Anaplasma phagocytophilum infection in horses in the Netherlands

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EQUINE granulocytic anaplasmosis is a tickborne disease caused by the obligate intracellular bacterium *Anaplasma phagocytophilum* (previously *Ehrlichia equi*), which can elicit febrile disease in animals and human beings (Dumler and others 2001). The disease has previously been referred to as equine granulocytic ehrlichiosis, and is transmitted in Europe by *Ixodes ricinus* ticks. Ticks of the *I ricinus* complex also act as vectors in the spread of *Borrelia burgdorferi* from one animal to another, and co-infections of *A phagocytophilum* and *B burgdorferi* have been confirmed in horses (Chang and others 2000a, Magnarelli and others 2000).

Equine granulocytic anaplasmosis was first described in the USA in 1969 (Gribble 1969), and has since been reported in other countries, including Switzerland, Sweden, France, Germany, Italy and the UK (Korbutiak and Schneiders 1994, Artursson and others 1999, Bermann and others 2002, Von Loewenich and others 2003, Alberti and others 2005). Following an incubation period of approximately 10 days (Gribble 1969, Pusterla and others 1999b, 2002), infected horses may experience subclinical disease or develop overt signs that include fever, depression, inappetence, a reluctance to move and distal limb oedema. The disease can be self-limiting when untreated, and clinical signs usually last from seven to 14 days (Gribble 1969). These signs, however, are not pathognomonic for the disease, and demonstration of granulocytic inclusions, either morulae or initial bodies, in Wright-Giemsa- or haematoxylin and eosin-stained blood smears can confirm a clinical diagnosis (Gribble 1969, Engvall and Egenvall 2002). These organisms can be found microscopically in peripheral blood only for a few days in the acute stage of the disease (Gribble 1969, Stannard and others 1969, Rikihisa 1991), and molecular techniques like PCR (Engvall and others 1996, Pusterla and others 1999a) or serology (Van Andel and others 1998, Artursson and others 1999) can be valuable tools in the confirmation of infection. This short communication describes the analysis of 61 blood samples obtained from horses with fever of unknown origin, for the presence of *A phagocytophilum*, *B burgdorferi*, *Babesia caballi* and *Theileria equi*.

Blood samples from 61 horses, which had been admitted to the authors' clinic with fever of unknown origin between 2002 and 2005, were collected in EDTA tubes. Quick stain haematoxylin and eosin blood smears were made of buffy coat and analysed microscopically for granulocytic and erythrocytic inclusions. In addition, Wright-Giemsa-stained blood smears were made and analysed at the veterinary diagnostic laboratory, Faculty of Veterinary Medicine, Utrecht University. The remaining blood was stored at –20°C, until DNA was extracted using a DNA extraction kit (Qiagen), according to the manufacturer's instructions.

PCR products for the detection of *Anaplasma*, *Borrelia*, *Babesia* and *Theilera* species by reverse line blot (RLB) hybridisation were amplified in an automated thermocycler (Bio-Rad Laboratories) as described by Schouls and others (1999), Bekker and others (2002), Nijhof and others (2005). RLB hybridisation was performed as described by Nijhof and others (2005). Two additional and previously undescribed species-specific oligonucleotide probes for the detection of *B caballi* and *T equi* were incorporated in the RLB. They were deduced from gene sequences available from GenBank and synthesised by Isogen Life Science (Table 1).

TABLE 1: Oligonucleotide probes used for the detection of *Babesia caballi* and *Theileria equi*

Oligonucleotide probe specificity	18S rRNA sequence (5′ to 3′)	GenBank accession number	Melting point (°C)
B caballi	GTGTTTATCGCAGACTTTTGT	AY309955, AY534883 and Z15104	52·4
T equi	TTCGTTGACTGCG(CT)TTGG	AY150062, AY150063, AY150064 and Z15105	56·9

There were 10 stallions, 26 geldings and 25 mares of Dutch warmblood, quarter horse, Friesian horse, Icelandic horse, Shetland pony, Belgian draft horse breeds in the study, ranging in age from one to 16 years. Equine granulocytic anaplasmosis was diagnosed in six horses of different breeds, aged between four and 15 years. Clinical signs consisted of lethargy, pyrexia (38·7 to 41·1°C), oedema of the hindlegs or all four legs, and partial or total anorexia (Table 2). Haematology revealed a relatively low packed-cell volume (23 to 29 l/l, reference range 30 to 42 l/l), and in four of these horses a marked thrombocytopenia (22 to 26 x 10^9/l, reference range >100 x 10^9/l) was present (Table 2).

