STUDIES OF A HUMAN ISOLATE OF *ANAPLASMA PHAGOCYTOPHILUM* IN CULTURED TICK CELLS, TICKS AND SHEEP

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TICKS AND SHEEP

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CHAPTER I

INTRODUCTION AND LITERATURE REVIEW

Introduction

Anaplasma phagocytophilum (Order Rickettsiales, Family Anaplasmataceae) is a pathogen transmitted by ticks, most notably of the genus *Ixodes*, to a wide range of hosts, including birds, small and large mammals and humans (Goodman 2005; Woldehiwet 2010). This organism is the etiologic agent of a febrile illness of humans (human granulocytic anaplasmosis, HGA), ruminants (tick-borne fever, TBF), horses (equine granulocytic anaplasmosis) and dogs (canine granulocytic anaplasmosis, CGA). *A. phagocytophilum* is a well established pathogen of small ruminants in Europe (Stuen 2007) and, more recently has been shown to be the cause of an emerging tick-borne disease of humans in the United States, Europe and Asia (Goodman 2005). The wide host range of *A. phagocytophilum* and the extensive distribution of tick vectors will likely contribute to an increase in the number of reservoir hosts. In addition, the expanding range of tick vectors and the movement of human populations from urban to rural areas will also impact reservoir host and human contact with infected ticks, all of which will likely increase the risk of acquiring *A. phagocytophilum* infections. Therefore, the continued emergence of diseases associated with *A. phagocytophilum* infection is a growing concern for human and animal health in the United States and other parts of the world. The recognition of the broad distribution of *A. phagocytophilum* and it emergence

as a tick-borne pathogen has created renewed interest and has accelerated research on this organism, particularly on the molecular relationship of the pathogen with it's vertebrate and tick hosts (Woldehiwet 2010).

Literature Review

Historical Background

A. phagocytophilum was first recognized over 70 years ago in Europe as the causative agent of tick-borne fever (TBF) in domestic ruminants, primarily sheep (Gordon et al. 1940; Woldehiwet 2010). The prototype of *A. phagocytophilum,* the agent of TBF in ruminants, was first named *Rickettsia phagocytophila* (Foggie 1949), only to be subsequently renamed *Cytoecetes phagocytophila* (Foggie 1962) based on morphological similarities to *Cytoecetes microti* (Tyzzer 1938). The organism was then listed in the tribe Ehrlichieae as a separate species, *E. phagocytophila*, by Ristic and Huxsoll (1984), but this designation was not adopted by researchers in Europe who continued to refer to the pathogen as *C. phagocytophila* (Woldehiwet and Scott 1993).

In the United States, the first case of equine granulocytic anaplasmosis (EGA) in horses was reported in California in 1969 (Gribble 1969) and was presented as a separate species, *E. equi*. Canine granulocytic anaplasmosis (CGA), another emerging disease caused by *A. phagocytophilum,* was first detected in a German shepherd dog in Arkansas in 1971 (Madewell and Gibble 1982). At this time the pathogen was thought to be maintained in a transmission cycle between domestic animals and free-living reservoirs (Ogden et al. 1998 a,b).

During the early 1990's, an emerging disease of humans in the United States was found to be caused by a granulocytic agent similar to *A. phagocytophila* and *E. equi*

(Chen et al. 1994). The disease caused by this newly-discovered organism was named human granulocytic ehrlichiosis (HGE) but was later designated human granulocytic anaplasmosis (HGA) after the pathogen was classified in the genus *Anaplasma* and named *A. phagocytophilum* (Dumler et al. 2001).

Through the comprehensive reclassification of the Family Anaplasmataceae in 2001, these organisms were all classified as *A. phagocytophilum* (Dumber et al. 2001). Since that time, the number of strains of *A. phagocytophilum* that vary in host preferences and other aspects are continuing to increase in the U.S. and Europe and are considered to be variants of the same organism.

Classification

The reclassification of *A. phagocytophilum* (Table 1), in which three previous species (*Ehrlichia phagocytophilia*, *Erhlichia equi* and the previously unnamed agent of HGA) were combined as one, was part of a comprehensive review of the families Rickettsiaceae and Anaplasmataceae (Dumler et al. 2001). This reclassification was based on genetic and antigenic similarities, a preference for granulocytes as host cells, development of the organism within parasitophorous vacuoles, similar developmental cycles involving reticulated and dense forms and by being vectored by ticks. In this reorganization, the tribes *Rickettsieae, Ehrlichieae, Wolbachieae,* and *Anaplasmataceae* were eliminated and the species were moved to the family level based on their molecular and phenotypic similarities (Dumler et al. 2001). Therefore, the family *Anaplasmataceae* was broadened to include all species of the *α-Proteobacteria* listed previously in the genera *Ehrlichia, Anaplasma, Cowdria, Wolbachia,* and *Neorickettsia* (Dumler et al. 2001). This reorganization was based on biological characteristics and genetic analyses of

16S rRNA genes, *groESL*, and surface protein genes (Dumler et al. 2001). These phylogenetic analyses consistently supported the formation of four distinct genera within the family *Anaplasmataceae*: (i) *Anaplasma*, with a 96.1% minimum similarity; (ii) *Ehrlichia*, with a 97.7% similarity; (iii) *Wolbachia*, with a 95.6% similarity; and (iv) *Neorickettsia*, with a 94.9% similarity (Table 2). While organisms placed in the family *Anaplasmataceae* are obligate intracellular organisms, they are found exclusively within membrane-bound vacuoles in the host cell cytoplasm. Furthermore, most all organisms in the family *Anaplasmataceae* multiply in both vertebrate and invertebrate hosts (primarily ticks or trematodes). In contrast, organisms classified within the family *Rickettsiaceae* (genera *Rickettsia* and *Orientia*) are obligate intracellular bacteria that grow freely within the cytoplasm of eukaryotic cells. *A. phagocytophilum* was grouped with the organisms that infect ruminants (*Cytocecetes phagocytophila*, *Ehrlichia phagocytophila*) and horses (*Ehrlichia equi)* (Dumler et al. 2001). A common feature is that bacterial survival of organisms in the genera *Anaplasma* and *Ehrlichia* depends on mammalian hosts because transovarial transmission does not occur.

Epidemiology, geographic distribution and emergence of granulocytic anaplasmosis in the United States and other areas of the world

Anaplasma phagocytophilum has been detected in mammals and ticks throughout the United States and in nearly all-European countries, but the strains or variants and the resulting diseases vary with the geographic location (de la Fuente et al. 2005b; Stuen 2007; Woldehiwet 2010).

Anaplasma phagocytophilum has been reported to be the most widespread tick-borne infection in animals in Europe (Stuen 2007) and is an emerging tick-borne

pathogen of humans in the U.S. Sequence analysis of the *A. phagocytophilum msp4* gene that was performed on 50 samples from the United States, Germany, Poland Norway, Italy, and Switzerland and 4 samples of *A. phagocytophilum*-like organisms obtained from white-tailed deer in the United States provided evidence of sequence variation that was higher than observed among *A. marginale* strains (de la Fuente et al. 2005b). The overall analysis did not provide phylogeographic information but did differentiate strains of *A. phagocytophilum* obtained from ruminants, horses and dogs. The organisms from white-tailed deer were found to be more diverse. These results were corroborated by similar phylogenetic studies using the *msp2* gene. It is therefore likely that the strains from humans, which differ from ruminant strains, may be maintained in nature in different reservoir hosts (de la Fuente et al. 2005e).

Among free-living ruminants in the U.K., *A. phagocytophilum* was detected in feral goats and in red, fallow and rose deer (Foster and Greig 1969; McDiarmid 1965; Alberdi et al. 2000). In Norway, Slovenia, Switzerland and Austria *A. phagocytophilum* was detected in a variety of cervid ruminants, including roe deer, moose and chamois (as reviewed by Woldehiwet 2010). The epizootiology of *A. phagocytophilum* has also been reported to involve wild rabbits, birds and cats (Bjoersdorff et al. 2001; Daniels et al. 2002; Goehert and Telford, 2003; Lappin et al. 2004; de la Fuente et al. 2005c).

Recent studies have contributed to the understanding of the epidemiology of *A. phagocytophilum* in southern Europe. Throughout Sicily, which represents a typical Mediterranean ecosystem, *A. phagocytophilum* was detected by PCR of the 16S rRNA gene in a broad host range including cattle, goats, sheep horses, dogs and mice (de la Fuente et al. 2005d; Torina et al. 2008a; 2010). Five genotypes of the pathogen were

identified: two were found in various hosts only the western region, one with 98.9% identity to a sequence isolated from a human case was identified only in cattle in the eastern region and two genotypes were found in sheep or mice in the eastern region. Higher host and regional specificity was found for *A. phagocytophilum* than for *A. marginale* and *A. ovis,* which was most likely related to the pathogen's wider host range (Torina et al. 2008a). Domestic animals, such as cattle, horses, donkey sheep, dogs and cats may serve as reservoir hosts for *A. phagocytophilum,* but ruminant variants were found to differ from human ones (Torina et al. 2008b). The low abundance of the *I. ricinus* in these areas suggests that other ticks may be involved in transmission of *A. phagocytophilum*.

In central Spain, *A. phagocytophilum* infections occur in humans and are maintained in cattle, donkeys, deer and birds. The presence of concurrent infections of *A. marginale* and *A. phagocytophilum* in cattle and deer suggests that these pathogens may multiply in the same reservoir host and also illustrates the complexity of the epidemiology of bovine and human anaplasmosis in the country (Naranjo et al. 2006). In Northern Spain, *A. phagocytophilum* infections were demonstrated in cattle, sheep and humans, while in central and southern Spain, wild rabbits, birds and cats were also been implicated in the epizootiology of *A. phagocytophilum* (de la Fuente et al. 2005c). In southern Spain, European roe deer were found by PCR analyses and serologic studies to be infected with *A. phagocytophilum* (de la Fuente et al. 2008d). Two different *A. phagocytophilum msp4* genotypes were identified in the roe deer that were 99.5-99.9% identical to sequences reported previously from northern Spain, while 89.9-90.1% of the sequences were identical to roe deer from Germany (de la Fuente et al. 2008d). Roe deer

are, therefore, likely to be involved in the natural cycle of *A. phagocytophilum* in Spain by serving as reservoir hosts.

Canine granulocytic anaplasmosis has a wide geographic distribution, and has also been reported throughout Europe, Australia and Canada and in all states of the U.S., except Mississippi and Nebraska (as reviewed by Tsachev 2009; Woldehiwet 2010).

Since the first report of HGE in horses in California, the emergence of *Anaplasma phagocytophilum*-associated diseases has continued to spread throughout the United States and other countries. Equine granulocytic anaplasmosis was subsequently reported in horses from Florida, Colorado, New Jersey and Connecticut (as reviewed by Woldehiwet 2010). *E. equi* was also reported in horses from areas in Europe, including Scandinavia (Engvall and Egenvall 2002), Switzerland (Pusterla et al. 1998) and the UK (McNamee et al. 1989; Korbutiak and Schneiders 1994; Shaw et al. 2001). Canine granulocytic anaplasmosis (CGA) was also first recognized in the USA before its more recent discovery in Europe.

