

# On *Anaplasma phagocytophilum* in Horses

Peter Franzén

*Faculty of Veterinary Medicine and Animal Science  
Department of Clinical Sciences  
Uppsala*

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### Abstract

Equine Granulocytic Anaplasmosis (EGA) is an acute tick-borne infection caused by *Anaplasma phagocytophilum*. The bacterium can infect various animal species and humans. Persistence of infection is documented in ruminants and is shown experimentally in dogs, but it is unknown whether it occurs in horses or if EGA induces longer-term clinical changes in horses.

This thesis includes results and analyses of a serosurvey of the exposure level and association with clinical disease (excluding acute EGA) in over 2000 horses throughout Sweden. It also includes an experimental infection study of six horses using a Swedish isolate of the bacterium.

Overall 17% of the horses were seropositive with large geographical variations. However, seropositivity was not statistically associated with any increase in clinical sign or disease in the animals.

In the experimental study acute disease was readily induced and the clinical disease was similar to EGA described from USA. Laboratory data showed that the PCR test had the widest diagnostic window for EGA with positive signals days before onset of clinical signs. Clinically, diagnostic inclusions (morulae) in leukocytes in blood smears were visible first after a few days into clinical disease and lasted shorter time than did the PCR signal.

One horse died suddenly 2 days into the acute disease, with post mortem changes of general vasculitis and hyaline thrombi in the kidneys suggesting disseminative intravascular coagulation (DIC). This was the first documented case of death attributed probably solely to EGA.

The remaining horses were closely monitored for more than 3 months after spontaneous recovery from EGA. Some of the horses became transiently PCR positive during this period, mainly close in time after selected interventions to mimic stress. However no detectable clinical abnormalities were found during this follow-up period. At post mortem there were no macroscopic or microscopic changes in any of the tissues that could be associated with persistence of infection.

In conclusion this thesis shows that for EGA; seropositive horses in Sweden are common but do not have increased levels of clinical disease; PCR provides the earliest laboratory diagnosis for acute EGA-infection;

sudden death due to EGA can occur; and the organism can persist in some horses for up to 3 months post recovery but was clinically and pathologically silent.

*Keywords:* *Anaplasma phagocytophilum*, horse, serosurvey, PCR, experimental infection, persistence.

*Author's address:* Peter Franzén, Department of Clinical Sciences, SLU  
Box 7054 SE-750 07 Uppsala Sweden  
*E-mail:* peterfransen.pf@gmail.com

To my mother



# Contents

<b>List of Publications</b>	<b>9</b>
<b>Abbreviations</b>	<b>10</b>
<b>Introduction</b>	<b>11</b>
Historical background	11
The bacterium <i>Anaplasma phagocytophilum</i>	12
The vector and the reservoir	15
Clinical signs – different species	17
Subclinical infection	18
Diagnosis	18
Treatment, immunity and prevention	18
Seroprevalence studies	20
Experimental infection in horses and different strains	21
Persistence of infection	24
<b>Aims of the studies</b>	<b>27</b>
<b>Comments on materials and methods</b>	<b>29</b>
The seroepidemiologic study (Paper I)	29
Sampling	29
Questionnaires	29
Serologic test to <i>Anaplasma phagocytophilum</i>	30
Healthy group of horses	30
Statistical analysis	30
Experimental infection (Papers II, III and IV)	31
Animals	31
Experimental infection and inoculate	32
Clinical evaluation and sampling of horses during the acute stage (Paper II)	32
Clinical evaluation, sampling and experimental design during the follow-up period (Paper IV)	33
Serology, PCR and hematology	33
Post mortem examination	35

Immunohistochemistry and DNA extraction for PCR on tissue samples	36
<b>Comments on results and discussion</b>	<b>39</b>
Serosurvey (Paper I)	39
Experimental infection	42
Acute stage (Paper II)	42
Death due to <i>A. phagocytophilum</i> (Paper III)	45
Convalescent phase (Paper IV)	47
<b>Summary and conclusions</b>	<b>55</b>
<b>References</b>	<b>57</b>
<b>Acknowledgements</b>	<b>66</b>

## List of Publications

This thesis is based on the following papers which will be referred to by their Roman numerals:

I Egenvall, A., Franzén, P., Gunnarsson, A., Engvall, E.O., Vågsholm, I., Wikström, U-B., Artursson, K. (2001) Cross-sectional study of the seroprevalence to *Borrelia burgdorferi sensu lato* and granulocytic *Ehrlichia* spp. and demographic, clinical and tick-exposure factors in Swedish horses. *Prev. Vet. Med.* 49, 191-208.

II Franzén, P., Aspan, A., Egenvall, A., Gunnarsson, A., Åberg, L., Pringle, J. (2005). Acute clinical, hematologic, serologic, and polymerase chain reaction findings in horses experimentally infected with a European strain of *Anaplasma phagocytophilum*. *J. Vet. Intern. Med.* 19, 232-9.

III Franzén, P., Berg, A-L., Aspan, A., Gunnarsson, A., Pringle, J. (2005). Death of a horse infected experimentally with *Anaplasma phagocytophilum*. *Vet. Rec.* 160, 122-5.

IV Franzén, P., Aspan, A., Egenvall, A., Gunnarsson, A., Karlstam, E., Pringle, J. (2008) Molecular evidence for persistence of *Anaplasma phagocytophilum* in the absence of clinical changes in horses following recovery from acute experimental infection. Submitted.

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## Abbreviations

ab	antibody
AP	alkaline phosphatase
BCIP	5-bromo-4-chloro-3'-indolyphosphate p-toluidine salt
C	Celsius
CBC	complete blood count
DNA	deoxyribonucleic acid
DIC	disseminative intravascular coagulation
EDTA	ethylenediaminetetraacetic acid
EGA	equine granulocytic anaplasmosis
EGE	equine granulocytic ehrlichiosis
E. spp.	<i>Ehrlichia</i> species
ELISA	enzyme-linked immunosorbent assay
FITC	fluorescein-isothiocyanate
GMT	geometric mean titer
HGE	human granulocytic ehrlichiosis
IFA	indirect immunofluorescence assay
M	Molar
mM	millimolar
NBT	nitro-blue tetrazolium chloride
NSAID	non-steroid anti-inflammatory drug
OR	odds ratio
PCR	polymerase chain reaction
p.i.	post inoculation
rRNA	ribosomal ribonucleic acid

# Introduction

## Historical background

Tick borne fever in domestic ruminants in Europe has been known for at least 200 years (Stuen 2007) though it was not until 1932 the disease was associated with actual tick infestation and the infective agent involved was assumed to be a *Rickettsia* (Gordon et al. 1932a, Gordon et al. 1932b, Gordon et al. 1940). The organism was classified as *Rickettsia phagocytophila* (Foggie 1951) and later named *Ehrlichia phagocytophila* in Bergeys manual of determinative bacteriology (Philip 1974). The genus *Ehrlichia*, within the family *Rickettsiaceae* was named to honour the German bacteriologist Paul Ehrlich. Ehrlichial organisms grow strictly intracellularly, with different target cells for different species of *Ehrlichia*. *Ehrlichia phagocytophila* has predilection for granulocytic leukocytes, with aggregations of bacteria visible in the cytoplasm of neutrophils and occasionally in eosinophils on blood smears from infected animals. These form inclusion bodies also named “morulae”. The disease has also thus been named “granulocytic ehrlichiosis”.

During the last 40 years granulocytic ehrlichiosis has subsequently been recognized in a number of other animal species. In the horse it was first described from California as a febrile disease with inclusion bodies detectable in the cytoplasm of granulocytes (Gribble 1969), and resembling the clinical disease in ruminants. The agent infecting horses was called *Ehrlichia equi*.

Later a febrile disease with detectable morulae in granulocytes was recognized in dogs (Madevall and Gribble 1982, Bellström 1988). The first confirmed clinical case in cats was reported in 1999 (Bjöersdorff et al. 1999). Further, a similar infection with clinical febrile disease and inclusion

bodies visible in neutrophilic granulocytes of febrile humans was also described (Bakken et al. 1994, Chen et al. 1994). The causative agent in humans was called “the human granulocytic ehrlichiosis-agent” - the HGE-agent since it was not known if it was identical to *E. phagocytophila* or *E. equi*. The infection has also been recognized in the wild fauna, for example in rodents and roe-deer. Moreover, several other mammalian species have been shown to be both seropositive and PCR positive (Stuen 2007).

### The bacterium *Anaplasma phagocytophilum*

The genera *Anaplasma*, *Ehrlichia*, *Cowdria*, *Neorickettsia* and *Wolbachia* are all intracellular bacteria infecting eukaryotic cells. Historically they have been placed in taxa based on morphological epidemiological and clinical characteristics. The “*Ehrlichia* group” consisted of a number of bacteria infecting different species and gave rise to different diseases, apart from *E. phagocytophila*, *E. equi* and the HGE-agent, including Potomac horse fever in horses (*Ehrlichia risticii* - monocytic), monocytic senettsue fever in man (*Ehrlichia senettsu*), *Ehrlichia ewingi* causing (another) granulocytic ehrlichiosis in dogs and humans and *Ehrlichia canis* causing chronic monocytic infection in dogs (Table 1). Recent genetical analyses of 16S rRNA, groESL and surface protein genes have resulted in phylogenetic trees showing the genetic “relationship” among different genera. This has led to a recent reorganization of taxonomy (Dumler et al. 2001) resulting in a new classification and new names for certain bacteria based on the genetic similarity (Table 1). In this new classification, *Ehrlichia equi*, *Ehrlichia phagocytophila* and the HGE-agent, were deemed to be so closely related genetically that they were amalgamated into the same and “new” species, named “*Anaplasma phagocytophilum*” (Dumler et al. 2001). The new species *A. phagocytophilum* is described as being a gram-negative coccoid to ellipsoidal, often pleomorphic bacterium. Two ultra-structural morphologies may occur; a larger reticulate form and a smaller dense form containing condensed protoplasm (Dumler et al. 2001). The bacterium is an obligate intracellular organism that infects predominantly blood cells, principally granulocytes and especially neutrophils (Rikihisa 1991). In the target cell, bacteria are internalised in separate phagosomes and avoid degradation by inhibiting the phagosome from fusing with lysosomes (Rikihisa 1991, Carlyon and Fikrig 2003, Carlyon and Fikrig 2006). The vacuole enlarges as the bacteria multiply by binary fission and eventually forms inclusion bodies in the cytoplasm. These inclusions or “morulae” can be identified on microscopic evaluation of blood smears from infected

individuals in the acute febrile stage of disease, within the cytoplasm of granulocytes (especially in neutrophils) (Gribble 1969, Gribble 1970, Rikihisa 1991) Figure 1.

