhf respectively. This assay may provide valuable information on the significance of a positive PCR result and may prove useful in the assessment of response to treatment with antibiotics.

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