Prior to discovery of human granulocytic anaplasmosis in the United States, the disease was thought to be limited to domestic and wild animals. Since the early 1990s when a Wisconsin patient with the index case of HGA died with a severe febrile illness after a tick bite, the yearly incidence of HGA in one Wisconsin county increased by 1995 to approximately 58 cases per 100,000 in one county (Dumler et al. 2005). Many of these newly described strains may be distinct and prove to have a limited host range. For example, the variant (Ap-variant 1) was shown to be infective for goats and deer but was not associated with human disease or infective for hamsters, mice and gerbils (Massung et al. 2006b; Reichard et al. 2009). Furthermore, ticks that were allowed to feed on deer

inoculated with the Ap-1 or the NY-18 isolate only became infected on deer inoculated with the Ap-1 variant (Reichard et al. 2009). These and other studies suggested that strains from ruminants might share common characteristics, which differ from strains that infect humans (Reichard et al. 2009; de la Fuente et al. 2005b). While most all strains of *A. phagocytophilum* identified thus far appear to be serologically cross-reactive, phylogenetic analysis of major surface protein (MSP) sequences, primarily *msp4*, provided strain differentiation (de la Fuente et al. 2005b).

The majority of human granulocytic anaplasmosis cases in the United States have been reported in the northeastern and upper mid-western states including Massachusetts, Connecticut, New York, Minnesota and Wisconsin (Rikihisa 2006). The yearly incidence rate in Connecticut from 1997 to 1999 was 24 to 51 cases per 100,000 population (Dumler et al. 2005). A total of 3,637 cases of HGA have been reported in the USA from 2003 to 2008, with most case reports (834) occurring in 2007 (Thomas et al. 2009). Cases of HGA have been reported in areas with a high incidence of human babesiosis and Lyme disease and also have coincided with the distribution of the tick vector, *Ixodes* spp. (Goodman 2005; Nadelman et al. 1997). Sero-epidemiology studies have suggested that many infections may not be diagnosed and that an estimated 15% to 36% of the population may have been infected (Aguero-Rosenfeld et al. 2002; Bakken et al. 1998). The incidence of *A. phagocytophilum* was recently reported to have increased between 2000 and 2007 from 1.4-to 3.0-cases/million persons/year (Dahlgren et al. 2011).

In Europe, the first cases of HGA were reported in Slovenia in 1997. The first human case of HGA was documented in Sicily, Italy, in 2005 (de la Fuente et al. 2005d). In Canada, the first case of HGA was reported in 2009 in an 82-year old man in which

infection was demonstrated by PCR and morulae were also identified in peripheral neutrophils (Parkins et al. 2009). Subsequently, HGA has been reported in The Netherlands, Spain, Sweden, Norway, Croatia, Poland, Slovenia and Greece (Thomas et al. 2009). Studies done in Europe suggest that the pathogen is an important tick-borne disease in Slovenia, Denmark and Sweden (Goodman 2005). Serologic studies also support the presence of HGA infection in potentially exposed adults in Germany, Bulgaria, Spain, Italy, Estonia and Greece but these cases were more likely to be asymptomatic (Woldehiwet 2010).

Tick vectors, transmission and the tick developmental cycle

Ticks, the arthropod vector of *Anaplasma* spp., are ectoparasites of wild, domestic animals and humans which transmit pathogens that impact human and animal health, and are considered to be the most important arthropod vector in some regions (de la Fuente et al. 2008b; Dumler et al. 2001). Ticks are classified in the subclass Acari, order Parasitiformes, suborder Ixodida and are dispersed worldwide from arctic to tropic areas (de la Fuente et al. 2007a). *A. phagocytophilum* is transmitted primarily by ticks of the *Ixodes persulcatus* complex, which are distributed mainly in the northern hemisphere (Woldehiwet 2010). In Europe *I. ricinus* appears to be the main vector of *A. phagocytophilum* (Strle 2004). However, several other ticks may also be vectors, including *Haemaphysalis punctata*, *I. persulcatus*, *I. trianguliceps* and *Rhipicephalus sanguineus* (as reviewed by Stuen 2007).

Ticks are a necessary biological vector of the *A. phagocytophilum* life cycle. The transmission of *A. phagocytophilum* was first studied experimentally in *I. ricinus* during the 1930s by MacLeod (MacLeod and Gordon 1933; MacLeod 1932; 1936). While

transmission of *A. phagocytophilum* was shown to be transstadial, in which infection is acquired by the feeding of larvae or nymphs on infected hosts and transmission occurring by the next tick stage, nymphs or adults, transovarial transmission was not demonstrated from the female adult tick to her progeny. Therefore, transmission of *A. phagocytophilum* within a generation of ticks is dependent on acquisition of the pathogen by feeding on an infected host (Hodzic et al. 1998a).

The tick species that vector *A. phagocytophilum* varies with the geographic region. In Europe, *I. ricinus* has been shown to be the main vector of *A. phagocytophilum* (Blanco and Oteo 2002; as reviewed by Thomas et al. 2009). Two *Ixodes* sp. are vectors of *A. phagocytophilum* in the United States: *I. scapularis* is the tick vector in the eastern and midwestern states (Pancholi et al. 1995; Goodman 2005), while *I. pacificus* is the vector in the western coast and mountain areas (Richter et al. 1996; Reubel et al. 1998).

While *Ixodes* ticks are considered to be the primary vectors of *A. phagocytophilum*, use of molecular technologies has provided evidence that other tick species are also infected with this pathogen and may therefore be involved in the pathogen transmission cycle. In a study of ticks collected in central Spain from European wild boar (*Sus scrofa*) and Iberian red deer (*Cervus elaphus hispanicus*), *A. phagocytophilum* was detected in *D. marginatus*, *Rhipicephalus bursa* and *Hyalomma m. marginatum* (Naranjo et al. 2006; de la Fuente et al. 2004; de la Fuente et al. 2005c,f). The low abundance of *I. ricinus* in areas of central and southern Spain suggests that other tick species are likely to be vectors of *A. phagocytophilum* and therefore could likely contribute to increased risk of the emergence and spread of granulocytic anaplasmosis. Tick transmission of the pathogen therefore may occur in the absence of adequate

populations of *Ixodes* sp. Other studies demonstrated *A. phagocytophilum* in *Dermacentor*, *Hemaphysalis* and *Rhipicephalus* ticks (MacLeod 1962; Holden et al. 2003; Alberti et al. 2005, Cao et al. 2006; Barandika et al. 2008). Tick transmission of *A. phagocytophilum* variants by these tick species may have different transmission patterns and target hosts which have not been reported thus far. Notably, Baldridge et al. (2009) demonstrated transovarial transmission of *A. phagocytophilum* variants in *D. albopictus,* which is of interest because this mode of transmission is not considered to occur with other *Anaplasma* spp. Transovarial transmission of *A. phagocytophilum* variants in nature, would reduce their dependence on mammalian reservoirs. Continued studies on tick transmission patterns of *A. phagocytophilum* strains and variants are needed to more fully define the role of ticks in the transmission of these variants. However, for the purpose of this review, transovarial transmission will not be considered as a characteristic of *A. phagocytophilum*.

Mammalian host infection levels at the time of tick feeding were found to influence tick infection rates. The transmission rate of *A. phagocytophilum* in nymphal *I. scapularis* from mice correlated with the bacteremia level in the mouse blood (Hodzic et al. 1998b). During the early stages of infection when infection of peripheral blood granulocytes was detected, ticks readily acquired the infection. Later in infection the number of ticks that became infected was reduced when the parasitemias were low (Hodzic et al. 1998a,b). However, once ticks become infected, even with a low number of bacteria, biological replication of the agent in tick appeared to compensate for the low infection rates and enhanced transmission which has also been shown with *A. marginale* (Eriks et al. 1993; Hodzic et al. 1998a). Transmission of *A. phagocytophilum* by ticks was

shown to occur between 24 and 48 h after tick attachment (Sukumaran at al. 2006). Hodzic et al. (1998b) reported that approximately one-third of the ticks became infected within 24 hours of attachment and that the frequency of tick infections and the tick infection rates increased over time. Ticks that were allowed to feed for over 48 hrs transmitted infection to mice, and ticks that fed to repletion proved to have higher infection rates which most likely resulted from both acquiring a great number of organisms and from replication of the pathogen within ticks during feeding (Hodzic et al. 1998b; Katavolos et al. 1998).

Vertebrate hosts, reservoir hosts and transmission cycle

Although *A. phagocytophilum* transmission was thought previously to be primarily between ticks and ruminants (Woldehewit 2010), this host range is now known to vary with the geographic region and includes various hosts such as rodents, birds, cats, deer, humans, horses, cattle, dogs, and sheep. However, only those hosts that develop persistent infections have been considered as a potential reservoir species (as reviewed by Woldehiwet 2010). In some regions small mammals may be less important reservoirs hosts because they develop low-level infections and have short life cycles while in other areas, rodents are important reservoir. The occurrence and severity of disease caused by *A. phagocytophilum* variants in hosts also appears to vary with the geographic region, which is thought to be influenced by a combination of factors including the variant, incidental and reservoir hosts, tick vectors and their capacity to transmit the pathogen (Woldehiwet 2010).

Reservoir hosts of *A. phagocytophilum* in Europe have been more completely defined than those in the United States. In Europe wood mice, yellow-necked mice,

voles, roe and red deer are the major reservoirs (Liz et al. 2000; Petrovec et al. 2002; Silaghi et al. 2008). In the U.S. reservoirs of *A. phagocytophilum* are hosts parasitized by *I. scapularis,* and include white-tailed deer (*Odocoileus virginianus*), raccoons (*Procyonw lotoa*), while footed mice (*Peromyscus leucopus*), gray squirrels (*Sciurus carolinensis*) and chipmunks (Levin et al. 2002; Nieto and Foley 2009; Telford et al. 1996; as reviewed by Woldehiwet 2010). In the eastern United States the major reservoir host is the white-footed mouse, while in the western part of the country wood rats and the Western gray squirrel serve as reservoir hosts (Nicholson et al. 1999; Foley et al. 2002; Nieto and Foley 2008; 2009). Other closely related species, including Douglas squirrels, flying squirrels and chip-monks, become infected with *A. phagocytophilum* and harbor ticks that may be capable of transmitting the pathogen to other wildlife species and humans (Foley et al. 2007; 2008 a,b; Nieto and Foley 2009).

While birds have not been clearly implicated as reservoir hosts of *A. phagocytophilum*, two species of birds in the U.S. may be hosts for variants and a source for infection for larval ticks (Daniels et al. 2002). Infected nymphal *I. ricinus* collected from migrating birds in Sweden were also shown to be infected with *A. phagocytophilum* (Bjoersdorff et al. 2001). Birds could contribute to the epidemiology of HGA by both serving as reservoir hosts and by spreading infected ticks during migrations. In Spain, *A. phagocytophilum* was detected in birds by PCR, with high prevalence in blackbirds (n=3), and *Turdus spp.* has been suggested previously to play a role in the epidemiology of HGA (de la Fuente et al. 2005c). In a study conducted on a 900-hectare hunting estate in the province of Ciudad Real, Castilla-La Mancha, central Spain of the bird species

analyzed, the highest prevalence of *A. phagocytophilum* infection was detected in blackbirds by PCR (de la Fuente et al. 2005c).