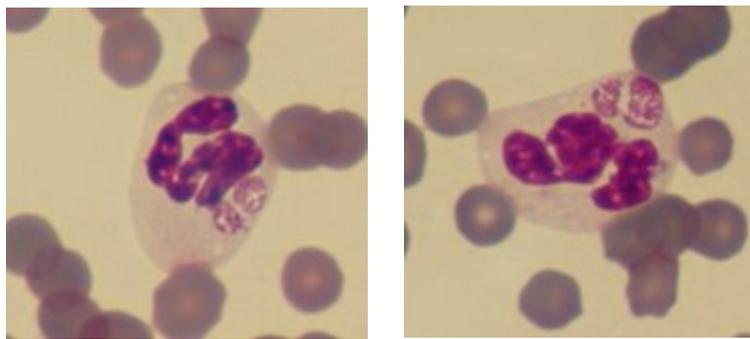


Figure 1. Bloodsmear with neutrophil containing two morulae in the cytoplasm. (Pictures courtesy of Dr. Harold Tvedten).

The morulae in equine neutrophils can vary from 0,5 to 5  $\mu\text{m}$  in diameter, (Gribble 1969 and 1970, Lewis 1976, Sells et al. 1976), and may contain more than one and up to 20 bacteria. On blood smears morulae may be seen in from less than 1% up to 20-30% of the neutrophils (Madigan and Pusterla 2000). They stain deep blue to pale blue grey with giemsa and are fluorescent with acridine orange stains (Gribble 1969). More than one morula is often in a single infected cell. It is not possible to grow the bacterium in standard cell free media. Successful cultivation may be achieved through the use of the human promyelocytic leukemia cell line HL-60, the tick cell lines IDE8 and IDE6 and monocytic and granulocytic progenitors from human bone marrow (Munderloh et al. 1996, Klein et al. 1997b, Munderloh et al. 1999). This is mainly performed in research facilities and such techniques are seldom available for use in routine diagnostic laboratories.

The bacterium is sensitive to oxytetracycline. Although not commonly used clinically, rifampicin, rifabutin and trovafloxacin have also been reported to be effective *in vitro* whereas other antibiotics, such as those in the beta lactamase group, are reported to be ineffective against this bacterium (Brouqui and Raoult 1992, Brouqui and Raoult 1993, Klein et al. 1997a, Horowitz et al. 2001, Davidson and Bjöersdorff 2001).

Table 1. Bacteria previously in the “*Ehrlichia* group” according to Rikihisa (2000), with their new names, and which species, disease name and cell type infected for each of the pathogens

<i>Ehrlichia</i> group and Pathogen	Affected species	Disease	Target cell
1. <i>Ehrlichia canis</i>	Dog	Canine ehrlichiosis	Monocyte
	Human	Rare, no disease	
1. <i>E chaffensis</i>	Human	Human monocytic ehrlichiosis	Monocyte
	Dog	Unnamed	
	Goat	Unnamed	
1. <i>E ewingi</i>	Human	Human ehrlichiosis	Granulocyte
	Dog	Canine granulocytic ehrlichiosis	
2. <i>Anaplasma phagocytophilum</i> (formerly the HGE-agent) (formerly <i>Ehrlichia equi</i> )	Human	Human granulocytic ehrlichiosis	Granulocyte
	Horse	Equine granulocytic ehrlichiosis	
	Dog	Canine granulocytic ehrlichiosis	
	Cat	Feline granulocytic anaplasmos	
(formerly <i>Ehrlichia phagocytophila</i> )	Ruminant	Tick-borne fever	
2. <i>Anaplasma platys</i> (formerly <i>Ehrlichia platys</i> )	Dog	Canine cyclic thrombocytopenia	Thrombocyte
2. <i>Anaplasma marginale</i>	Cattle	Anaplasmosis	Erythrocyte
3. <i>Neorickettsia sennetsu</i> (formerly <i>Ehrlichia sennetsu</i> )	Human	Sennetsu fever	Mononuclear leukocyte
3. <i>Neorickettsia risticii</i> (formerly <i>Ehrlichia risticii</i> )	Horse	Potomac horse fever	Monocyte

### The vector and the reservoir

The tick responsible for transmitting *A. phagocytophilum* in Europe is predominantly *Ixodes ricinus*, whereas in the US the western black-legged tick *Ixodes pacificus* and also *Ixodes scapularis* seem to be the primary vectors. Tick transmission is believed to be the only epidemiologically important means of acquiring infection. However, blood contamination may be a rare way for transmission as human infection through blood transfusion has been reported (McQuiston et al. 2000).

The tick has a four stage life cycle: egg, larvae, nymph and adult (male or female) (Figure 2.) The larvae and the nymph require one blood meal each to develop into the next stage. The adult female needs one large blood meal to produce eggs. The larvae and the nymphs normally suck blood for 3-5 and 5-7 days respectively from mammals and birds of all sizes. The adult females suck blood during 7-13 days from medium to large mammals including the cat, dog, hare, deer, human, sheep and horse. Birds are believed to be more seldom involved as a reservoir. Each stage of development lasts approximately one year and the entire life cycle often takes 3 years, but may vary from 2-6 years (Balashov 1972). Transovarial transmission of the bacteria from an adult tick to eggs is not believed to occur. Therefore, immature ticks (larvae and nymphs) must feed on infected animals to acquire and transmit infection to the successive life stages (nymphs and adults respectively). This phenomenon is known as trans-stadial transmission (Telford et al. 1996, Hodzic et al. 1998). However, trans-stadial maintenance of *A. phagocytophilum* appears to be ineffective and may need a large load of bacteria to persist through the moult (Ogden et al. 2002).



Figure 2. The stages (except the egg stage) of the life cycle of *Ixodes ricinus* in their relative size. The nymph is approximately 1.5 mm long. The larvae have three pairs of legs, whereas later stages have four leg pairs. (Picture courtesy of Jan Chirico and Gunilla Olsson).



Figure 3. *Ixodes* tick on the leg belonging to the main supervisor.

The adult female is known to infect recipients. Whether the earlier stages and the adult male are able to infect recipients is not known at present time (J. Chirico, SVA, Uppsala, Sweden personal communication).

The maintenance of anaplasma infection in nature is dependent on the existence of animal hosts that can harbour anaplasma organisms for sufficient periods of time. The host of the infectious agent must also act as a feeding host for the vector. Wild rodents have been suggested as reservoirs for *A. phagocytophilum* (Ogden et al. 1998a, Liz et al. 2000) and also sheep and deer (Stuen et al. 1998b, Alberdi et al. 2000, Ogden et al. 2002). Birds are believed to act as carriers of infected ticks, rather than serving as reservoir themselves (Bjöersdorff et al. 2001).

Serologic evidence of host reservoir capacity has further been reported in rodents including rats, mice and voles, elk and roe-deer, foxes, raccoons, opossums, chipmunks and coyotes (MacLeod and Gordon 1933, Telford et al. 1996, Walls et al. 1997, Lockhart et al. 1997, Ogden et al. 1998a, Foley et al. 1998, Alberdi et al. 2000, Liz et al. 2000).

### Clinical signs – different species

Among **sheep, goats and cattle** in Europe tick-borne fever is mainly reported as a febrile disease. Clinical signs may vary from mild detectable illness to severe febrile disease, sometimes accompanied by sequelae such as secondary or opportunistic infections, hemorrhage, abortions and sterility (Foggie 1951, Ervin 1981, Dumler et al. 2001). In ruminants the disease is also called pasture fever. In lambs there is often fever and acute disease, even sudden death can eventually occur (Stuen et al. 2003). However, the infection in lambs is also associated with predisposition for other infections (Foggie 1956, Foggie 1957) such as bacterial pneumonia (i.e. staphylococcosis and pasteurellosis), and with reduced weight gain (Stuen et al 2002b). Classical signs in lactating cows include high fever, often more than 41°C and more than 50% reduction in milk production (Cranwell and Gibbons 1986, Larsson et al. 2006).

In **humans** the infection manifests as an acute nonspecific febrile illness characterized by high fever (temperature >39°C), rigor, generalized myalgia, severe headache and malaise (Bakken et al. 1994, Agüero-Rosenfeld et al. 1996, Bakken et al. 1996, Wallace et al. 1998, Bakken and Dumler 2000), and has sometimes been called “summer influenza”. The illness in humans often lasts only for a few days. Rarely, however, patients have been ill for several weeks in the absence of appropriate antibiotic therapy (Bakken and Dumler 2000) especially in the 90’s when the disease

was “new” in humans and awareness of this infection in humans was not wide-spread. Fatalities are rare but may occur in less than 1% of cases, especially in elderly and immuno-compromised persons (Bakken and Dumler 2000, Olano and Walker 2002).

In the **horse** EGA is described as an acute disease with an incubation period of less than 14 days, characterized by high fever, depression, inappetence, staggering or ataxia, distal limb edema and hematologic alterations, such as thrombocytopenia, neutropenia, lymphopenia and mild anaemia (Gribble 1969, Madigan 1993). Petechiation of mucous membranes and icterus may also occur. Clinical signs usually disappear without treatment within 7-14 days in horses but a more rapid recovery will occur with oxytetracycline treatment (Madigan and Pusterla 2000).

In the **dog** the typical clinical signs of granulocytic ehrlichiosis include anorexia, depression, fever, and reluctance to move (Greig et al. 1996, Egenvall et al. 1997).