Blood smears revealed neutrophils with cytoplasmic inclusions (Fig 1) consistent with *A phagocytophilum* infection in five of the six horses, and PCR-RLB confirmed the diagnosis by showing positive results for *A phagocytophilum* in these five horses plus one other. The clinical signs were most severe in a four-year-old Friesian gelding (horse 2), which showed ataxia, became recumbent and was euthanased on humane grounds due to the deterioration in its condition despite intravenous treatment with 7 mg/kg oxytetracycline every 24 hours. The other five horses showed mild clinical signs comprising pyrexia, lethargy, partial anorexia and oedema of the hindlimbs. Clinical disease in these horses resolved without treatment.

PCR-RLB and stained blood smears for all the horses were negative for all other tickborne pathogens.

It has been suggested that horses younger than three to four years of age generally experience less severe clinical disease (Gribble 1969, Madigan and Gribble 1987) when

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TABLE 2: Clinical signs, haematology and PCR-reverse line blot (RLB) results for horses infected *Anaplasma phagocytophilum*

Horse	Age (years)	Breed	PCV (l/l)	Platelet (x 10^9/l)	Lethargy	Temperature (°C)	Oedema	Ataxia	Anorexia	Blood smear result	PCR-RLB for *A phagocytophilum*
1	10	Dutch warmblood	29	100	Yes	40·4	None	No	Partial	–	+
2	4	Friesian	28	344	Yes	41·1	All legs	Yes	Total	+	+
3	14	Dutch warmblood	23	22	Yes	38·7	Hindlegs	No	Partial	+	+
4	15	Dutch warmblood	24	26	Yes	40·6	Hindlegs	No	Partial	+	+
5	15	Dutch warmblood	25	23	Yes	41·0	Hindlegs	No	Partial	+	+
6	10	Dutch warmblood	29	22	Yes	41·0	Hindlegs	No	Partial	+	+

PCV Packed-cell volume, – Negative, + Positive

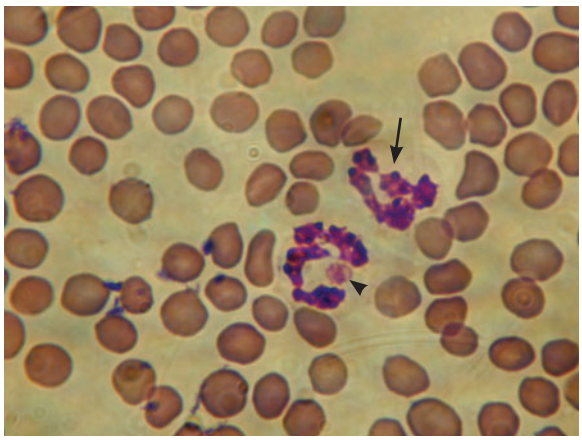

FIG 1: Blood smear from horse 4 showing a neutrophil with cytoplasmic inclusion (arrowhead) of *Anaplasma phagocytophilum* and one normal neutrophil (arrow)

infected with *A phagocytophilum*. However, in the present study, the youngest positive horse, which was four years of age, showed severe clinical signs and did not survive, whereas the other positive horses were aged between nine and 15 years and their infections resolved without treatment. To confirm an infection with tickborne pathogens in horses, molecular tests such as PCR in combination with RLB could improve the sensitivity as well as the specificity because, compared with stained blood smears, both live and dead organisms, as well as intact and fragmented DNA of multiple parasites can be detected in one test (Chang and others 2000b). In the present study, however, five of the 61 horses were diagnosed as being positive for *A phagocytophilum* using a haematoxylin and eosin-stained as well as a Wright-Giemsa-stained blood smear, and only one further positive horse was identified using the more sensitive and specific, but also expensive and time consuming PCR-RLB. Stained blood smears and PCR-RLB were also used to detect *B caballi* and *T equi*, but for the detection of *B burgdorferi* only PCR-RLB was used. No other tickborne pathogens (except *A phagocytophilum*) were found in any of the blood samples from the 61 horses.

Clinical anaplasmosis in horses is probably still underdiagnosed in the Netherlands as most horses recover spontaneously and clinical signs are similar to those caused by infections with other pathogens such as *B burgdorferi*, *B caballi*, *T equi*, equine herpesvirus, equine infectious anaemia virus, equine arteritis virus and Leptospiraceae.

In the authors' experience, microscopic interpretation of a buffy coat haematoxylin and eosin-stained blood smear is a sensitive and practical diagnostic tool for veterinarians considering possible infection with *A phagocytophilum* in horses with pyrexia, but some cases may require PCR testing for diagnosis.

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