Pathogenesis and clinical presentation

In the mammalian host, *A. phagocytophilum* is one of only four bacteria known to survive within human neutrophils where it multiplies within parasitophorous vacuoles called “morulae”, a designation that was derived from the Latin word ‘morus’, meaning mulberry. Morulae of *A. phagocytophilum* are approximately 1.5 µm to 2.5 µm in diameter but have been reported to be as large as 6 µm (Popov et al. 1998). *A. phagocytophilum* infection of mammalian cells is mediated by pathogen recognition of a host cell receptor. However, the mechanisms by which the organism infects vertebrates at dermal tick bite site, the cells in which it multiplies, the cause of clinical symptoms or, in the case of severe illness, the duration of tissue damage, are not well understood (Goodman 2005).

A. phagocytophilum’s strict intracellular location provides a mechanism for evading host defenses and also promotes chemotactic mechanisms (IL-8) that assist the attraction of neutrophils to the tick bite site (Granquist at al. 2010). Neutrophils have been thought to be a poor host cell for intracellular bacteria because they are short-lived and are a principal defense cell involved in natural immunity by their ability to ingest and kill invading microorganisms. However, *A. phagocytophilum* infection changes neutrophil functions, which, in turn, contribute to the clinical disease. Apoptosis of infected cells is an immune response against most intracellular pathogens, including viruses, bacteria, and parasites. Neutrophils typically undergo spontaneous apoptosis within 6-12 h after being released into the peripheral blood from the bone marrow, and

this is an important process in the maintenance of homeostatic levels of neutrophils and resolution of inflammatory responses. However, *A. phagocytophilum* isolated in human peripheral blood neutrophils was shown to inhibit spontaneous and induced apoptosis of neutrophils for up to 48 h. This effect was also demonstrated by morphological evaluation for up to 96 h of neutrophils from peripheral blood leukocyte cultures (Niu et al. 2010). After infection of neutrophils within *A. phagocytophilum* morulae, as seen in stained blood smears, the organism proceeds to divide with the parasitophorous vacuole until the cell lyses and the released organisms infect other cells.

The early development of *A. phagocytophilum* pathogenesis in mammalian hosts remains to be clearly elucidated. A prepatent period of 4-7 days occurs between transmission from the tick bite and development of rickettsemia during which the host cell and site of development during this time have not been described. When susceptible animals are inoculated intravenously (IV) with infective blood, rickettsemia was not detected for up to 72-96 hours post inoculation (PI) (Woldehiwet 2010). Therefore, prior to the appearance of morulae, the organism remains undetectable and could possibly replicate in a different host cell. Evidence has suggested that *A. phagocytophilum* may be present in the lungs and spleen prior to being detected in blood, but the host cells during this time have not been identified (Snodgrass 1974; Woldehiwet 2010). *A. phagocytophilum* has been reported to likely infect myeloid precursors in the bone marrow rather than mature neutrophils (Walker and Dumler 1996; Woldehiwet 2010). However, an earlier study on *A. phagocytophilum*-infected sheep failed to provide evidence that immature neutrophils from the bone marrow were infected (Woldehiwet and Scott 1982; Woldehiwet 2010). When sheep infected with a variant of TBF were

treated with dexamethasone during the peak period of rickettsemia, the proportion of circulating granulocytes rose to over 90% within 2 hours, but the percentage of infected neutrophils was reduced. These results suggested that immature neutrophils mobilized from the bone marrow reserve were not infected prior to entering peripheral blood (Woldehiwet 2010).

During the rickettsemia, the main targets for *A. phagocytophilum* infection include the eosinophils, monocytes, and primarily neutrophils, and infection of these cells occurs by the end of the initial rickettsemia (Woldehiwet 1987; 2010). In *A. phagocytophilum* infection in sheep, goats and cattle, as many as 90% of the granulocytes may become infected, but the severity of infection and the febrile reaction is influenced by the *A. phagocytophilum* strain and the susceptibility and immune status of the host (Foggie 1951; Tuomi 1967a,b; Woldehiwet and Scott 1982; 1993; Woldehiwet 2010). In sheep, cattle and horses rickettsemia is accompanied by fever that can last for 7 days or longer (Tuomi 1967a,b; Gribble 1969; Woldehiwet 1987; Woldehiwet 2010). The first indication of TBF in animals that have been moved into tick-infested pastures is presence of high fever. However, other clinical signs, such as pyaemia in lambs, respiratory signs in cattle and secondary infections that appear days after being introduced to tick-infested pastures are good indicators of TBF (Woldehiwet 2006). A drop in milk yield is another clinical sign in dairy cattle. The severe leukopenia and especially the prolonged neutropenia that accompanies the disease are also good indicators of TBF. In some cases abortions may occur, especially when pregnant ewes or cows are moved to tick-infested pastures during the last stages of their pregnancy (Woldehiwet 2006). Equine and canine

infections are characterized by fever, depression, anorexia, leukopenia, and thrombocytopenia (Dumler et al. 2005).

The symptoms of *A. phagocytophilum* infection most commonly demonstrated by human patients are fever; chills, headache and myalgias (Bakken and Dumler 2006), but the duration and magnitude of the initial *A. phagocytophilum* infection in human patients have not been clearly established. The clinical presentation of HGA ranges in severity from asymptomatic, mild or a severe acute febrile illness that lead to death in some cases (Goodman 2005). Most HGA patients have reported exposure to tick bites 1 to 2 weeks prior to the onset of symptoms (Bakken and Dumler 2006). Infection of humans may not be apparent, and Dumler et al. (2005) estimated that as much as 15% to 36% of the population in endemic areas may have been infected without apparent clinical symptoms.

When *A. phagocytophilum* was propagated in the human promyelocytic cell line, HL-60 cells, the heavy infection developed rapidly and after 4 dpi most of the host cells died or were degenerating (de la Fuente et al. 2005a). While the host cellular mechanisms involved in *A. phagocytophilum* infection in vivo delays neutrophil apoptosis, infected HL-60 cells were found to be considerably more apoptic than uninfected cells. Thus, the *A. phagocytophilum* induced apoptosis delay appears to be a neutrophil-specific process and not a global consequence (de la Fuente et al. 2005a).

Clinical and Laboratory Diagnosis

In clinical human *A. phagocytophilum* infections, morulae can often be demonstrated in peripheral blood neutrophils. While *A. phagocytophilum* infection can be confirmed in stained blood smears by demonstration of morulae, these infected cells are often not easily seen in some hosts. Therefore, both serology and polymerase chain

reaction (PCR) amplification of *A. phagocytophilum* DNA from acute-phase blood analysis may be required to provide diagnosis in both early and late infections. Diagnosis can also be done by isolation of *A. phagocytophilum* in HL-60 cultured cells inoculated with acute-phase blood. However, tests done using blood samples must be conducted before patients begin antibiotic therapy because treatment will rapidly reduce the rickettsemia (Bakken and Dumler 2006). The ability to accurately and rapidly determine exposure to a tick-borne pathogen may improve the understanding of the clinical signs presented by a patient (Chandrashekar et al. 2010).

For serologic diagnosis, Chandrashekar et al. (2010) evaluated a commercially available cELISA for the detection on *A. phagocytophilum*. The *A. phagocytophilum* antigen used in the cELISA was a peptide derived from the immunodominant P44 protein. Experimentally infected dogs were found to be seropositive as early as 8 days PI (Chandrashekar et al. 2010). However, while these dogs remained persistently infected, morulae were not observed in neutrophils and these dogs did not show clinical signs (Chandrashekar et al. 2010). A positive ELISA was an indicator that the dog was exposed to *A. phagocytophilum* but did not confirm active infection. Therefore, PCR was required for confirmation of an infection through amplification of *A. phagocytophilum* DNA (Chandrashekar et al. 2010). These experimentally infected dogs also had *A. platys* antibodies that cross-reacted with the *A. phagocytophilum* ELISA (Chandrashekar et al. 2010). The cELISA was 99.1% specific for the detection of *A. phagocytophilum,* but it was important to note that the cELISA could be serorologically cross-reactive with antibodies to other *Anaplasma spp*. (Chandrashekar et al. 2010). In general, the serologic cross reaction of *Anaplasma* spp was shown to result from the presence of conserved

proteins, most notably major surface protein 5 (MSP5). A monoclonal antibody against MSP5 is a component of the *A. marginale* cELISA currently approved for use in the United Stated and Canada (Dreher et al. 2005; Strik et al. 2007). The *A. marginale* cELISA is cross-reactive with *A. phagocytophilum* antibodies.

Serologic diagnosis of *A. phagocytophilum* can also be done by indirect fluorescent antibody (IFA) tests at most reference laboratories (Chandrashekar et al. 2010). By use of IFA dogs were found to seroconvert as early as 2 to 5 days after morula appear in the blood (Chandrashekar et al. 2010).

Propagation in cell culture

A. phagocytophilum has been propagated in human, monkey and bovine capillary endothelial cell lines (Munderloh 2004). The human promyelocytic leukemia cell line, HL-60, allows for direct isolation and cultivation of *A. phagocytophilum* from humans and mice (Goodman et al. 1996; Blas Machado et al. 2007). In addition, two tick cell lines, IDE8 and ISE6, isolated originally from embryos of *I. scapularis,* have been used for propagation of human and variant strains of *A. phagocytophilum* (Woldehiwet and Horrocks 2005; Munderloh et al. 1996a,b; 1999; Massung et al. 2006a; Reichard 2009). These cell culture systems have been used for research on elucidating mechanisms of adhesion and for identification of *A. phagocytophilum* receptors (Goodman et al. 1999; Herron et al. 2000) and pathogen gene expression (Jauron et al. 2001; Woldehiwet and Horrocks, 2005), as well as gene expression in human and tick cells in response to *A. phagocytophilum* infection (de la Fuente et al. 2010; Villar et al. 2010; Zivkovic et al. 2009; 2010; de la Fuente et al. 2007b).

Genetic variants of *A. phagocytophilum*

Many variant strains of *A. phagocytophilum* occur in nature and have been described, most of which are serologically cross-reactive because serologic tests are often group-specific for highly conserved outer membrane proteins (Dumler et al. 1995; Zhi et al. 1997; 1998). *A. phagocytophilum* variants can be differentiated by sequence analysis of key genes. Variants of TBF were differentiated from HGA ones by comparing the sequences of the 16S rRNA gene, in which TBF variants differ in three positions (Chen et al. 1994). HGA variants that cause disease in humans in the United States were reported to have identical 16S rRNA sequences (Belongia et al. 1997; Massung et al. 2002; 2003; 2005). de la Fuente et al. (2005b) differentiated *A. phagocytophilum* variants from different hosts, including dogs, horses and humans by sequencing the *msp4* gene that encodes for the major surface protein 4. Five distinct variants of *A. phagocytophilum* were described in dogs. In Norway, Stuen et al. (2002) demonstrated that multiple variants of *A. phagocytophilum* occurred in the same flock of sheep.

Variants of *A. phagocytophilum* differ in their ability to infect hosts (Gabriel et al. 2009; Madigan et al. 1995; Morissette et al. 2009; Foley et al. 2002; 2007; 2008 a,b; Nieto and Foley 2008; 2009; Goodman 2005). Many of the factors that influence host and host cell specificity are yet to be identified (Rikihisa 2011). These differences are important to determine in order to define the epidemiology and ecology *A. phagocytophilum* variants in nature and to predict the risk of disease outbreaks.