### Subclinical infection

Infection without development of clinical signs of disease appears to exist in sheep (Foggie 1951, Stuen and Bergström 2001b), cattle (Hudson 1950, Larsson et al. 2006), horses (Madigan et al. 1990, Madigan and Pusterla 2000), dogs (Egenvall et al. 2000a, Egenvall et al. 2000b) and humans (Dumler et al. 2005, Dumler et al. 1997). The proportion of infected cases that actually develop clinical disease is not known.

### Diagnosis

Diagnostic criteria for EGA include presence of typical clinical signs of acute disease, demonstration of inclusion bodies in neutrophils and presence of antibodies in serum (most often using IFA-test but also ELISA-tests are available) and a positive PCR signal for DNA from *Anaplasma phagocytophilum*; the latter being the most sensitive diagnostic tool (Pusterla and Madigan 2007). Differential diagnoses to EGA include viral arteritis, purpura heemorrhagica, encephalitis, liver disease (Madigan and Pusterla 2000) or any systemic infection with fever and no localized signs.

### Treatment, immunity and prevention

Effective treatment for EGA is accomplished using oxytetracycline 6-7 mg/kg i.v. once daily for 5-7 days (Madigan 1993, Madigan and Pusterla

2000). Fever will usually disappear within 12-24 hours and other accompanying signs abate within a few days. A prompt recovery after oxytetracycline injection is considered to support the diagnosis. Mortality is very low but fatalities may occur from trauma due to incoordination of the limbs in the acute stage or due to secondary infection (Gribble 1969, Madigan and Pusterla 2000). In rare cases fever with anaplasma bacteremia has recurred some weeks after first infection (Madigan 1993). Possible explanations, although not well evaluated, may include exacerbation of persisting bacterial infection following a too short course of antibiotic treatment (Madigan 1993) or possibly reinfection through new tick-bites before immunity has fully developed.

The time of immunity after clinical disease is not completely known. In inoculation studies horses have been refractory to develop disease again when challenged 2, 5, 6, 12 and in one case after a 20-month period (Gribble 1969) after initial clinical disease. This is consistent with another report where ponies that had recovered from clinical disease after experimental infection, failed to develop detectable reinfection upon challenge (Nyindo et al. 1978). Antibodies have been described to persist at least for 5-12 months after natural infection (Van Andel et al. 1998, Artursson et al. 1999) and at least 300 days after experimental infection (Nyindo et al. 1978).

The time for transmission of the infective agent has been studied in experimental infections and transmission is thought to occur within a window of 40-48 hours of feeding. It has thus been suggested that through removal of ticks prior to 36 hours of feeding only few clinically apparent anaplasma infections would occur (Hodzic et al. 1998, Katavolos et al. 1998). Further, according to Lotric-Furlan et al. (2001) the time from initial tick infestation to transmission of the HGE-agent is at least 24 hours.

Continuous removal of ticks and use of insect repellents appears to be of some use to prevent infection. These aspects are similar to the situation of the tick-borne infection with *Borrelia* species where transmission of the infective agent is known not to, or only rarely to occur within the first 24 hours (Piesman et al. 1987, Kahl et al. 1998, Ramamurthy et al. 2002, Hojgaard et al. 2008) as opposed to the chain of infection for the tick-borne virus of tick borne encephalitis in humans where the agent may be transferred earlier after tick bite. There is presently no vaccine available against *A. phagocytophilum* (Pusterla and Madigan 2007).

## Seroprevalence studies

A number of seroprevalence studies of antibodies to *A. phagocytophilum* in horses have been reported from different parts of the world with seroprevalence varying from 0% to 17% in investigated horses (Table 2) (Madigan et al. 1990, Bullock et al. 2000, Egenvall et al. 2001, Teglas et al. 2005, Leblond et al. 2005, Levi et al. 2006, Amusatogui et al. 2006). Inclusion criteria and clinical information on the material have varied. It has been recorded in endemic areas that at one farm 50% of healthy horses can be seropositive (Madigan et al. 1990).

In selected serosurveys of humans similar seroprevalences have been noted. Of 361 healthy German recruit soldiers 14.9% were seropositive and another 5.3% seroconverted during military service without developing signs of HGE (Woessner et al. 2001). In another study, of 159 adult blood-donors in New York state, 11.3% were seropositive to HGE and seropositivity was further noted in 21% of 42 patients with borrelia-caused erythema migrans (Aguero-Rosenfeld et al. 2002). In Sweden, 185 of the 356 permanent residents on the Koster islands coast were screened for HGE-antibodies and 11.4% tested seropositive (Dumler et al. 1997). Granulocytic anaplasmosis is considered to be an emerging disease in humans as well as in dogs and horses in the western hemisphere (Rikihisa 2000, Leblond et al. 2005, Blanco and Oteo 2006, Beltrame et al. 2006, Santos et al. 2006).

Table 2. Selected reports of serosurveys of antibodies to *Anaplasma phagocytophilum* in horses

Study	Year of publication	Nr of horses included	% sero-positive *	Geographic area	Population
Madigan et al.	1990	240	10.4	California US - foothills	“Routine health care” cases and competing endurance horses
Madigan et al.	1990	95	3.1	California US -non-foothills	“Routine health care” cases and hospital patients with non-medical problems
Bullock et al.	2000	741	10.8	Minnesota, Wisconsin US	Serobank -horses (543) originally tested for infectious anaemia- plus healthy horses sampled for this study (198)
Egenvall et al. #	2001	2018	16.6	Sweden	400 healthy horses and 1616 randomly picked patients visiting equine clinics with detailed clinical status
Teglas et al.	2005	74	13	Guatemala	Owners brought horses from 7 villages for “preventive veterinary care and minor medical treatment”
Leblond et al.	2005	424	11.3	France - southern	
Levi et al.	2006	300	0	Israel	Population “from farms throughout Israel”
Amusatogui et al.	2006	46	6.5	Spain - north-western	Not defined

\* IFA was used for antibody detection in all surveys except for Leblond’ et al. where ELISA was used.

# =included in this thesis

### Experimental infection in horses and different strains

The clinical and hematological findings on EGA were described in detail by Gribble in his pioneering work (Gribble 1969, Gribble 1970). He infected a large number of horses, followed them clinically and euthanized them at

different stages into clinical disease, describing the pathological changes in detail. The agent used in those studies was the strain found in naturally infected horses in California and it was by then named *Ehrlichia equi*. Later, Nyindo et al. (1978) performed experimental infections with the same agent, with the purpose of studying the immune response. Both humoral and cell mediated immunologic responses were demonstrated (Nyindo et al. 1978).

Development of new techniques has made it possible to analyze the DNA sequence of specific genes. The 16S rRNA gene has become a standard method to assess the phylogenetic relationship between bacterial genera and species, since it is a stable and well conserved gene. It was reported that the isolate of *A. phagocytophilum* (by then named *Ehrlichia* species) found in infected horses and dogs in Sweden (Johansson et al. 1995) differed in 3 positions in 16S rRNA gene from the deposited Californian *E. equi* agent and in two positions from the *Ehrlichia phagocytophila* deposited in Genbank by Anderson et al. (1991). However it was identical to the agent found in clinically diseased humans – the so-called “HGE-agent” deposited in Genbank (Chen et al. 1994) with respect to the 16S rRNA gene.

Later, more isolates from Swedish horses showed the same 16S rRNA sequence (Björnsdorff et al. 2002) as did a horse isolate from Switzerland (Pusterla et al. 1998a) as well as isolates from the middle and eastern USA (Madigan et al. 1996, Bullock et al. 2000). After it was found that the HGE-agent was infective to horses and gave rise to disease in horses clinically indistinguishable from EGA (Madigan et al. 1995), the horse has been used as an animal model for the disease in humans. Several experimental HGE-agent infections in horses are reported (Table 3).

Isolates pathogenic for cattle (formerly named *Ehrlichia phagocytophila*) have failed to induce clinical disease when inoculated in horses (Pusterla et al. 1998b) and conversely, isolates from horses have failed to induce clinical disease in lambs and cattle (Stuen et al. 1998a, Pusterla et al. 2001) despite seroconversion in both instances. These findings suggest that, although *E. phagocytophila*, *E. equi*, the Swedish isolate from horses previously named *Ehrlichia* species and the HGE-agent are all currently included as a single species *A. phagocytophilum*, there appears to be differences in biologic capacity to induce clinical infection in different animal species.

Several 16S rRNA gene variants of *A. phagocytophilum* with minor nucleotid differences has been reported in isolates from sheep (Stuen et al. 2002a, Stuen et al 2003). Further, *A. phagocytophilum* isolates from different geographic locations have been sequenced also concerning other genes and it appears to exist geographic differences in the Ep-ank and groESL heat

shock operon genes (Chae et al. 2000, Massung et al. 2000). Further, global diversity in the MSP2 (44) outer membrane protein have been reported (Barbet et al. 2006).

Table 3. Experimental infections in horses with strains currently included in the species *A.phagocytophilum*

Author, year	Agent	Donor	No.	Reason for study	Infective dose €
Gribble 1969	<i>E. equi</i>	horse	43	describe the disease	
Nyindo 1978	<i>E. equi</i>	horse		study immune response	
Barlough et al. 1995	<i>E. equi</i> (after HGE)	horse	1	does HGE give immunity to <i>E. Equi</i>	
Madigan et al. 1995	HGE	human	1	test if HGE is infective to horses	
Barlough et al. 1996	<i>E. equi</i>	horse	1	to test a PCR	4.5x10 <sup>7</sup>
Franzén et al. 1998	<i>E. species</i> (Swed. isolate)	horse	1	can persistence occur?	2 x 10 <sup>7</sup>
Pusterla et al. 1998b	<i>E. phag.</i> &	cow	4	does <i>E. phag.</i> give rise to EGE in horses	4.6x10 <sup>7</sup>
Chang et al. 1998	HE	human	2	produce material for an IFA-test	
Reubel et al. 1998	Not specified	ticks	3	can <i>I. pacificus</i> exp. infect horses?	
Pusterla et al. 1999a	HE	horse	3	compare tick infection route with i.v. inf. route regarding	6.5x10 <sup>6</sup>
“	HE	human	1	bacterial burdon in infected	
“	HE	ticks	4	recipients	
Pusterla et al. 1999b	<i>E. phag.</i> & Swiss isolate	cow	2	does <i>E.phag.</i> infection in a horse give rise to immunity to	4.5x10 <sup>7</sup>
“	"HGE-like" Swiss isolate	horse	2	HGE-like infection	4.5x10 <sup>7</sup>
Pusterla et al. 2000	HE	cell culture	4	is HGE from cell culture infective in horses and in different passages	1x10 <sup>6</sup>
Pusterla et al. 2001	HGE and <i>E. equi</i> resp.	cell culture	2	positive control for inf. in cattle	1x10 <sup>6</sup> or 15x10 <sup>6</sup>
Franzén et al. # 2005	<i>A.phag.</i> Swed.isolate	horse	6	describe signs of EGA with a Swedish isolate and may persistence occur?	2x 10 <sup>7</sup> or 6 x10 <sup>6</sup>

\*=no of horses inoculated in study, €=no of infected neutrophils, & = *E. phagocytophila*, #=included in this thesis

## Persistence of infection

Extended persistence of infection by *A. phagocytophilum* has been described in sheep and cattle (Foggie 1951, Ogden et al. 1998b, Stuen et al. 1998b, Stuen et al. 2001a, Stuen och Bergström 2001b), dogs (Egenvall et al. 2000a, Egenvall et al. 2000b, Alleman et al. 2006) and calves (Larsson et al. 2006).

In dogs and calves persistence of infection appears to be clinically silent in the convalescent phase (Egenvall et al. 2000a, Larsson et al. 2006) whereas persistence of infection in lambs has been associated with predisposition to secondary infections such as pneumonia (Foggie 1951, Foggie 1956, Stuen 2007) and reduced weight gain (Stuen et al. 2002b).

In neither the horse nor humans has long term or persistence of the infection been described in the literature, either in case reports of more extensive studies or experimental infections (Nyindo 1978, Backen and Dumler 2000, Madigan and Pusterla 2000, Lotric-Furlan et al. 2001, Pusterla and Madigan 2007). There is however a single report on PCR-positive tissues 37 days after experimental infection with the HGE-agent in the horse (Chang et al. 1998) (with no clinical signs of disease after acute fever for 3–4 days). Moreover, in one own early equine challenge study PCR-positivity was demonstrated (without clinical signs after acute fever) 56 days after inoculation of a horse-derived isolate (Franzén et al. 1998).

However, among veterinarians in the field in Sweden and other European countries there are clinical suspicions that infection with *A. phagocytophilum* in the horse may also induce a milder but more chronic form of disease with other than the well described signs of acute disease. This is based predominately on associating seropositivity against *A. phagocytophilum* in horses with poorly defined clinical abnormalities such as fatigue, unwillingness to be ridden, poorly localised lameness or polyarthritis, behavioural problems, stiffness and musculoskeletal soreness (Bjöersdorff and Myrin-Carlsson 1998) and in selected cases, apparent improvement in clinical signs following treatment with tetracyclines.

Currently in Sweden it appears that a large number of horses are treated with oxytetracyclines because of suspected “chronic granulocytic anaplasmosis” despite the lack of substantial, scientific data available to support this. With this as a clinical background, and lack of data on longer

term effect of *A. phagocytophilum* infection in the horse, the studies in this thesis were performed.



## Aims of the studies

Aims of the studies were to  
through serosurvey:

- determine the seroprevalence of antibodies to *Anaplasma phagocytophilum* in a fairly large and defined population of horses in Sweden,
- evaluate in a large material of Swedish horses whether seropositivity to *Anaplasma phagocytophilum* was statistically associated with any sign of disease or any specific diagnosis (excluding acute granulocytic anaplasmosis),

through experimental infection:

- describe clinical signs and laboratory changes and their temporal changes in infection with a well defined Swedish isolate of *A. phagocytophilum*,
- study whether the infective agent may persist in horses after acute clinical disease had disappeared,
- evaluate whether clinical or pathological signs of eventual persistent infection with *A. phagocytophilum* was detectable after acute disease signs had abated.



## Comments on materials and methods

### The seroepidemiologic study (Paper I)

#### Sampling

A cross-sectional study was done using 17 equine clinics all affiliated to ATG (the Swedish Horse Race Totalizator board). The clinics were localized all over the country, from Malmö in the south to Boden in the north of Sweden. All kinds of horses (not only race horses) visit these clinics for a large variety of reasons. Some of the horses were not presented for disease problems but rather for prophylactic purposes or routine procedures (i.e. vaccination, teeth floating or pre-purchase examination).

Each clinic was randomly assigned (by lottery) 2 days each month during 12 months for sampling. There were from 2 to 19 samples per clinic per month. The number of requested samples from each clinic was proportional to the case-load for each clinic. During sampling days, the owners/trainers were informed and asked to participate in the study. Every horse visiting the clinic during sampling day was sampled (unless the owner did not want to participate) until the requested number of horses was reached.

#### Questionnaires

Participating owners/trainers were asked to complete a questionnaire regarding demographic parameters, tick exposure and health status of the horse (including if the horse was considered to have been healthy the previous year). Parameters asked for included; gender, age, breed, usage, geographic location, season, pasture access, outdoor confinement and tick exposure of the horse. Additionally, the veterinarians were asked to complete questions concerning the health status of the horse including

reason for visit and the final diagnosis. Recorded reasons for visit were pre-purchase examination, castration, vaccination, lameness examination, disease (specified by signs/diagnosis) or other. Recorded signs according to the owner (anamnestic details) or the veterinarian (clinical findings) included abortion, wasting, fever, diarrhoea, integumentary problems, unwillingness to be ridden, tendon injuries, stiffness, respiratory problems, fatigue, staggering, traumatic injuries and ophthalmologic disease, as well as referral for radiographic examination.

#### Serologic test to *Anaplasma phagocytophilum*

Two-fold dilutions of fresh sera were added to slides precoated with *E. equi* antigen. Bound antibodies were visualised by fluorescein-isothiocyanate (FITC)-conjugated rabbit-anti-horse immunoglobulin (*Ehrlichia equi* antigen Protate, St Paul, MN, USA) when slides were examined by fluorescence microscopy. In this test, an IFA titer  $\geq 1:40$  was considered positive. The test was accredited according to the EN ISO /IEC 17025 (International Organisation of Standardization) with inclusion of positive and negative controls. The limit for seropositivity was originally set by calibrating the test according to results obtained from external laboratories and the test is continuously checked in ring controls with other laboratories to ensure same levels of results. The IFA-test is the most used test according to the literature for serologic testing of *A. phagocytophilum*.

#### Healthy group of horses

The owner/trainer was asked whether the horse was considered totally fit for the task requested at the time of sampling, and also if this had been the case for the previous 12 months. The questions were designed to construct a “healthy group” of horses in the study. A horse was placed in the healthy group of horses if it was considered fit at the time of sampling as well as during the previous year and visited the clinic for procedures not associated with any prevalent disease (i.e. pre purchase examination, vaccination etc) with one exception. Horses were included in the healthy group also if they were considered healthy during the past 12 months and visited the clinic due to acute traumatic injuries such as wound injuries, splint bone fractures, sore sole as it was considered that acute traumatic events were unlikely to be associated with *Anaplasma* infection.

#### Statistical analysis

Geometric mean titers (GMT) were determined for all seropositive horses, for seropositive horses in the healthy group and for the remaining

seropositive horses i.e. the seropositive horses that were not considered healthy. For clinical signs, disease variables and for whether the horses were in the healthy group or not, the chi-square test was used to investigate associations and Fischer's exact test was used when expected values were too low for the chi-square test to be valid. To compare titers in healthy and "non-healthy" horses the continuity corrected Wilcoxon two-sample test was used.

Logistic regression was used to model seropositivity. Included factors in the unreduced models were: age, gender, breed, racing, region and season. A disease model (n=1983) was developed to evaluate associations between disease factors with respect to positive *Anaplasma* serotiter and a tick exposure model (n=1754) for tick exposure factors. Factors associated with disease status were included in the disease model if at least 50 horses were affected. Disease variables were also tested in the multivariable models if they were significantly associated (two-sided tests  $P < 0.05$ ) with the outcomes in the unconditional analyses. In the tick exposure model two variables associated with exposure to ticks were included: pasture access and outdoor confinement. The question about tick exposure according to trainer/owner responses was not included since many were unaware of exposure level. Model building was by backward elimination. Full main-effect models were reduced until the  $P \leq 0.05$  (change in deviance between one model and another model with one less variable), after which all possible two-way interactions were tested. Confidence limits (95%) were constructed around prevalence odds ratios using the profile likelihood.  $P \leq 0.05$  (two-tailed) was considered significant. The Hosmer-Lemeshow test was used to evaluate goodness-of-fit. SAS (SAS Institute, Inc., Cary, NC) was used to perform all analyses with PROC GENMOD used for the multivariable modeling and PROC LOGISTIC for the Hosmer-Lemeshow goodness-of-fit-test.

## Experimental infection (Papers II, III and IV)

### Animals

Five Standardbred horses, aged 5–10 years, and one 19-year-old Warmblood riding horse were used in this study. The Standardbreds had recently retired from racing careers. All horses were clinically healthy based on a detailed clinical examination and routine blood chemistry and CBC. Horses were negative for antibodies to *A. phagocytophilum* as well as *Borrelia burgdorferi* (National Veterinary Institute, Uppsala, Sweden). The horses

were housed in individual stalls in a shared room, without direct contact with other horses, in the research animal area of the Department of Clinical Sciences, Faculty of Veterinary Medicine and Animal Science, Swedish University of Agricultural Sciences, Uppsala, Sweden. Hospital staff attended the horses, which were allowed daily access to outdoor, vegetation-free, sand paddocks. The study was carried out in winter-spring seasons.