Genomics

The *A. phagocytophilum* genome, as determined by use of the human HZ isolate, was found to be 1.47 Mb, which is approximately one fourth the size of the *Escherichia coli* genome (Rikihisa et al. 1997). The number of open reading frames (ORFs; 1,369) is

also proportionately the same in comparison with *E. coli* (Dunning Hotopp et al. 2006). The G-C content of the DNA of this strain is 41.6 mol%. Plasmid, intact prophage or transposable elements were not found in the genome (Rikihisa 2011). Genes required for the biosynthesis of lipopolysaccharide and peptidoglycan are also absent from the genome (Lin and Rikihisa 2003; Dunning Hotopp et al. 2006).

The genome of *A. phagocytophilum* contains numerous repeats with over 100 p44 /*msp2* genes, and genes containing tandem repeats (Dunning Hotopp et al. 2006; Storey et al. 1998; Zhi et al. 1999). The 44-kDa immunodominant major surface proteins of *A. phagocytophilum* are encoded by the *p44/msp2* multi-gene family (Wurytu et al. 2009), and the Omp-1/P44/Msp2 superfamily has been the most intensively studied outer membrane protein. P44 and MSP2 proteins are homologous yet distinct groups of proteins (Lin et al. 2004). The transcription of the various p44 genes allows for antigenic variation of *A. phagocytophilum.* P44 proteins play an important role in the pathogenesis of *A. phagocytophilum* in the mammalian host. The diversity of these genes and the surface protein they encode for may also reflect differences among variants in geographic regions and host specificities (Lin et al. 2004).

The genetic basis of the ability of *A. phagocytophilum* to adapt to different environments is by gene duplications that contribute to the diversification of the genes, often from development of novel gene function or pseudogenes (Lin et al. 2004). The *A. phagocytophilum* genome has 121 genes belonging to this superfamily: one *msp2*, two *msp2* homologs, one *msp4*, 113 *p44*, and three *omp-1* genes. *A. phagocytophilum* genes are differentially expressed in HL-60 and ISE6 cultured cells (Wang et al. 2007; Nelson

et al. 2008; Galindo et al. 2008; Zivkovic et al. 2009), suggesting that the host cell environment regulates the transcription of *A. phagocytophilum*.

Molecular interactions of *A. phagocytophilum* and vertebrate host cells

Intracellular pathogens, through a long-term association with host cells, have developed mechanisms that allow them to survive within the hostile environment of the host cells (Galan and Cossart 2005; Garcia-Garcia et al. 2009). These mechanisms have resulted in transcriptional changes in the infected host cells and regulation of cell functions, both of which have the potential to eventually contribute to disease (Garcia-Garcia et al. 2009). Major pathways affected during infection have been identified by global analysis of mammalian gene expression in response to infection with intracellular bacteria (Bryant et al. 2004; Garcia-Garcia et al. 2009). Intracellular bacteria have limited genetic and metabolic resources and therefore most likely have evolved global and efficient mechanisms for host cell gene regulation.

The first studies reported on the impact of *A. phagocytophilum* infection on host cell gene expression were done using the human promyelocytic cell line, HL-60. Microarrays of synthetic polynucleotides of 21,329 genes were studied in order to identify genes differentially expressed in response to pathogen infection, and the microarray results were then confirmed by RT-PCR (de la Fuente et al. 2005a). Genes found to be differentially regulated infected cells were those essential for cellular mechanisms including growth and differentiation, cell transport, signaling and communication, protection and some of these genes may be required for infection and multiplication with the host cell (de la Fuente et al. 2005a).

The presence of morulae of *A. phagocytophilum* within the cytosol of peripheral granulocytes is the hallmark of an HGA infection (Troese and Carlyon 2009). The infection and multiplication of *A. phagocytophilum* in human neutrophils results in changes of this host cell's function which contributes to cell longevity, thus maintaining the pathogen's niche within the parasitophorous vacuole by delaying apoptosis, inhibiting NADPH oxidase activity, and subverting phagolysome biogenesis in order to reside in an inclusion that does not fuse with lysosomes (Caryon and Fikrig 2003a; Sultana et al. 2010). The *A. phagocytophilum* AnkA protein is tyrosine phosphorylated by Abl-1 kinase to facilitate infection (Lin et al. 2007; IJdo et al. 2007; Sultana et al. 2010). *A. phagocytophilum* AnkA protein also binds to granulocytic DNA as well as nuclear proteins, and this interaction leads to speculation about the functional nature of AnkA-host cell DNA interactions (Park et al. 2004; Sultana et al. 2010). The agent of HGA also induces the tyrosine phosphorylation of ROCK1 in human neutrophils, which aids in intracellular survival (Thomas and Fikrig, 2007; Sultana et al. 2010). The studies done by Sultana et al. (2010) demonstrated the uniqueness of *A. phagocytophilum* as an obligate intracellular pathogen because of the development of mechanisms for persistence within mammalian cells. Tyrosine phosphorylation of proteins appears to play a significant role in the manipulation of host cellular events that promotes the survival of *A. phagocytophilum*.

The *A. phagocytophilum* infection cycle in mammalian host cells is initiated when the organism binds to the host cell receptor, Sialyl Lewis x (sLe×) – modified P-selectin glycoprotein ligand 1 (PSGL-1) (Yago et al. 2003; Carlyon and Fikrig 2006; Sukumaran et al. 2011). The binding to PSGL-1 requires cooperative recognition of the N-terminal

primary amino acid sequence as well as the α2,3-linked sialic acid and α1,3 linked fructose of sLex (Carlyon et al. 2003b; Yago et al. 2003; Troese and Carlyon 2009). Infection of bone marrow progenitors and myeloid cell lines occurs after *A. phagocytophilum* binds to the host cell receptor (Goodman et al. 1999; Herron et al. 2000; Troese and Carlyon 2009). Once within the parasitophorous vacuole, *A. phagocytophilum* undergoes a two-phase developmental cycle. The first intracellular stage is the reticulated form, which is a larger, pleomorphic, electron-lucent form that has a dispersed nucleoid. The reticulated form subsequently transforms into a smaller, round electron dense form, which has a dense nucleoid (Heimer et al. 1997; Munderloh et al. 1999; Munderloh et al. 2004; Munderloh et al. 1996b; Popov et al. 1998; Rikihisa et al. 1997; Webster et al. 1998; Troese and Carlyon 2009). The dense form is eventually released from the host cell and then is infective for other susceptible cells.

Sp110, which is a member of the nuclear body (NB), functions as a nuclear hormone receptor transcriptomal co-activator (de la Fuente et al. 2007b; Bloch et al. 2000). Sp110, as well as other NB proteins, play a role in IFN response and virus replication (de la Fuente et al. 2007b; Regad and Chelbi-Alix 2001). The expression of Sp110 is induced in human peripheral blood leukocytes and the spleen and is not seen in any other tissues (de la Fuente et al. 2007b; Regad and Chelbi-Alix 2001). Recently Sp110 was shown to control susceptibility to *Mycobacterium tuberculosis* in the mouse Sp110 homologue (de la Fuente et al. 2007b; Pan et al. 2005). de la Fuente et al. (2007b) hypothesized that Sp110 may be involved in the infection of Hl-60 cells with *A. phagocytophilum*. Sp110 mRNA levels were found to increase in HL-60 cells after 24 hr PI. This increase in Sp110 mRNA levels coincided with pathogen multiplication and

increasing infection levels (de la Fuente et al. 2007b). The silencing of Sp110 by RNAi resulted in decreased infection levels and suggested that Sp110 was required for *A. phagocytophilum* infection and or multiplication in the HL-60 cells (de la Fuente et al. 2007b).

The over expression of host PLIN in HL-60 cells infected with *A. phagocytophilum* suggested a mechanism by which lipolysis and cholesterol synthesis are affected during pathogen infection and multiplication within human cells (Manzano-Roman et al. 2008). The total cholesterol content was also found to be higher in *A. phagocytophilum* infected cells as compared to the uninfected (Manzano-Roman et al. 2008). The hormone-sensitive lipase (HSL) catalyses the formation of cholesterol in macrophages and other cells (Manzano-Roman et al. 2008; Yeaman 2004). PLIN was shown recently to promote HSL-mediated adipocyte lipolysis (Moore et al. 2005; Miyoshi et al. 2006; Granneman et al. 2007; Manzano-Roman et al. 2008).

The binding of *A. phagocytophilum* to HL-60 cells is dependent on the expression of PSGL-1 and α1-3 fucosyltransferase (Herron et al. 2000; Rikihisa 2010). Troese and Carlyon (2009) tested the bacterial populations enriched with dense and reticulated forms for their abilities to adhere to HL-60 cells and these studies demonstrated that only the dense form of *A. phagocytophilum* was able to bind to and infect HL-60 cells. After infection of cells, the reticulated forms became apparent within the morula, which initiate intracellular multiplication of the organism and subsequently transform into dense forms. Studies using Chinese hamster ovary cells that were transfected with PSGL-1 further confirmed that only the dense forms were able to bind to the PSGL-2 receptor further confirming their role as the infective stage (Troese and Carlyon 2009).

Following binding to the host cell receptor, *A. phagocytophilum* enters into the cell by caveolae/lipid raft-mediated endocytosis, a vesicle trafficking system used for cell entry by a wide variety of pathogenic microorganisms which bypasses phagolysosmal pathways (Lafont and van der Goot 2005; Rikihisa 2010). Lipid rafts are located on the cell surface and are specialized lipid microenvironments that are enriched with cholesterol, glycosphingolipid, GM1 ganglioside, glycosylphosphatidylinositol-anchored proteins (GAPs) and several other types of membrane proteins involved in signal transduction including receptors, signal transducers and membrane transporters (Simmons and Toomre 2000; Rikihisa 2010). Another important part of internalization of *A. phagocytophilum* is ROCK1 phosphorylation-induced signaling (Thomas and Fikrig 2007; Sukumaran et al. 2011). Gaps are required for the internalization of *A. phagocytophilum* (Rikihisa 2010). Lipid rafts form caveolae when caveolae-specific proteins or caveolins accumulate (Anderson 1998; Rikihisa 2010). Caveolae are also involved in compartmentalization of signaling activities (Anderson 1998; Simmons and Toomre 2000; Rikihisa 2010).

Neutrophils function to kill invading microorganisms by a variety of processes (Cohen 1994). *A. phagocytophilum* infection interferes with vesicular trafficking for avoidance of lysosomes. The inclusion compartment is not acidic, does not undergo lysosomal fusion or acquire late endosomal/lysosomal markers (Webster et al. 1998; Mott et al.1999). The parasitophorous vacuole avoids fusion with secretory vesicles and specific granules containing NADPH oxidase and proteolytic enzymes (Mott et al. 1999; IJdo and Mueller 2004; Carlyon et al. 2004). Cholesterol is required by *A. phagocytophilum* for survival and infection, which is incorporated from the host into its

membrane (Lin and Rikihisa 2003). The expansion of the parasitophorous membrane during infection also requires lipid, including cholesterol (Xiong et al. 2007).

Infection of *A. phagocytophilum* also results in inhibition of apoptosis, an important mechanism for killing intracellular pathogens. Peripheral blood neutrophils normally undergo apoptosis spontaneously or after being induced by the phagocytosis of microorganisms (DeLeo 2004; Scaife et al. 2003; as reviewed by Rikihisa 2011).