#### Experimental infection and inoculate

All infective blood used for the experiment originated from a clinical case of EGE in a Swedish horse. Blood for infection was collected from 2 experimentally infected horses during the acute phase of infection; donor 1 was infected with blood from the clinical case, as described by Franzén and others 1998, and donor 2 was the 1st horse infected in this current group of experimental horses. Horses 1 and 2 were infected with the blood from donor 1 and the other 4 horses were infected with blood from donor 2. Donor blood for the present study was harvested in sodium citrate-containing blood bags during the acute phase of illness, and held at  $-70^{\circ}\text{C}$ . The infective blood was thawed in cold running water and then infused into the recipient horses through the jugular vein (day 0). The dose was calculated to be  $2 \times 10^7$  infected neutrophils (horses 1 and 2) or  $6 \times 10^6$  infected neutrophils (horses 3–6). DNA sequencing for three genes was performed on the inoculum and from samples from all horses-16S rRNA (1428 bp), GenBank accession number AY527213, the *groESL* (1339 bp) GenBank accession number AY529489 and the *ankA* (1735 bp) GenBank accession number AY529487 - and confirmed that the organisms were identical in all instances. The 16S rRNA gene sequence proved to be identical to the *A. phagocytophilum* sequenced from Swedish horses as described by Johansson et al. (1995) and Engvall et al. (1996) and to the HGE agent.

#### Clinical evaluation and sampling of horses during the acute stage (Paper II)

All horses were examined clinically on a daily basis. The evaluation included assessment of general demeanour and behaviour, body temperature, appetite, and passage and consistency of faeces. Additionally the examination consisted of assessment of oral mucous membranes, auscultation of the cardio-respiratory system, recording of heart and respiratory rates, assessment of body posture and movements in box and during transfer to the paddock and in the paddock for any gait abnormality

or detectable lameness, and assessment of musculature, extremities, body and head for changes such as soreness, stiffness, lameness or edema.

Blood samples for microscopic examination for inclusions and for PCR evaluation were obtained on a daily basis, whereas samples for CBC and serology were obtained during the period when horses showed signs of disease, for the most part daily or on alternate days. Blood was drawn from the jugular vein via catheter or needle puncture to tubes containing ethylenediaminetetraacetic acid (EDTA) for CBC and into plain glass tubes for serology.

#### Clinical evaluation, sampling and experimental design during the follow-up period (Paper IV)

Following spontaneous recovery from acute experimental EGA (i.e. after day 21) p.i. horses were monitored until 98-129 p.i., after which they were euthanized and subjected to post mortem examination. During this period a complete clinical examination was performed every day in the same manner as during the acute phase. In addition the presence of lameness or ataxia was evaluated daily while the horses moved freely in the stall and/or in a paddock. A separate clinical lameness examination with trotting on a hard surface and flexion test of all four limbs was performed before the trial started and was repeated, on 2-3 occasions depending on the follow-up time. A final lameness examination was performed immediately prior to euthanasia and post-mortem examination.

Blood samples for analyses for PCR signal and inclusion bodies were collected on a daily or alternate day basis. Blood sampling for routine hematologic analysis, and serology against the EGA agent were obtained on a weekly basis.

After 60 days p.i. the horses were also subjected to periods of various “stress interventions” that could potentially hamper immune function. One horse (5) was trained on a treadmill at a walk and moderate trot for 15-25 minutes on three separate occasions. Horses 3 and 4 were placed in a trailer and transported on a major highway for four hours. Further, following day 72 p.i. all horses were administered dexamethazone (0.1 mg/kg) (Vorenvet Boeringer Ingelheim, Vetmedica, Copenhagen, Denmark) i.m. for three days on one occasion for horses 1 and 5 and on two occasions for horses 2, 3 and 4.

#### Serology, PCR and hematology

The serologic IFA-test for *A. phagocytophilum* is described and discussed earlier in comments on the seroepidemiologic article (Paper I).

DNA from EDTA blood samples was extracted by the QIAamp Blood Extraction Kit from QiaGen (Hilden, Germany). The PCR primers used were as described by Goodman et al. (1996a) in the Errata of this article (Goodman et al. 1996b).

A number of PCR primers for *A. phagocytophilum* detection have been published, of which several were evaluated by Massung and Slater (2003). They reported problems with the Goodman PCR and therefore specificity and sensitivity for this test was not presented in their paper. As the Goodman PCR performed well in our laboratory, we speculate that the primers tested by Massung and Slater might be the ones from the original Goodman paper, in which there was a misprint in the sequence, and not the amended primer sequences from the Goodman Errata (Goodman et al. 1996b).

Before the blood samples were analyzed from the experimental study, the PCR methods published at the time were compared. The Goodman method was found to be the most specific and sensitive method in our hands (data not shown). After the Massung and Slater paper was published we evaluated the method that was used (Goodman) by comparing it to two of the PCR methods they reported as being the most sensitive, i.e. with the primers ehr521-ehr790 as described by Kolbert (1996) and the primers msp2-3f-msp2-3r as described by Massung (Massung, presented at the EUWOG-ASR Joint Meeting, Marseille, France, 1999 Program and Abstracts, p. 6), (PCR methods not readily found when searching PubMed). We found the ehr521-ehr790 PCR less sensitive and the msp2-3f-msp2-3r more sensitive than the Goodman PCR (data not shown), the latter being about five to ten times more sensitive than the method used in this study. However, taking into account the amount of blood tested in the present study compared to Massung and Slater (1 mL and 0.2 mL respectively) the detection limit is approximately the same, corresponding to approximately 50 bacteria in one ml of blood (data not shown).

Total hemoglobin, red blood cell count, total and differential white blood cell and thrombocyte counts were analyzed electronically (Cell-Dyn 350, Abbot Laboratories, Abbot Park IL, USA). Microscopic evaluation of blood smears was used to detect inclusion bodies in the neutrophilic granulocytes indicative of *Anaplasma* bacteria within those cells. Blood smears were stained with Giemsa for light microscopy and with Acridin orange for fluorescent microscopy. At least 200 neutrophils in every blood sample were examined for presence of inclusions.

#### Post mortem examination

The horses in paper IV were euthanized by intravenous injection of an overdose (10 grams) of sodium thiopental (Pentothal® Natrium, Abbott Scandinavia AB, Solna Sweden). A full necropsy was carried out on all five horses within a few hours after euthanasia at the Department of Pathology, National Veterinary Institute, Uppsala, Sweden. Tissue samples from internal organs (Table 4) were fixed in 10% buffered formalin, processed routinely, embedded in paraffin, sectioned and stained with hematoxylin-eosin (HE)-stain. Tissue samples from lymph nodes, lung, liver, spleen and kidney were submitted for bacteriological examination. The horse in Paper III was examined within 10 hours of death. For bacteriological examination the same samples were collected as for the other horses with addition of synovial membrane and brain. Samples for histological examination were collected from kidneys, heart, lung, spleen, liver, muscle and muscle fascia, spinal cord, and brain (cortex, basal ganglia, hippocampus and mesencephalon) from this horse.

Table 4.

Tissue specimens from post mortem in five experimentally infected horses

\* = histopathologic examination

# = PCR analysis

& = three different specimens from the same organ analyzed with PCR

x = two different specimens from the same organ analyzed with PCR

Organ/tissue specimen	Horse number				
	1	2	3	4	5
Bone Marrow	* #	* #	* #	* €&	* #
Adrenal gland	*	* #	* #	*	*
Fascia	* #	* #	* #	* #	* #
Brain	* #&	* #	* #	* #	* #
Meninges	*	* #	* #	* #	* #
Choroid plexus	#				
Cerebrospinal fluid		#	#		#
Spinal cord	* #	* #	* #	* #	* #
Myocard	* #	* #	* #	* #	* #
Joint capsule	* #	* #	* #	* #	*
Synovia		#	#	#	#
Liver	* #	* #	* #	* #	* #
Lung	* #	* #	* #	* #	* #
Lymph node	* #	* #	* #	* #&	* #
Spleen	* #x	* #	* #	* #&	* #
Skeletal muscle	* #	* #	* #	* #	* #
Kidney	* #	* #	* #	* #	* #
Peritoneum	*	* #	* #	*	*
Intestine	* #	* #	* #	* #	* #
Urinary bladder	* #	* #	* #	* #	* #

#### Immunohistochemistry and DNA extraction for PCR on tissue samples

Immunohistochemistry for the detection of *A. phagocytophilum* was applied to tissue sections of the liver and kidneys of the horse in paper III, using a high-titre serum (1:10,000) from another experimentally infected horse as

the primary reagent; liver and kidney sections from an uninfected horse served as negative controls.

Samples from internal organs (Table 4) from all horses in Paper IV were frozen in liquid nitrogen and stored at  $-70^{\circ}\text{C}$  until processing. DNA from approximately 25 mg of each organ sample was extracted, and PCR analysis for *A. phagocytophilum* was performed as described earlier.



## Comments on results and discussion

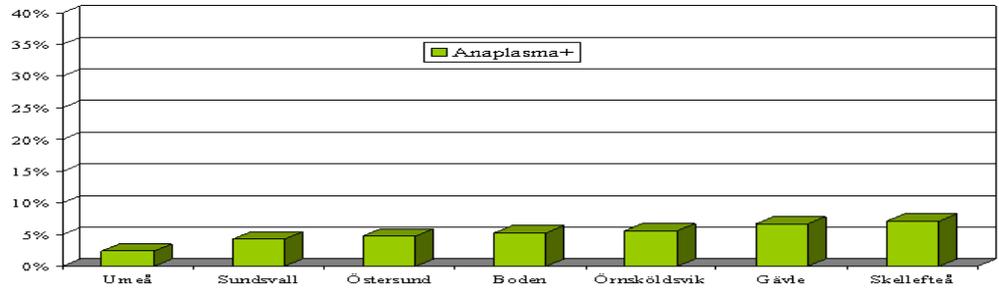
### Serosurvey (Paper I)

The population sampled was a cross-section of horses attended at the country's network of racing commission (ATG) equine clinics in 1997-1998 but does not represent the total Swedish horse population. Since the population is derived from horses visiting equine clinics, it is assumed there is overrepresentation of active, training and competition horses i.e. racehorses, dressage horses, show jumpers and riding horses and underrepresentation of foals and yearlings, broodmares, breeding stallions, draft horses and horses under more extensive management and usage conditions.

The cross-sectional study showed that 336 out of 2018 horses examined were seropositive to *A. phagocytophilum* corresponding to 16.6% of the horses. The geometric mean titer (GMT) was 1:86 and the range was 1:40-1:1280. Statistical differences in the seroprevalence were found for different regions of Sweden with the lowest prevalence in the northern part. This was not surprising as it is well known that ticks are more seldom found in the north of Sweden (Figure 4). In the group of healthy horses the seroprevalence was 15.5% and in the rest of the horses – from here-on called “the clinical signs group” 16.9%. The GMT for *A. phagocytophilum* in the healthy group was 1:94 as compared to 1:84 in the “clinical signs group”. Comparing healthy horses with “clinical signs horses” there was no statistical difference in the seroprevalence to *A. phagocytophilum* (Table 5).

Data on demographic variables were relatively similar between the both groups although age was slightly lower in the healthy group.

**Northern Region  
(Norrland)**



**Southern regions  
(Götaland and Svealand)**

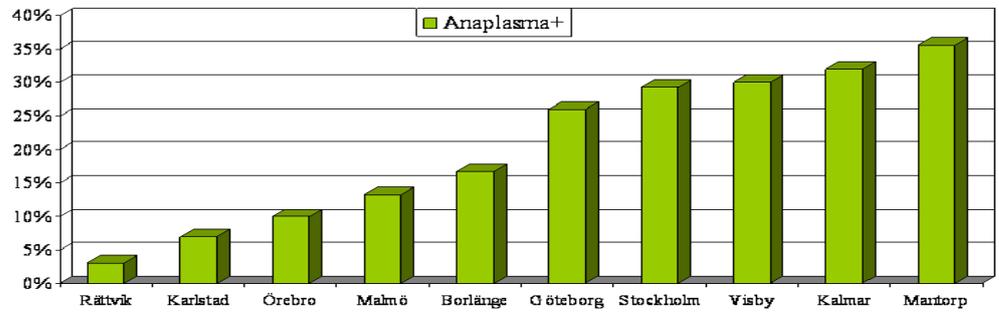


Figure 4. The seroprevalence of antibodies to *A. phagocytophilum* by clinic. The map shows the location of the clinics.



In the multivariable disease model, when controlling for demographic factors, no disease variables remained statistically significant with respect to seropositivity for *Anaplasma*. The factors with remaining association in both the multivariable models (disease model and tick exposure model) included age (higher when older), positive titer to *Borrelia* and season (lower odds ratios (OR) in April to September, compared to January–March). This finding is difficult to explain given that tick exposition is likely to be high during the summer.

The middle and south region had higher ORs compared to the north region. However there was an interaction with racing, so that racing horses in both the south and middle region had relatively low ORs (data not shown). The ORs also increased with increasing access to pasture the previous year.

Further in the tick exposure model pasture access was associated with seropositivity. Both these latter findings may be explained by that without access to pasture (which race horses seldom have as they commonly are located on trace tracks and racing camps) exposition to ticks and risk for infection is relatively low.

From a clinical point of view it was concluded that there was no association between for instance lameness, arthritis, fatigue, unwillingness to be ridden (which have been suggested to be linked to seropositive horses) or any other of tested clinical signs and a positive titer to *A. phagocytophilum*.

Together with the findings of high overall seropositivity in many geographical areas and the fact of no difference in seroprevalence in healthy and clinical signs group, this study gives no support to the suggested association between positive serotiter and any sign of disease that have been suggested associated to positive serotiters with suspicion of chronic persistent infection with *A. phagocytophilum*.

Table 5. Results of the IFA-test comparing the healthy and clinical signs groups

	Healthy group	Clinical signs group
No. of horses	400	1618
% seropositives	15.5 €	16.9 €
geometric mean titer	1:94 *	1:84 *
range	1:40-1:640	1:40-1:1280

€ non-significant difference Chi square test (P=0.49)

\* non-significant difference Wilcoxon two-sample test (P=0.55)

## Experimental infection

### Acute stage (Paper II)

In this study it was possible to induce acute disease in horses with a Swedish isolate of *A. phagocytophilum*. All horses developed typical signs of EGA including fever, depression, reduced appetite, distal limb edema, leukopenia and thrombocytopenia. However, one horse died suddenly and unexpectedly two days into clinical disease. This horse is discussed separately (Paper III). The clinical course in the 5 remaining horses was almost identical to what is described from the United States (Gribble 1969, Madigan 1993). However, icterus, as reported in earlier work, was not noted in these horses.

The PCR test turned positive several days before the first clinical signs appeared. The temporal events of fever, PCR positivity, neutrophil inclusions and seroconversion were similar in the different horses figure (Figure 5). One small, but clinically important difference between the present study and Gribbles work, (Gribble 1969, Gribble 1970) was that inclusion bodies were first noted several days into clinical disease. This was contrary to the report by Gribble where inclusion bodies were noted simultaneously with onset of fever. The discrepancy may be explained by variation in laboratory methods. In Gribble's work very small or single inclusions were also recorded as inclusion bodies.

In the current study inclusions were judged to be present when distinct morulae were seen, without strive to identify other possible small intracytoplasmatic changes that eventually may be mistakenly deemed as "small early inclusions". For example, false positive interpretations may occur due to toxic granulation, Döhle bodies, superimposed platelets or contaminant particles (Walker 1999). This minor difference is clinically important, because if inclusions are used as the definite diagnostic criterium, the diagnosis may be missed during the first couple of days of high fever, which often is when the veterinarian is requested to examine the horse.

It is obvious that PCR has a wider diagnostic window for detection of the bacterium in peripheral blood than do inclusion bodies and accordingly PCR appears to be the best tool for a definite diagnosis of acute EGA. Fever was the first sign and distal limb edema the last to occur. It appears that limb edema should not be expected during the first couple of days of fever. Further, the clinical signs of disease disappeared without antibiotic therapy in less than 14 days in all horses. All horses developed mild and transient leukopenia, neutropenia, lymphopenia, thrombocytopenia and anaemia seen after day 9 p.i. and for approximately 7-10 days with anaemia

and thrombocytopenia lasting longer than the reduction in the white blood cell count. The hematological changes are consistent with what has previously been described for the disease in horses in California (Gribble 1969, Madigan 1993).

It is not known whether intravenous experimental infection directly mimics natural infection especially regarding temporal changes of signs and development of serotiters to *Anaplasma*. The intravenous and tick induced experimental infection routes with the HGE-agent in horses have been compared (Pusterla *et al.* 1999a) where a longer incubation period was found in tick-induced infection compared to intravenous infection.

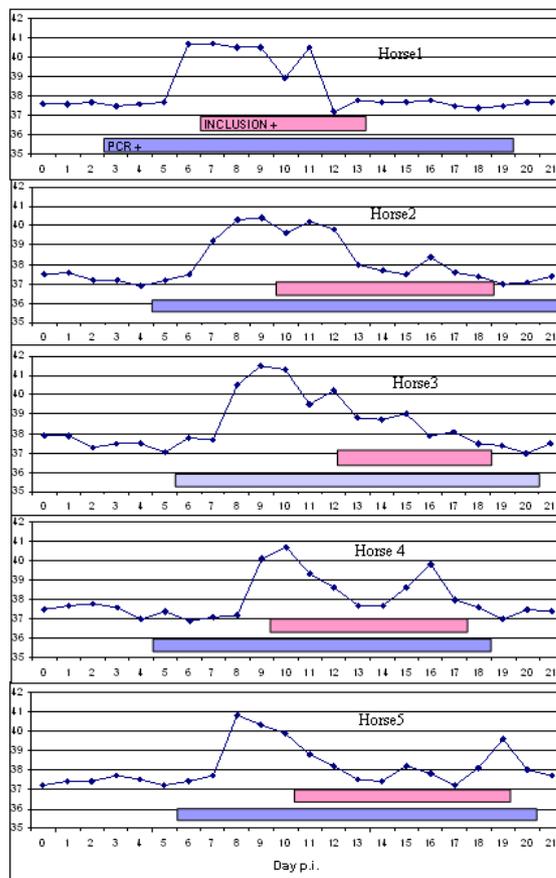


Figure 5. Body temperature ( $y$ -axis,  $^{\circ}\text{C}$ ), PCR-signal (blue bar) and visible inclusions (pink bar) in neutrophils in peripheral blood in five horses inoculated with *A. phagocytophilum*. The figure shows the measurements from day 0 to 21 p.i., through the acute EGA-infection.

However the severity of clinical signs, the course of disease and the serotiter 30 days after infection was similar regardless of infection route. Further, using real-time Taqman-PCR for quantitative analysis of the anaplasma burden in the analyzed peripheral blood, there were significantly higher bacterial loads noted in the intravenously infected horses in the first days after inoculation, whereas the mean anaplasma load in the later part from day 7 to day 9 was significantly higher in the tick-infected group. That study concluded similar severity in experimental i.v. infection and experimental tick infection (Pusterla et al. 1999a). Whether this holds true also for natural tick infection is not known but the results indicate that it may indeed be the case.

The horses in this study (Paper II) seroconverted by day 12-16 p.i. which was 6-8 days after onset of clinical signs. At the point of detection of seroconversion all horses still had positive PCR, all had still inclusions and two of the five were still febrile. Three of five horses had the peak in titer while still showing inclusions. The high and uniform anaplasma dose may have influenced the timing of the serologic reaction, which was very similar in all these horses.

A  $\geq$  fourfold rise in titer has been suggested as one way for defining a definite diagnosis (Madigan et al. 1990). However, if the change in serotiter after clinical signs occurs as fast in naturally infected horses as in the experimentally infected horses in this thesis, the first of paired samples needs to be taken early in the disease for detection of titer change. In the above cited comparison between i.v. and tick experimental infection (Pusterla et al. 1999a) seroconversion occurred significantly later in the tick infested group when counting from day of inoculation. When comparing from when clinical signs appeared there was no difference in time for seroconversion. Moreover, the 30 day convalescent titer was similar in both groups.