Molecular interactions of *A. phagocytophilum* and tick host cells

Ticks and the pathogens that they vector have evolved molecular interactions that benefit their mutual survival (Dale and Moran 2006; Kikuchi et al. 2007; Neelakanta et al. 2010). Evidence of co-evolution of ticks and tick-borne pathogens includes pathogen ligands and tick receptors which enable pathogens to infect tick cells (Pal et al. 2004; de la Fuente et al. 2010). Some microbes have been found to favor the host survival, while many are parasitic or commensal (Braendle et al. 2003; Kikuchi et al. 2007; Paris et al. 2008; Koropatnick et al. 2004; Baumann 2005; Hurst and Werren 2001; Scarborough et al. 2005; Hance and Bolvin 1993; Block et al. 1987; Neelakanta et al. 2010). Infection of ticks with most pathogens does not appear to be detrimental to the tick's survival and the tick immune response may function to limit pathogen infection levels to promote tick survival. Recent evidence suggests that pathogen infection may enhance tick fitness and survival (Busby et al. 2011). Characterization of molecular mechanisms that mediate tick-pathogen and tick-host interactions are important to identify because they will likely provide new targets for vaccines for control of tick infestations and for reduction of the tick vector competency (de la Fuente et al. 2008b; Kocan et al. 2008).

The interactions of *A. phagocytophilum* and tick host cells are not as well described as those between the organism and vertebrate host cells. While *A. phagocytophilum* has been shown to infect *I. scapularis* gut muscle cells Ap-V1 variant (Reichard et al. 2009) and salivary glands (Sukumaran et al. 2006; Kocan, unpublished results), the developmental cycle of this pathogen has not been completely described in ticks.

Transstadial transmission of the organism occurs but transovarial transmission does not appear to be a major means of transmission for most *A. phagocytophilum* variants. Uninfected larval *I. scapularis* can acquire infection within 2 days of tick feeding on an *A. phagocytophilum* infected mouse, and infection persists to the nymphal and adult stages (Hodzic et al. 1998a; Katavolos et al. 1998; Sultana et al. 2010). Once the infected tick feeds, transmission of *A. phagocytophilum* occurs between 24 to 48 hours (Hodzic et al. 1998a; Katavolos et al. 1998; Sultana et al. 2010).

Salp proteins were shown to be required for infection of *I. scapularis* with *A. phagocytophilum* (Schwalie and Schultz 2009). While proteins of the salp 15 family were found to be involved in *Borrelia burgdorferi* (the Lyme disease agent) tick infections, *A. phagocytophilum* was found to induce expression of Salp 16 in tick salivary glands (Sukumaran et al. 2006) and the protein was up-regulated in tick salivary glands during tick feeding (Sukumaran et al. 2006; Sultana et al. 2010). Silencing of the salp 16 gene by RNA interference (RNAi) resulted in a significant decrease in salivary glands infections (Sukumaran et al. 2006; Sultana et al. 2010). While *A. phagocytophilum* infected tick guts in the salp-silenced ticks, most of the organisms that infected the gut cells were unable to migrate to, infect or be transmitted from the salivary glands. *A.*

phagocytophilum therefore has a specific requirement for this salivary gland protein in order to complete its developmental cycle.

A. phagocytophilum selectively altered tick gene expression to promote its survival and transmission. In *I. scapularis*, the pathogen was found to induce the phosphorylation of actin and to selectively regulate gene transcription (Sultana et al. 2010). The phosphorylation of actin inhibited the nucleation and elongation of actin filaments, thus reducing actin polymerization. Pathogen induced actin phosphorylation was shown to be dependent on the p21activated kinase (IPAK1) –mediated signaling. The pathogen stimulated IPAK1 activity by G protein-coupled receptor Gβγ subunits, which mediates phosphoinositide 3-kinase (P13K) activation. Disruption of these pathways was found to impact the salp 16 gene which was shown to be crucial for *A. phagocytophilum* survival in tick salivary glands.

Infection of ticks with *A. phagocytophilum* also causes upregulated expression of alpha1, 3-fucosyltransferease. Interestingly, while the silencing of this gene was found to reduce infection of ticks with *A. phagocytophilum*, transmission was not affected (Pedra et al. 2010). Therefore, expression of this gene appears to be targeted to colonization of ticks with *A. phagocytophilum*.

Studies done by Neelakanta et al. (2010) identified an arthropod antifreeze glycoprotein, IAFGP which is involved in a mutualistic interaction between the *A. phagocytophilum* and *I. scapularis* and facilitates tick survival at cold temperatures (Neelakanta et al. 2010). In *A. phagocytophilum* over-wintered in ticks, a significant increase in the expression of the *iafgp* gene was observed at cold temperatures as compared with uninfected ticks that were allowed to over winter. The interaction

between IAFGP and *A. phagocytophilum* therefore may enhance the long-term coexistence of both the pathogen and the vector (Neelakanta et al. 2010).

The gene expression responses of ticks and cultured tick cells in response to infection with *A. phagocytophilum* and *A. marginale* were recently studied by microarray and real time RT-PCR analysis (Zivkovic et al. 2009). The results demonstrated the modulation of tick cell gene expression by *A. phagocytophilum* that proved to differ from *A. marginale*. Genes differentially expressed in *I. scapularis* nymphs and tick ISE6 cells infected with *A. phagocytophilum,* included GST and ferritin, were also shown previously to affect *A. marginale* infection and/or multiplication in ticks and/or tick cells (de la Fuente et al. 2007a). GST, ferritin, and aspartic protease (C3B2), also found to be differentially expressed in *A. phagocytophilum*-infected ISE6 cells, have been reported to be regulated by tick feeding or infection with other pathogens (Blouin et al. 2003; Macalusa et al. 2003) GST, ferritin, and aspartic protease (C3B2), also found to be differentially expressed in *A. phagocytophilum*-infected ISE6 cells, have been reported to be regulated by tick feeding or infection with other pathogens (de la Fuente 2005e; 2008c). Other genes differentially expressed after *A. phagocytophilum* infection included U2A8 (signal sequence receptor delta), 1I5B9 (ixodegrin-2A RGD containing protein), 2I3A7 (NADH-ubiquinone oxidoreductase), 2IP10 (ubiquitin C variant 5-like), 2I3A3 (gamma actin-like protein), C4B10 (von Willebrand factor), C2E6 (troponin I), and R1E12 (ribosomal protein L32) and may be involved in infection and/or multiplication of the pathogen in ticks or may be part of tick cell immune response to moderate pathogen infection levels.

Expression of heat shock proteins (HSPs) and other stress response proteins (SRPs) was characterized in ticks and cultured tick cells in response to *Anaplasma* spp. infection by proteomics and transcriptomics analyses. Heat shock demonstrated that the stress response was activated in ticks and cultured tick cells after *A. phagocytophilum* infection (Villar et al. 2010). However, in the natural vector-pathogen relationship, HSPs and other SRPs were not strongly activated, which likely resulted from tick-pathogen co-evolution. These results also demonstrated pathogen- and tick-specific differences in the expression of HSPs and other SRPs in ticks and cultured tick cells infected with *Anaplasma* spp. and suggested the existence of post-transcriptional mechanisms induced by *Anaplasma* spp. to control tick response to infection.

The differential expression of the tick protective antigen, subolesin, in response to infection of HL-60 and the ISE6 cultured tick cells and nymphal ticks with *A. phagocytophilum* was also reported (de la Fuente et al. 2008a). While *A. phagocytophilum* infection did not affect the expression of subolesin in cultured tick cells or human HL-60 cells, other genes were differentially regulated. Subolesin levels were similar for *I. scapularis* nymphs and the HL-60 cells, which suggest that the mechanism by which subolesin is regulated is after infection with *A. phagocytophilum* (de la Fuente et al. 2008a).

Summary

Although *A. phagocytophilum* has been recognized as a pathogen of veterinary importance in Europe for over 70 years, the emergence of human granulocytic anaplasmosis in the United States, Europe and Asia has created renewed interest and accelerated research on this pathogen. Although the *A. phagocytophilum* infection cycle

was thought previously to be primarily between ticks and ruminants (Woldehewit 2010), this host range now includes a variety of pathogen variants and hosts including various rodents, birds, cats, deer, horses, cattle, dogs, sheep and humans.

Many variants of *A. phagocytophilum* have been described, and the occurrence and severity of the disease caused by these variants in their respective hosts appears to vary with the geographic region. *A. phagocytophilum* variants are not easily differentiated because they are serologically cross-reactive due to the presence of conserved and group specific proteins expressed on the bacterial outer membrane. Variant identification is therefore most accurately done by PCR and sequence analysis of key genes. In contrast to *A. marginale* which is host specific for ruminants, the wide host range of *A. phagocytophilum* and the expanding populations and distribution of *Ixodes* sp. vectors will likely contribute to the increasing risk of acquiring *A. phagocytophilum* infection and thus the spread of granulocytic anaplasmosis is a growing concern for human and animal health in the United States.

In the vertebrate host, *A. phagocytophilum* infects granulocytes where it multiplies in the cytoplasm in parasitophorous vacuoles known as "morulae". The early stages of *A. phagocytophilum* pathogenesis in mammalian hosts remain to be clearly elucidated. During early infection the organism remains undetectable and could possibly replicate in other host cells. The infection and adaptation of *A. phagocytophilum* to vertebrate host cells has resulted in transcriptional changes and in disregulation of cell functions, both of which may contribute to the disease process.

Ticks are the necessary biological vectors for the *A. phagocytophilum* life cycle. The developmental cycle of *A. phag*ocytophilum in ticks has not been fully described.

While recent research has contributed to our understanding of these tick cell/pathogen interactions, continued studies are needed on the molecular interactions at the tick-pathogen interface in order to identify the tick genes that are required for tick vector competency and pathogen transmission.

Recent research on *A. phagocytophilum* has been focused on the molecular relationship of the pathogen with its host cells. As an intracellular pathogen with a long-term association with both vertebrate and tick host cells, *A. phagocytophilum* has developed mechanisms for survival within the hostile environment of the host cells. Research done using the human promyelocytic leukemia cell line, as well as two tick cell lines, ISE6 and IDE8, have provided insight on mechanisms of adhesion and identification of receptors for *A. phagocytophilum* and pathogen antigen expression, as well as the gene expression of human and tick cells in response to *A. phagocytophilum* infection.

Characterization of the molecular interactions involved in the host-tick-pathogen interface which will likely provide new targets for vaccine development which would likely provide effective strategies for disease prevention and control. Vaccine development strategies targeted toward the pathogen-tick interface could result in both reduced tick vector competency and control of tick infestations.

RESEARCH PROBLEM

Anaplasma phagocytophilum, the focus of this research, is the causative agent human granulocytic anaplasmosis, which is an emerging tick-borne disease of humans in the U.S. (Dumler et al. 2001; Stuen 2007). Control of this and other pathogens will be enhanced by development of vaccines that avoid the drawbacks of tick control by acaricides and, in addition, which are designed with the dual effect of targeting a broad range of tick species and reducing the tick vector competency for pathogens. Definition of the molecular interactions between pathogens and tick host cells is required in order to discover antigens for vaccine development. While *A. phagocytophilum* is known to be transmitted by *Ixodes spp.,* information on the tick development cycle and pathogen/vector interactions is lacking. Therefore, this research is focused on development of model systems for characterization of the interactions of a human isolate of *Anaplasma phagocytophilum* and its tick vector, *Ixodes scapularis*. The overall hypothesis for this research is that genes expressed in tick cells in response to *A. phagocytophilum* infection will include those required for pathogen infection, development and transmission. The first part of this research involved use of a tick cell line in order to obtain preliminary data on tick gene expression in response to infection with a human NY-18 isolate of *A. phagocytophilum.* Genes of interest, identified previously in proteomic studies, were tested in gene expression studies and in gene silencing studies using RNA interference (RNAi) in order to determine whether they were involved in pathogen infection. In the second part of this research a sheep tick model system was developed to allow infection of ticks in order to characterize the molecular interactions between ticks and the human isolate of *A. phagocytophilum*. The

results of these studies will contribute to our understanding of molecular interactions at the host-tick-pathogen interface, and definition of genes involved in these interactions will be fundamental toward development of new and novel vaccines for control of both tick infestations and prevention of the transmission of pathogens.

The specific objectives of the research proposed herein are:

Part I: Studies of the human NY-18 isolate of *A. phagocytophilum* in cultured tick cells.

(1) To characterize the differential expression of selected tick genes in cultured cells in response to *A. phagocytophilum* infection.

(2) To characterize the effect of gene silencing by RNAi of these selected genes on *A. phagocytophilum* infections in cultured tick cells; and

Part II: Development of a sheep model for studying the molecular interaction between the human NY-18 isolate of *A. phagocytophilum* and tick cells:

(3) Test whether the human NY-18 isolate of *A. phagocytophilum* is infective for sheep.

(4) Define the infection parameters of this human isolate in sheep.

(5) Test whether tick stages will feed and acquire *A. phagocytophilum* infection from the experimentally-infected sheep.

(6) To test for transstadial transmission of *A. phagocytophilum* by adult ticks acquired infection as nymphs on an experimentally-infected sheep.

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CHAPTER II

ANAPLASMA PHAGOCYTOPHILUM INHIBITS IXODES SCAPULARIS TICK CELL GROWTH AND TRANSPORT THROUGH PROTEINS INVOLVED IN PATHOGEN INFECTION/MULTIPLICATION AND TICK RESPONSE TO INFECTION

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Abstract

In this research we characterized the proteome of the *Ixodes scapularis*-derived cell line, ISE6, after infection with the tick-borne pathogen, *Anaplasma phagocytophilum*. Proteomics results were correlated with mRNA levels by real-time RT-PCR. Gene silencing studies were conducted using RNA interference (RNAi) in order to characterize the function of genes differentially expressed in the cultured tick cells in response to *A. phagocytophilum* infection. The quantitative proteomics analysis demonstrated that expression of proteins involved in cell growth and transport were significantly reduced in *A. phagocytophilum*-infected cells, thus reflecting the effect of pathogen multiplication on these cell processes. The results of this study identified new genes, including spectrin alpha chain or alpha-fodrin and Voltage-dependent anion-

selective channel or mitochondrial porin, to be involved in the tick cell infection/multiplication of *A. phagocytophilum* and the tick cell response to infection, thus contributing to our understanding of molecular events at the tick-pathogen interface. These results have increased our understanding of the role of tick genes in *A. phagocytophilum* infection and multiplication, and constitute a fundamental contribution toward the development of novel tick control measures.

Introduction

Ticks are ectoparasites of animals and humans and are considered to be the most important arthropod vector of pathogens in some regions (de la Fuente et al., 2008a). *Ixodes scapularis* Say (Acari: Ixodidae) is an important vector of pathogens that infects and cause disease in humans and domestic animals in the United States. *Anaplasma phagocytophilum* (Rickettsiales: Anaplasmataceae), the focus of this study, is the causative agent of human, canine and equine granulocytic anaplasmosis and tick-borne fever of ruminants (Dumler et al., 2001; Stuen, 2007).

Anaplasma phagocytophilum is an intracellular bacterium that infects vertebrate host neutrophils where it multiplies within a parasitophorous vacuole, thus evading host defenses while promoting chemotactic mechanisms that contribute to the attraction of neutrophils to the tick bite site (Popov et al., 1998; Granquist at al., 2010). Tick-*A. phagocytophilum* interactions are not as well characterized as those between *A. phagocytophilum* and vertebrate host cells (Rikihisa, 2011). While this pathogen has been shown to infect *I. scapularis* gut muscle cells (Ap-V1 variant) (Reichard et al., 2009) and salivary glands (Sukumaran et al., 2006; our unpublished results), the developmental cycle of this pathogen has not been described in ticks. Tick proteins such as Salp 16,

subolesin, antifreeze glycoprotein IAFGP and alpha1-3-fucosyltransferease were shown to be differentially regulated and required for infection of *I. scapularis* by *A. phagocytophilum* (Sukumaran et al., 2006; de la Fuente et al., 2006a; 2008b; 2010; 2011; Zivkovic et al., 2009; 2010; Schwalie and Schultz, 2009; Sultana et al., 2010; Pedra et al., 2010; Neelakanta et al., 2010). Expression of heat shock proteins and other stress response proteins was also characterized by proteomic and transcriptomic analyses in ticks and cultured tick cells in response to *Anaplasma* spp. infection. The stress response was activated in *I. scapularis* tick cells after *A. phagocytophilum* infection but at a lower level when compared with non-natural tick-pathogen relationships, probably reflecting tick-pathogen co-evolution (Villar et al., 2010a).

Tick cell lines were developed at the University of Minnesota, USA, in the 1990s (Munderloh et al., 1996; 1999) that were derived originally from *I. scapularis* embryos. These cell lines, particularly the IDE8 and ISE6 cell lines, were subsequently found to support the growth and multiplication of several tick-borne pathogens, including *A. phagocytophilum* (Munderloh et al., 1996; 1999; Woldehiwet and Horrocks, 2005; Woldehiwet et al., 2002; Massung et al., 2006; Bell-Sakyi et al., 2007; Reichard et al., 2009). Despite differences reported in the expression of some tick genes between *in vitro* and *in vivo* studies, cultured tick cells rapidly became a valuable tool for the study of tick-pathogen interactions (Bell-Sakyi et al., 2007; de la Fuente et al., 2007; 2010; Zivkovic et al., 2009; Villar et al., 2010a).

The overall goal of our research is to characterize molecular interactions at the vector-pathogen interface and then to use these results to develop vaccines for the control of tick infestations and pathogen infection/transmission. Proteomics studies provide

information on the cell protein content that may differ from results at the transcriptomic level and may be more relevant for tick vaccine antigen discovery (Villar et al., 2010a). Our hypothesis was that tick proteins differentially expressed in response to pathogen infection would include those involved in pathogen infection, multiplication and transmission, as well as being involved in the tick protective response against infection.

Few studies have characterized tick proteome in response to pathogen infection (de la Fuente et al., 2007; Villar et al., 2010a; 2010b; Stopforth et al., 2010). Herein, we characterized the *I. scapularis* proteome in early and late *A. phagocytophilum* infections of ISE6 tick cells. The proteomics results were correlated with mRNA levels by real-time RT-PCR. Functional studies were then conducted by RNA interference (RNAi) in order to characterize the function of differentially expressed genes in response to *A. phagocytophilum* infection of ISE6 tick cells. These experiments are a fundamental contribution towards the understanding of the tick-pathogen interface and may contribute to the development of new generation pathogen transmission-blocking vaccines designed to prevent transmission and reduce exposure of vertebrate hosts to *A. phagocytophilum*.

Materials and methods

Cultured tick cells

The ISE6 tick cell line, derived originally from *I. scapularis* embryos (provided by U.G. Munderloh, University of Minnesota, USA) was cultured in L15B medium as described previously (Munderloh et al., 1999). The ISE6 cells were inoculated with the NY-18 isolate of *A. phagocytophilum* propagated in HL-60 cells and maintained according to the procedures of de la Fuente et al. (2005). Uninfected cells were cultured in the same way, except with the addition of 1 ml of culture medium instead of infected

cells. Uninfected and infected cultures (five independent cultures with approximately 10^7 cells each) were sampled at 6 days post-infection (dpi) (early infection; percent infected cells 11-17% (Ave±SD, 13±2)) and 13 dpi (late infection; percent infected cells 26-31% (Ave±SD, 28±2)). Collected cells were centrifuged at 10,000 x g for 3 min and cell pellets were frozen in liquid nitrogen until used for protein, DNA and RNA extraction.

Proteomics analysis of infected and uninfected ISE6 tick cells

Proteomics analysis of *I. scapularis* ISE6 tick cells in response to *A. phagocytophilum* infection was done using protein one-step in-gel digestion, peptide iTRAQ labeling, IEF fractionation, LC-MS/MS analysis and peptide identification. Five independent cultures with approximately 10^7 ISE6 cells were pooled from each condition and lysed in 350 µl lysis buffer (PBS, 1% Triton X-100, 1 mM sodium vanadate, 1 mM NaF, 1 mM PMSF, 1µg/ml leupeptin, 1µg/ml pepstatin) for 30 min at 4ºC. Total cell extracts were centrifuged at 200×g for 5 min to remove cell debris. The supernatants were collected and protein concentration was determined using the Bradford Protein Assay (Bio-Rad, Hercules, CA, USA) with BSA as standard.

Protein extracts from the four experimental conditions (100 µg each): control early (CE), infected early (IE), control late (CL) and infected late (IL) were resuspended in up to 300 µl of sample buffer and applied using a 5-well comb on a conventional SDS-PAGE gel (1.5 mm-thick, 4% stacking, 10% resolving). The run was stopped as soon as the front entered 3 mm into the resolving gel, so that the whole proteome became concentrated in the stacking/resolving gel interface. The unseparated protein bands were visualized by Coomassie Brilliant Blue R-250 staining, excised, cut into cubes (2 x 2 mm) and digested overnight at 37ºC with 60 ng/µl trypsin (Promega, Madison, WI, USA)

at 5:1 protein:trypsin (w/w) ratio in 50 mM ammonium bicarbonate, pH 8.8 containing 10% (v/v) ACN and 0.01% (w/v) 5-cyclohexyl-1-pentyl-ß-D-maltoside (CYMAL-5) (Katayama et al., 2004; Shevchenko et al., 2006). The resulting tryptic peptides from each proteome were extracted by 1hr incubation in 12 mM ammonium bicarbonate, pH 8.8. TFA was added to a final concentration of 1% and the peptides were finally desalted onto C18 OASIS HLB Extraction cartridges (Waters, Milford, Massachusetts, USA) to remove the amine-containing buffers and dried-down.

Dried peptides were taken up in 30 µl of iTRAQ dissolution buffer provided with the kit (Applied Biosystems, Madrid, Spain) and labeled by adding 70 µl of the corresponding iTRAQ reagent in ethanol and incubating for 1hr at room temperature in 70% ethanol, 180 mM triethylammoniumbicarbonate (TEAB), pH 8.53. CE was labeled with 114, IE was labeled with 115, CL was labeled with 116 and IL labeled with 117 iTRAQ tags. After quenching the reaction with 100 µl 0.1% formic acid for 30 min, samples were brought to dryness to completely stop the labeling reaction. This quenching process was repeated once more to promote TEAB volatilization. The four labeled samples were resuspended in 100 µl 0.1% formic acid and combined into one tube. The mixture was dried down, redissolved in 3.3 ml 5 mM ammonium formiate, pH 3, cleaned up with SCX Oasis cartridges (Waters) using as elution solution 1 M ammonium formiate pH 3, containing 25% ACN, and dried down. The peptide pools were resuspended in 0.5 ml 0.1% TFA, desalted onto C18 Oasis cartridges using as elution solution 50% ACN in 5 mM ammonium formiate, pH 3 and dried down.