In another report (Reubel et al. 1998) three horses were experimentally infected via infected ticks. In two out of three horses seroconversion was detected 4 and 7 days into clinical disease respectively (the third horse did not develop clinical signs of disease and did not seroconvert despite positive PCR in blood). In a study on kinematics of antibody after natural infection Artursson et al. (1999) found 44% of 25 horses to be seropositive in the acute bacteraemic stage of disease after natural infection and 3 out of 10 horses had reached maximal or next to maximal titer already at the first occasion of blood sampling in the acute illness and with inclusions present.

Combining these various other studies and our findings supports the suggestion that, to avoid missing the diagnosis in a paired sampling for

detection of a fourfold titer rise, the first sample must be taken early in the clinical course. An additional limitation for use of serology as the sole diagnostic tool is the high prevalence of seropositivity in horses where EGA is endemic despite absence of history of clinical disease.

#### Death due to *A. phagocytophilum* (Paper III)

Unexpectedly, one of the 6 experimentally infected horses died suddenly two days after onset of clinical signs, at 8 days p.i. The clinical course and severity of clinical signs did not differ from the rest of the horses until the death occurred. Fatalities in association with *A. phagocytophilum* infection have rarely been reported in the horse. Serious sequelae that may potentially be fatal, such as secondary infections or trauma related to ataxia or staggering, caused by the acute illness have been reported. However, fatalities due to sequelae appear to be uncommon (Gribble 1969, Gribble 1970, Madigan 1993, Madigan and Pusterla 2000). To the best of our knowledge, this report was the first description of a case of equine granulocytic anaplasmosis where death was most likely attributed solely to *A. phagocytophilum* infection.

The pathological macroscopic findings in this horse included haemorrhage and edema in the limbs, ventral abdominal wall and prepuce as well as in the thoracic and abdominal organs including lungs and kidneys. This is in accordance with what is previously described in the pioneering work by Gribble (1969 and 1970) on the pathology of this disease in horses with the exception that cases in Gribble's studies lacked extended hemorrhage and vasculitis in internal thoracic and abdominal organs. Gribble euthanized the horses in different stages of the acute clinical disease and described the changes seen. In our horse, because the horse actually died, the infection was probably more severe and it is therefore not surprising that the same type of changes were more pronounced or more widely distributed in this case.

Microscopically the pathological changes appeared as perivascular and/or interstitial infiltrations of mononuclear cells in several organs including lung, kidney and liver. The type of changes were similar to those described by Gribble (1969, Gribble 1970) and later reexamined by Lepidi et al. (2000). The most prominent microscopic lesions in this horse were found in the kidneys with necrotising inflammation in the walls of scattered arterioles and capillaries. Further, there were swelling of the glomeruli and numerous intraluminal hyaline thrombi or globules within the arterioles and capillaries. This is suggestive of disseminative intravascular coagulation (DIC) which may partly explain the fatality. Although uncommon, consumptive

coagulopathy and DIC may occasionally occur both in human granulocytic anaplasmosis and in human monocytic ehrlichiosis (Bakken et al. 1994, Behl et al. 2000).

Immunohistochemically, the high-titre horse serum labelled occasional cells (presumably neutrophils) within the lumen of vessels in the kidneys and liver, and within the hepatic sinusoids (Figure 6). Parenchymal cells showed no immuno-reactivity. The direct cause of death in the present horse was most likely circulatory shock. Negative bacterial cultures, as well as the absence of morphological evidence ruled out secondary bacterial infections in this case.

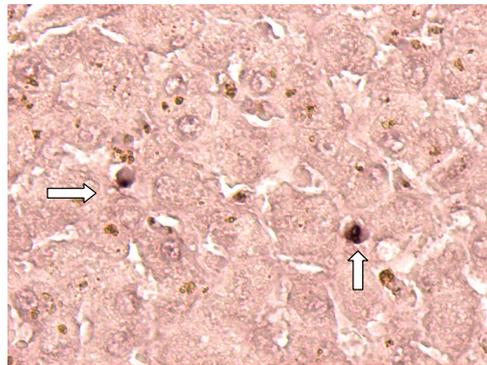


Figure 6. Two leukocytes containing *A. phagocytophilum* antigen (arrows) within the hepatic sinusoids. Immunohistochemistry with primary horse serum and AP-conjugated secondary antibody, NBT/BCIP as chromogen and light green as counterstain. x 400.

The reason for that the infection attained such a fulminant course in this particular horse is not clear. In EGA experimental infections in horses, where the infective doses have been clearly defined, doses used have ranged from  $1 \times 10^6$  to  $4.5 \times 10^7$  infected neutrophils (Table 3). In this fatal case, the dose was approximately  $2 \times 10^7$  infected cells. This dose was selected based on previous experiences with a similar dose (Franzén et al. 1998). In the earlier study, the horse developed clinical EGE and recovered uneventfully (Franzén et al. 1998). Apart from shorter incubation period, the other horse in the present series of 6 infected horses that was also given this higher infective dose did not differ clinically when compared to horses with a lower infective dose (Paper II). Thus, it is unlikely that the infective dose alone was the reason for the fatality. However, for the rest of the horses the infection dose was reduced to  $6 \times 10^6$  infected cells. Although uncommon, fatal cases of infection with *A. phagocytophilum* also occurs in sheep (Stuen et al. 2003).

Bakken and Dumler (2000) estimated the fatality rate in humans to be less than 1% based on previously reported studies (Bakken et al. 1994, Agüero-Rosenfeld et al. 1996, Bakken et al. 1996, Dumler and Bakken 1998, Wallace et al. 1998) and their own clinical experience, and with a clear increased risk for mortality in elderly people which is consistent with other reports (Olano and Walker 2002). Of four fatalities in man reported by Lepidi, three were over 70 years old (Lepidi et al. 2000).

Although 19 years old, our horse was in good physical condition and showed no overt signs of any other disease. However, it cannot be ruled out that failure to limit the infection and hence the development of DIC was at least partly due to an age-related decreased immunocompetence. In acute and sudden death cases of horses, for example on pasture, in areas where *A. phagocytophilum* is endemic it is suggested that EGA should be included in the list of possible differential diagnosis.

Sudden death due to EGA have been discussed among clinicians although not proven. In a recent case a horse was found acutely recumbent on pasture, unwilling to stand with no previous known trauma or signs of colic noticed. Fever (41.5°C), staggering movements, heavy breathing and a pulse of 50 beats per minute were the main findings at examination. Despite initial treatment with oxytetracyclines, i.v.-fluids- and an NSAID, the horse died a few hours later. Laboratory data verified a positive PCR signal in blood and inclusions in 5% of neutrophils. Unfortunately no post mortem was performed to verify or reject the suspicion of EGA as the cause of death.

#### Convalescent phase (Paper IV)

##### *Clinical signs*

After acute clinical signs had disappeared in the horses experimentally infected, no significant clinical signs were detectable in the horses during the follow-up period, apart from some transient subtle clinical changes such as mild nasal serous discharge or mild coughing on sporadic days. Mild distal limb swelling was found occasionally in the mornings. This can be expected as part of normal clinical variation in horses being thoroughly examined daily for extended time periods. One horse developed thrombophlebitis and in one instance embolic pneumonia occurred, of which both most probably were iatrogenic and coupled to the long-term catheterization of the animals and not to the EGA-infection. Based on extended clinical experience, it is likely catheter-related complications may

eventually occur in any situation with long-term iv-catheterised horses (Gulick and Meagher 1981, Bayly and Vale 1982, Lankveld et al. 2001).

It has been known for long and is well demonstrated that infection with *A. phagocytophilum* can predispose for secondary infection in sheep and enhanced infection susceptibility is also mentioned frequently in the human literature. This is not thoroughly documented in the horse apart from some cases in Gribble's series of experimental infections (1970).

#### *PCR and inclusions in blood*

The results of all PCR samples in blood are shown in Figure 7. From days 22 (when all signs of acute disease had disappeared) and onward during the following months three of five horses were sporadically PCR-positive.

Further on, after horses were subjected to stress interventions, periodic PCR positivity was observed occasionally in some of the horses. After dexamethasone administration on days 73-75, horse 1 turned PCR-positive for four consecutive days (days 76-79), and horse 5 was positive on day 4-5 and 12 after challenge (days 102-103, 110) whereas horses 2-4 remained negative on PCR after dexamethasone challenges. After transport intervention both horse 3 and 4 turned positive for four days one week after transport. Stress events in the form of treadmill exercise and rectal palpation was not followed by PCR-positivity. In one horse (2) the bacterium appear to have been cleared after day 33 p.i. whereas in the other four horses the last days of PCR-positivity were days 70, 79, 110 and 129 for individual horses (day 129 also being the last day of the trial).

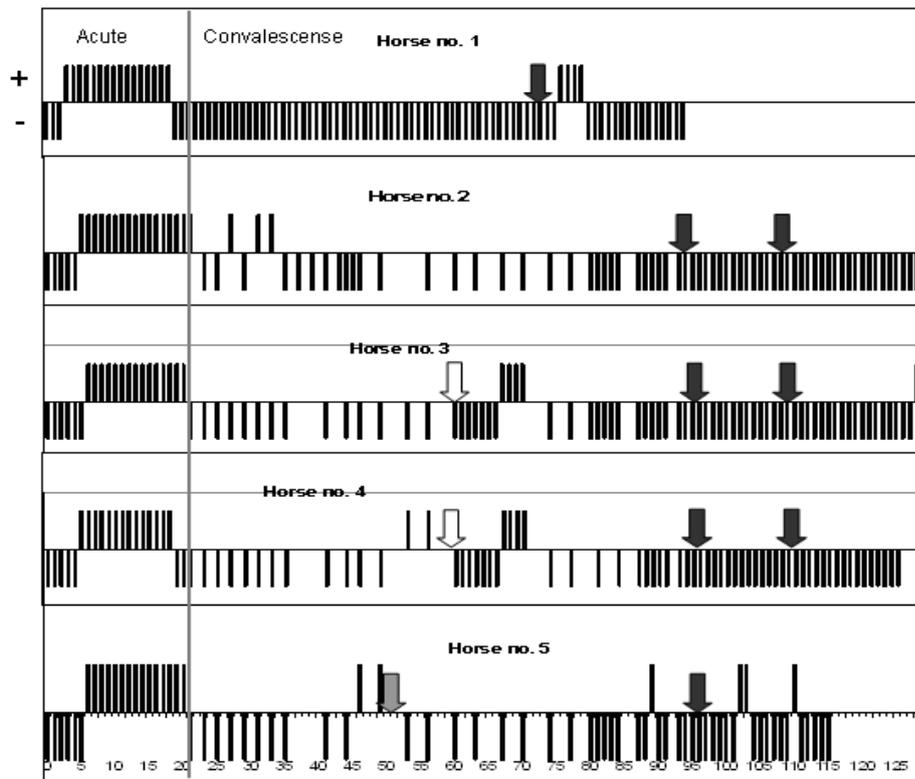


Figure 7. PCR results in blood from five horses during ~4 months after i.v. inoculation (day 0) with *A. phagocytophilum*. Positive/negative bars indicates positive/negative PCR signal. Black arrow indicates a three-day dexamethazone treatment, white arrow indicates a four-hour trailer transportation, grey arrow indicates repeated treadmill exercise.