The sample was taken up in focusing buffer (5% glycerol and 2% IPG buffer pH 3-10 (GE Healthcare, Madrid, Spain) loaded onto 24-wells over a 24 cm-long Immobiline

DryStrip, pH3-10 (GE Healthcare) and separated by IEF on a 3100 OFFgel fractionator (Agilent, Santa Clara, CA, USA), using the standard method for peptides recommended by the manufacturer. The recovered fractions were acidified with 20 µl of 1 M ammonium formiate, pH 3, and the peptides were desalted using OMIX C18 tips (Varian, Palo Alto, CA, USA). After elution with 50% ACN in 5 mM ammonium formiate, pH 3, the peptides were dried-down prior to RP-HPLC-LIT analysis.

All samples were analyzed by LC-MS/MS using a Surveyor LC system coupled to a linear ion trap mass spectrometer model LTQ (Thermo-Finnigan, San Jose, CA, USA) as described previously (Lopez-Ferrer et al., 2004; Ortega-Perez et al., 2005). The LTQ was programmed to perform a data-dependent MS/MS scan on the 15 most intense precursors detected in a full scan from 400 to 1600 amu (3 µscans, 200 ms injection time, 10,000 ions target). Singly charged ions were excluded from the MS/MS analysis. Dynamic exclusion was enabled using the following parameters: 2 repeat counts, 90 s repeat duration, 500 exclusion size list, 120 s exclusion duration and 2.1 amu exclusion mass width. PQD parameters were set at 100 ms injection time, 8 microscans per scan, 2 amu isolation width, 28% normalized collision energy, 0.6 activation Q, 0.3 ms activation time. For PQD spectra generation 10,000 ions were accumulated as target and automatic gain control was used to prevent over-filling of the ion trap.

Protein identification was carried out as described previously (Lopez-Ferrer et al., 2004) using SEQUEST algorithm (Bioworks 3.2 package, Thermo Finnigan), allowing optional (Methionine oxidation) and fixed modifications (Cysteine carboxamidomethylation, Lysine and N-terminal modification of +144.1020 Da). The MS/MS raw files were searched against the alphaproteobacteria combined with the

arachnida Swissprot database (Uniprot release 15.5, 7 July, 2009) supplemented with porcine trypsin and human keratins. This joint database contains 638,408 protein sequences. The same collections of MS/MS spectra were also searched against inverted databases constructed from the same target databases. The alphaproteobacteria Swissprot database was used to identify and discard *Anaplasma* and possible symbiotic bacterial sequences from further analyses.

RNA interference in ISE6 tick cells

Oligonucleotide primers homologous to *I. scapularis* genes containing T7 promoters (Table 1) were used for *in vitro* transcription and synthesis of dsRNA as described previously (de la Fuente et al., 2006b), using the Access RT-PCR system (Promega, Madison, WI, USA) and the Megascript RNAi kit (Ambion, Austin, TX, USA). *I. scapularis* subolesin (de la Fuente et al., 2006a) and the unrelated Rs86 (de la Fuente et al., 2006c) dsRNAs were synthesized using the same methods described previously and used as positive and negative controls, respectively. The dsRNA was purified and quantified by spectrophotometry.

RNAi experiments were conducted in cell cultures by incubating ISE6 tick cells with 10 µl dsRNA ($5x10^{10}$-$5x10^{11}$ molecules/µl) and 90 µl L15B medium in 24-well plates using 10 wells per treatment (de la Fuente et al., 2008b). Control cells were incubated with subolesin and the unrelated Rs86 dsRNAs. After 48 hours of dsRNA exposure, tick cells were infected with cell-free *A. phagocytophilum* (NY-18 isolate) obtained from approximately $5x10^6$ infected HL-60 cells (90-100% infected cells) and resuspended in 24 ml culture medium, resulting in 1 ml/well (Thomas and Fikrig, 2007) or mock infected by adding culture medium alone. Cells were incubated for an additional

72 hours, collected and used for DNA and RNA extraction. RNA was used to analyze gene knockdown by real-time RT-PCR with respect to Rs86 control. DNA was used to quantify the *A. phagocytophilum* infection levels by *msp4* PCR.

Real-time RT-PCR

Total RNA was extracted from cultured ISE6 tick cells using TriReagent (Sigma, St. Louis, MO, USA) following manufacturer's recommendations. Real-time RT-PCR was performed on tick RNA samples with gene specific primers (Table 2) using the iScript One-Step RT-PCR Kit with SYBR Green and the iQ5 thermal cycler (Bio-Rad, Hercules, CA, USA) following manufacturer's recommendations. A dissociation curve was run at the end of the reaction to ensure that only one amplicon was formed and that the amplicons denatured consistently in the same temperature range for every sample (Ririe et al., 1997). The mRNA levels were normalized against tick 16S rRNA (Zivkovic et al., 2009) using the genNorm method (ddCT method as implemented by Bio-Rad iQ5 Standard Edition, Version 2.0) (Livak and Schmittgen, 2001).

Determination of *A. phagocytophilum* infection levels

DNA was extracted from cultured tick cells using TriReagent (Sigma, St. Louis, MO, USA) following manufacturer's recommendations. *A. phagocytophilum* infection levels were characterized by *msp4* real-time PCR normalizing against tick 16S rDNA as described previously (de la Fuente et al., 2006a) but using oligonucleotide primers MSP4-L (5'-CCTTGGCTGCAGCACCACCTG-3') and MSP4-R (5'-TGCTGTGGGTCGTGACGCG3') and PCR conditions of 5 min at 95°C and 35 cycles of 10 sec at 95°C, 30 sec at 55°C and 30 sec at 60°C.

Sequence analysis

Protein ontology for biological process (BP) of differentially expressed proteins was done using the human protein databases at http://www.hprd.org/ and http://www.ebi.ac.uk/interpro/. Blasting against nonredundant sequence database (nr) and databases of tick-specific sequences (http://www.ncbi.nlm.nih.gov and http://www.vectorbase.org/index.php) was done using tblastx and blastn (http://blast.ncbi.nlm.nih.gov/Blast.cgi). Nucleotide sequences were aligned using the program AlignX (Vector NTI Suite V 5.5, InforMax, North Bethesda, MD, USA) and protein sequences were aligned using the CLUSTAL 2.1 multiple sequence alignment tool (EMBL-EBI; http://www.ebi.ac.uk/Tools/). Conserved protein domains (cd) were analyzed using Conserved Domains and Protein Classification at ncbi (http://www.ncbi.nlm.nih.gov/Structure/cdd/cdd.shtml; Marchler-Bauer et al., 2009). Phylogenetic analyses of protein sequences were conducted in MEGA5 (Tamura et al., 2007) using the Neighbor-Joining (Saitou and Nei, 1987), Maximum Likelihood (Jones et al., 1992) and Minimum Evolution (Rzhetsky and Nei, 1992) methods. A bootstrap analysis with 1,000 replicates was conducted to calculate the percentage of replicate trees in which the associated taxa clustered together (Felsenstein, 1985).

Statistical analysis

Statistical analysis of proteomics data and determination of error rates were performed with the Probability Ratio Method (Martínez-Bartolomé et al., 2008). False Discovery Rate (FDR) was used as a measure of statistical significance of peptide identification and was calculated using the refined method proposed by Navarro and Vázquez (2009) and Jorge et al. (2009). Differential protein expression in early versus

late infections was compared using Venn diagrams to demonstrate shared and distinct protein expression. Significance of overlaps was calculated using hypergeometric distributional assumption (Ivanova et al., 2002) and P-values were adjusted using Bonferroni correction for multiple comparisons (Schaffer, 1995). The proportion of up and down represented proteins was statistically analyzed separately for early and late infections and between early and late infections for each BP protein ontology category by a Fisher two tailed test (P=0.05) using Statistica 6.0 software (StatSoft Inc., Tulsa, OK, USA). To analyze gene expression and *A. phagocytophilum* infection levels, normalized Ct values were compared between test dsRNA-incubated tick cells and controls incubated with Rs86 dsRNA or between infected and uninfected tick cells by Student's t-test (P=0.05).

Results

Proteomics analysis of *I. scapularis* ISE6 cells infected with *A. phagocytophilum*

A total of 83 proteins were identified as being differentially expressed in *I. scapularis* ISE6 cells infected with *A. phagocytophilum* (Fig. 1A). Of them, 50 proteins were under-represented and 33 over-represented in infected cells. Early stages of infection (11-17% infected cells) resulted in 13 and 8 under- and over-represented proteins, respectively, while as infection advanced to 26-31% infected cells, the number of differentially expressed proteins increased to 50 and 31 under- and over-represented proteins, respectively (Fig. 1B). Comparison of differential protein expression in early and late infections with Venn diagrams demonstrated a significant overlap (Fig. 1A). Most of the proteins differentially expressed during early infection were also found during late infection. However, the number of differentially expressed proteins increased

as infection proceeded. Analyses of protein ontology for differentially expressed proteins demonstrated that biological processes (BP) such as cell growth, protein and nucleic acid metabolism, and transport were affected during early and late infections (Fig. 1B). However, significant differences were observed between under- and over-represented proteins in both early and late infections for cell growth and transport BP and between early and late infections for cell growth BP (Fig. 1B). While cell growth proteins were significantly over-represented during early infection, they were under-represented in late infected cells. Transport was significantly under-represented during both early and late infections (Fig. 1B). Proteins in cell growth and transport BP were selected for the characterization of mRNA levels during *A. phagocytophilum* infection in *I. scapularis* ISE6 tick cells (Table 3).

Changes in mRNA levels of genes encoding for *I. scapularis* cell growth and transport proteins affected in response to *A. phagocytophilum* infection

The mRNA levels of genes encoding for proteins in cell growth and transport BP were analyzed by real-time RT-PCR in early and late-infected cells and compared with the proteomics results (Table 4). The results showed differences between protein and mRNA levels. However, in 6 of the analyzed genes (CG1, CG8, CG10, T1, T2, T3), differential expression in late-infected cells was similar at the protein and mRNA levels, and these results were also similar for T1 and T2 genes in early-infected cells (Table 4). The genes with significant differences in protein and mRNA levels between infected and uninfected cells (CG2, CG8, CG10, T1, T2 and T3) (Table 4) were selected for analysis of mRNA levels in ISE6 cells and for functional studies by RNAi.

mRNA levels of selected genes in *I. scapularis* ISE6 cells

The mRNA levels of CG2, CG8, CG10, T1, T2 and T3 genes were characterized in ISE6 tick cells (Fig. 2). The results revealed differences in mRNA levels between genes. Lowest and highest mRNA levels were found for CG2 and T1 genes, respectively with 400-fold difference between them (Fig. 2). CG8 mRNA levels were only 10-fold higher than CG2 mRNA levels (Fig. 2). The mRNA levels for CG10, T2 and T3 were similar between them and approximately 100-fold higher than CG2 mRNA levels (Fig. 2).

Effect of gene knockdown on *A. phagocytophilum* infection of *I. scapularis* ISE6 cells

Gene knockdown by RNAi was used for functional characterization of CG2, CG8, CG10, T1, T2 and T3 genes during *A. phagocytophilum* early infection of ISE6 tick cells (Table 5). Significant gene knockdown was obtained for 4 genes, CG2, CG8, T2 and subolesin (Table 5). However, gene knockdown produced significant differences in *A. phagocytophilum* infection levels only for CG8, T2 and subolesin. Pathogen infection levels were lower and higher in CG8 and T2 knockdown cells, respectively (Table 5). Subolesin knockdown also resulted in higher pathogen infection levels in ISE6 tick cells (Table 5).