Once the acute disease phase had resolved inclusions in neutrophils were not observed at any of the sampling occasions, including when the PCR signal was positive. Sheep can be PCR-positive and show inclusions in neutrophils when persistently infected (Stuen et al. 2001a, Stuen and Bergström 2001b, Stuen et al. 1998b). Two experimentally infected dogs in the convalescence phase, when given corticoids three and five months p.i. respectively turned PCR-positive and at a few occasions inclusions were also detected (Egenvall et al. 2000a) several months p.i., the latter contrary to the horses in this study. This suggests that horses may be less prone than sheep and dogs to develop inclusions after the acute stage of febrile disease, despite the agent still apparently being present in the body. The PCR signal became positive only intermittently after corticosteroid challenge and was

also only intermittently positive on other occasions. This could be due to the number of bacteria in peripheral blood being intermittently under (or above) the detection level of the PCR used (one mL of EDTA blood was used for each DNA extraction). Since the cell reservoir of the bacteria is not known, it is possible that shedding into the peripheral blood is intermittent (given that the reservoir is not the circulating neutrophils themselves).

#### *Post mortem findings*

The catheter-related complications noted clinically were confirmed as jugular vein thrombophlebitis and embolic staphylococcal pneumonia post mortem. Further, four of five horses had mild to moderate degenerative joint lesions characterized by linear grooving and small erosions of the articular cartilage in the fetlock joint of three horses, elbow joint of two horses and in the hock joint of one horse. Taking into account that four of five horses had long-lasting (and successful) histories as racehorses and the fifth horse had been used as a riding-school horse for more than 12 years, such joint lesions can be expected.

Microscopically, mild perivascular infiltration of mononuclear inflammatory cells was sporadically observed in lung, liver portal tracts, kidney and joint capsule in selected horses. Similar discrete, nonspecific mild infiltrates of small numbers of mononuclear cells are often seen in adult horses, whether healthy or diseased, when performing such a thorough post mortem examination. Accordingly, association of these changes to the possible persistence of *A. phagocytophilum* can neither be definitely included or excluded.

The absence of specific post mortem changes is in accordance with results reported from long term follow-up of experimental infection in dogs with the same agent where molecular evidence for persistent infection in blood was demonstrated (Egenvall et al. 2000a). Routine bacteriology from internal organs of the horses was negative in all instances apart from the staphylococcal pneumonia as described above.

All tissue samples in all horses proved negative for *A. phagocytophilum* by PCR. As immunochemistry is less sensitive than PCR for identification of the bacterium and as all tissues were PCR-negative, immunochemistry was not performed.

#### *Experimental infection versus natural infection*

This was an i.v. inoculation study. However, according to the literature it is probably safe to assume that the difference between i.v. inoculation and

tick-borne infection is limited (Pusterla et al. 1999a). There are both strengths and weaknesses with experimental infections. A possible limitation is that rapid i.v. inoculation does not mimic natural tick bites, where the infection likely is transferred over time. Further, it is likely that the i.v. infection dose is much higher in experimental infection. The intravenous infection route has been compared to the tick infection route in an experimental infection with the HGE-agent to horses (Pusterla et al. 1999a). The i.v. route had shorter incubation time and shorter time to seroconversion.

However, once clinical signs had appeared, the course of clinical disease was similar in both groups. This is also consistent with the course of events observed after experimentally induced *Anaplasma* infection with horsederived isolates and similar to natural infection (Gribble 1969, Madigan and Gribble 1987). Surprisingly the tick infected group had a higher total bacterial load and a longer bacteraemia as evaluated by real-time quantitative PCR. Nonetheless, the study was an experimental tick infection which is not likely identical in all aspects to natural tick infection.

It is further not known if natural infection is comparable to experimental i.v. infection with respect to persistence of bacteremia. However, these observations suggest that if there are biological differences between i.v. inoculation and tick infestation-infection they may be small. One strength with experimental infection, compared to following clinical cases, is that it is much simpler to have predefined sampling intervals, and follow parameters and changes longitudinally, as timing of infection is predefined and the animals are available for sampling over the entire course of infection. It could be considered a weakness that the study not included control horses. This was due mainly to economic reasons and also ethical reasons.

#### *The question of persistent Anaplasma infection and “chronic” disease*

This study, on a few experimentally infected horses, found evidence for the persistence of the *A. phagocytophilum* organism for up to four months in the blood may occur in horses recovering from acute EGA. However, no evidence was found to support the existence of clinically detectable “chronic” form of disease meaning persistence of infection together with clinical manifestations. After the acute phase with typical clinical signs of EGA-disease no further signs of illness were found connected to the infection in any of the horses despite thorough daily complete examination.

No specific findings were noted on post mortem in any of the horses, nor were there any PCR positives in the tested organs from the horses.

When comparing with other species these results are in accordance with what is shown in experimentally infected dogs. Persistence of *A. phagocytophilum* in blood, shown as a positive PCR-signal, was shown up to 6 months without detectable clinical signs, bearing in mind the long term follow-up included only three dogs (Egenvall et al. 2000a). Alleman et al. (2006) experimentally infected two dogs with a human isolate. The authors found persistence (via PCR) of infection up to 340 days p.i. during intermittent periods following heavy corticosteroid medication, however in the absence of clinical signs during the whole year follow up.

In contrast, in ruminants and especially lambs persistent infection appears to be common and with documented increased risk for secondary infection and persistent infection in lambs has also been associated with reduced weight gain. The effect of chronic infection and secondary problems in lambs due to *A. phagocytophilum* infection has been known for long and is well documented.

The potential for chronicity in humans with HGE have been mentioned (Dumler and Bakken 1996) but the same authors later stated that “there is currently no clinical evidence in the literature that untreated HGE may evolve into a chronic illness as have been seen with Lyme *Borrelia*”. They further stated that “persistently elevated antibody titers should therefore be interpreted as evidence of post infection rather than as proof for ongoing unresolved infectious process”, and that “unlike Lyme borreliosis there does not appear to be a chronic form of HGE” (Bakken and Dumler 2000).

This is in accordance with a study where no clinical abnormalities were found in a long term follow-up of 30 patients diagnosed with HGE, whereas patients who had had additional tick bites after primary infection had significantly higher antibody titers in the follow up 24 month period (Lotric-Furlan et al. 2001).

A report on antibiotic-treated HGE-patients on average 2 years after onset of illness had lower health status scores for bodily pain and health relative to one year earlier documented via retrospective telephone-interviews and statistical preparation of the answers. However, there was no significant difference in physical functioning, general health or vitality measures as compared to controls (Ramsey et al. 2002).

In the discussion of possible persistence causing clinical disease in horses there is anecdotal experience by veterinarians suggesting improvement of health of horses after treatment with oxytetracyclines, which has been interpreted that *Anaplasma*-infection was present in these horses. While this may be true, it is no proof for that *A. phagocytophilum* was present or that signs were caused by this infection. One reason for this is that

oxytetracycline is a broad-spectrum antibiotic with a wide range of microorganisms being sensitive.

Further, tetracyclines have several pharmacological effects other than the antimicrobial effect (Sapadin and Fleischmajer 2006), such as anti-inflammatory effects probably mediated through inhibiting matrix metalloproteinases ( Ramamurthy et al. 2002, Vidal et al. 2007) and are currently under study for the treatment of osteoarthritis (Vidal et al. 2007). Doxycycline, another tetracycline antibiotic, has been shown to reduce severity of cartilage lesions in dogs with osteoarthritis (Hanemaaijer et al. 2001) as well as have positive clinical effect in humans with knee osteoarthritis (Brandt et al. 2003a, Brandt et al. 2003b). Other non-antibiotic use of tetracyclines includes treatment of rheumatoid arthritis and rosacea in humans (Voils et al. 2005) and treatment of contracted tendons in foals (Lokai and Meyer 1985).

In conclusion, this study showed molecular evidence for persistence of the *A. phagocytophilum* organism for up to three months in the blood of horses after recovery from acute EGA, but found no evidence to support persistence together with clinical manifestations of disease. After the acute phase with typical clinical signs of EGA-disease no further signs of illness were found that could be connected to the infection in any of the horses, despite daily complete examination. Neither were specific findings noted in any of the horses on post mortem, nor were the PCR test positive in any tested organ from the horses.



## Summary and conclusions

- The overall seroprevalence of antibodies to *A. phagocytophilum*, as determined from a population of horses visiting equine clinics in all parts of Sweden was 16.6% with large differences dependent on geographic location within the country.
- In this population there was no statistically significant correlation between seropositivity and any disease parameter or diagnosis recorded.
- In the healthy group of horses the seroprevalence of antibodies to *A. phagocytophilum* did not significantly differ from that in the group of horses with clinical signs.
- PCR is a more sensitive diagnostic tool than inclusions in the early phase of clinical disease.
- Death due to equine granulocytic anaplasmosis may occur and should possibly be included as a differential diagnosis in conjunction with cases of acute death on pasture in endemic areas.
- Molecular proof for persistence *A. phagocytophilum* for up to four months after experimental infection was demonstrated in certain individuals.
- The persistence of *A. phagocytophilum* was not coupled to any detectable clinical signs of disease or any specific pathological changes at post mortem.



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