Sequence analysis of *I. scapularis* genes functionally relevant for *A. phagocytophilum* infection of ISE6 tick cells

Additional sequence analysis was conducted on *I. scapularis* genes affecting *A. phagocytophilum* infection after gene knockdown in ISE6 tick cells. The *I. scapularis* spectrin alpha chain or alpha-fodrin (CG8) sequence contained 24 spectrin (cd00176) repeats. The *I. scapularis* voltage-dependent anion-selective channel (VDAC) or mitochondrial porin (T2) contained one VDAC (cd07306) domain. Sequence databases

were searched for CG8 and T2 orthologs. Sequences homologous to *I. scapularis* CG8 (Genbank accession number XP_002433506) were not identified in other tick species but *I. scapularis* T2 (XP_002408065) orthologs were found in *Rhipicephalus (Boophilus) microplus* (ADT82652) and *Amblyomma variegatum* (DAA34069). Phylogenetic analysis of CG8 (Fig. 3) and T2 (Fig. 4A) sequences showed that tick sequences clustered together in a clade close to water flea *Daphnia pulex* (Arthropoda: Crustacea) and insect sequences. Similar results were obtained with all methods used to infer evolutionary histories (data not shown). Alignment of T2 tick ortholog protein sequences revealed differences in sequence length that could be due to incomplete sequence information or result of evolution with a 79% homology in shared 234 amino acids (Fig. 4B).

Discussion

In this work we characterized *I. scapularis* proteins differentially expressed in response to *A. phagocytophilum* infection by proteomic analysis of infected and uninfected ISE6 tick cells. The quantitative proteomics analysis demonstrated that expression of proteins involved in cell growth and transport were significantly reduced in *A. phagocytophilum*-infected cells, thus reflecting the effect of pathogen multiplication on these cell processes. Proteins in the two most affected BP, cell growth and transport, were further characterized at the mRNA level by real-time RT-PCR. Genes confirmed as differentially expressed in infected tick cells at both protein and mRNA levels were functionally characterized by RNAi to analyze their role during pathogen infection of ISE6 tick cells. Several studies have characterized the *A. phagocytophilum*-tick interface at the molecular level (Sukumaran et al., 2006; de la Fuente et al., 2006a; 2008b; 2010; 2011; Zivkovic et al., 2009; 2010; Schwalie and Schultz 2009; Sultana et al., 2010; Pedra

et al., 2010; Neelakanta et al., 2010; Villar et al., 2010a). However, this appears to be the first report of the analysis of global protein changes in tick cells in response to *A. phagocytophilum* infection. Furthermore, genes confirmed to be differentially expressed at both protein and mRNA levels with predicted function suggested that these genes are involved in pathogen infection/multiplication and tick response to infection.

Spectrin alpha chain or alpha-fodrin (CG8).

The spectrin repeats in *I. scapularis* spectrin alpha chain or alpha-fodrin (CG8) sequence are found in several proteins involved in cytoskeletal structure (Pascual et al., 1997; Viel, 1999). CG8 is an actin cross linking and molecular scaffold protein that links the plasma membrane to the actin cytoskeleton and functions in the determination of cell shape, arrangement of transmembrane proteins and organelles organization, all necessary for cell growth and/or maintenance. In other systems, fodrin-mediated actin rearrangements occur during pathogen infection of host cells (Shimada et al., 1999) but fodrin activation could result in cell apopotosis (Dhermy, 1991). These results suggested a dual role for CG8 by inducing cytoskeleton rearrangements necessary for pathogen infection while at the same time acting as a host cell defense mechanism to control pathogen infection through induction of cell apoptosis.

This dual effect of CG8 was also suggested in tick cells infected with *A. phagocytophilum*. CG8 was downregulated in infected tick cells, likely manipulated by the pathogen, because the gene is involved in host cell response to infection. However, CG8 knockdown resulted in lower *A. phagocytophilum* infection levels because CG8 is also required for pathogen infection. In fact, downregulation of CG8 was more pronounced in late *A. phagocytophilum*-infected tick cells (Table 4), when inhibiting cell

apoptosis is crucial to increase infection but rearrangement of actin filaments that are required for pathogen infection may be less relevant at this infection stage. When RNAi produced gene knockdown before infection, the effect on cytoskeleton rearrangement occurred at early infection stages and thus resulted in lower pathogen infection levels. Interestingly, certain spectrin mutations or polymorphisms have been shown to constitute new factors of innate resistance to malaria *in vitro* (Dhermy et al., 2007).

Voltage-dependent anion-selective channel (VDAC) or mitochondrial porin (T2).

The VDAC domain present on *I. scapularis* voltage-dependent anion-selective channel (VDAC) or mitochondrial porin (T2) suggested that this molecule is the channel known to guide the metabolic flux across the mitochondrial outer membrane that plays a key role in mitochondrially-induced apoptosis (Colombini et al., 1996; Bayrhuber et al., 2008). T2 is the most abundant protein in the mitochondrial outer membrane and regulates the flux of mostly anionic metabolites through the outer mitochondrial membrane, which is highly permeable to small molecules (Colombini et al., 1996; Bayrhuber et al., 2008). T2 binds to and is regulated in part by hexokinase, an interaction that renders mitochondria less susceptible to pro-apoptotic signals, most likely by interfering with T2's capability to respond to Bcl-2 family proteins (Colombini et al., 1996; Bayrhuber et al., 2008). However, while T2 appears to play a key role in mitochondrially-induced cell death, a proposed involvement in forming the mitochondrial permeability transition pore, which is characteristic for damaged mitochondria and apoptosis, has been challenged by more recent studies (Colombini et al., 1996; Bayrhuber et al., 2008). Epithelial cell invasion by Group A *Streptococcus pyogenes* results in downregulation of VDAC1 and VDAC2 genes, apoptosis and stress (Nakagawa et al.,

2004). Upon viral infection, cells undergo apoptosis as a defense against viral replication and viruses have evolved mechanisms to subvert apoptotic processes through expression of viral proteins that interact with Bcl-2 and VDAC1 (Feng et al., 2007).

T2 was downregulated in both early and late *A. phagocytophilum*-infected tick cells (Table 4), and was likely manipulated by the pathogen because the gene is involved in cell response to infection as knockdown resulted in higher infection levels. These results suggested that T2 downregulation in tick cells was produced by *A. phagocytophilum* infection to induce mitochondrial dysfunction and inhibit mitochondrial apoptosis-mediated intracellular innate immunity as a mechanism to subvert host cell defense against pathogen infection.

Subolesin is a candidate tick protective antigen initially discovered in *I. scapularis* and subsequently found to be conserved in many tick species and other eukaryotes where it is the ortholog of insect and mammalian akirins (Almazán et al., 2003; Goto et al., 2008; Galindo et al., 2009; Mangold et al., 2009; de la Fuente et al., 2011). Subolesin plays an important role in tick immune response to pathogen infection through the regulation of genes involved in innate immunity (Galindo et al., 2009; Mangold et al., 2009; Zivkovic et al., 2010). Subolesin expression is induced in response to pathogen infection in ticks (Zivkovic et al., 2010; Merino et al., 2011). As in previous experiments, subolesin knockdown resulted in higher *A. phagocytophilum* infection levels in tick cells (Busby et al., 2011).

Functional studies are essential to understand the role of differentially expressed genes. dsRNA-mediated RNAi was used in this study to analyze the effect of knockdown of selected genes on *A. phagocytophilum* infection in ISE6 tick cells. The possibility of

RNAi off-target effects (Scacheri et al., 2004) could not be ruled out in our gene knockdown experiments. Although off-target effects may be minimal for some genes in ticks (de la Fuente et al., 2008c), their effect has been documented in *R. microplus* (Lew-Tabor et al., 2011). Additionally, due to differences between *in vitro* and *in vivo* studies (de la Fuente et al., 2008b; Villar et al., 2010a; Busby et al., 2011), results in cultured tick cells should be corroborated in ticks.

Conclusions

The present study had allowed for identification of new genes involved in the tick infection/multiplication of *A. phagocytophilum*, thus advancing our understanding of the molecular events at the tick-pathogen interface. The results reported herein contribute to our understanding of the role of tick genes in *A. phagocytophilum* infection/multiplication, which is fundamental toward development of novel tick control measures.

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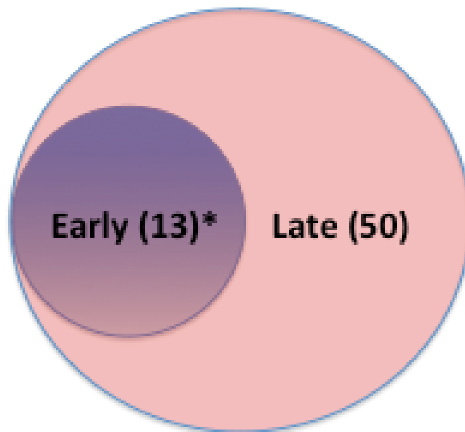
A

Down in Early vs. Late infections (63 proteins)

Up in Early vs. Late infections (39 proteins)



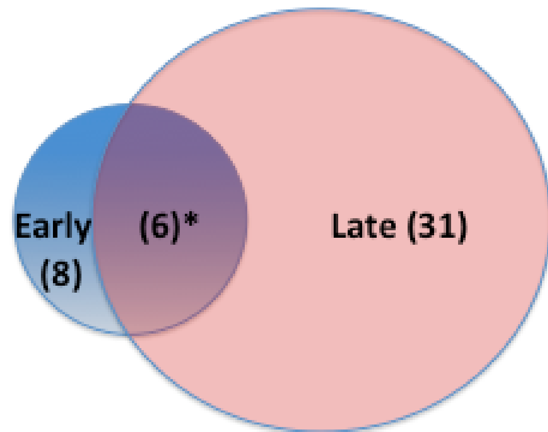


Fig. 1A

B

EARLY INFECTION

Down in infected cells	N=13
Cell growth	7.7%*, **
Protein metabolism	38.5%
Nucleic acid metabolism	23.1%
Transport	15.4%*
Unknown	15.3%
Up in infected cells	**N=8**
Cell growth	12.5%*, **
Protein metabolism	37.5%
Nucleic acid metabolism	25.0%
Transport	0.0%*
Unknown	25.0%

LATE INFECTION

Down in infected cells	N=50
Cell growth	20.0%*, **
Protein metabolism	30.0%
Nucleic acid metabolism	14.0%
Transport	6.0%*
Energy metabolism	16.0%
Cell communication	6.0%
Lipid metabolism	0.0%
Unknown	8.0%
Up in infected cells	**N=31**
Cell growth	3.2%*, **
Protein metabolism	38.7%
Nucleic acid metabolism	25.8%
Transport	0.0%*
Energy metabolism	9.7%
Cell communication	3.2%
Lipid metabolism	3.2%
Unknown	16.2%

Fig 1B

Figure 1. Overlapping protein expression in early versus late *A. phagocytophilum* infection of ISE6 tick cells. (**A**) Venn diagram detailing shared and distinct protein expression (*indicates significant overlaps ($p<10^6$)). (**B**) A summary of biological process (BP) protein ontology of differentially expressed proteins between infected and uninfected tick cells during early and late infections (* and ** indicate significant differences ($p<0.05$) between under- and over-represented proteins in both early and late infections and between early and late infections, respectively).

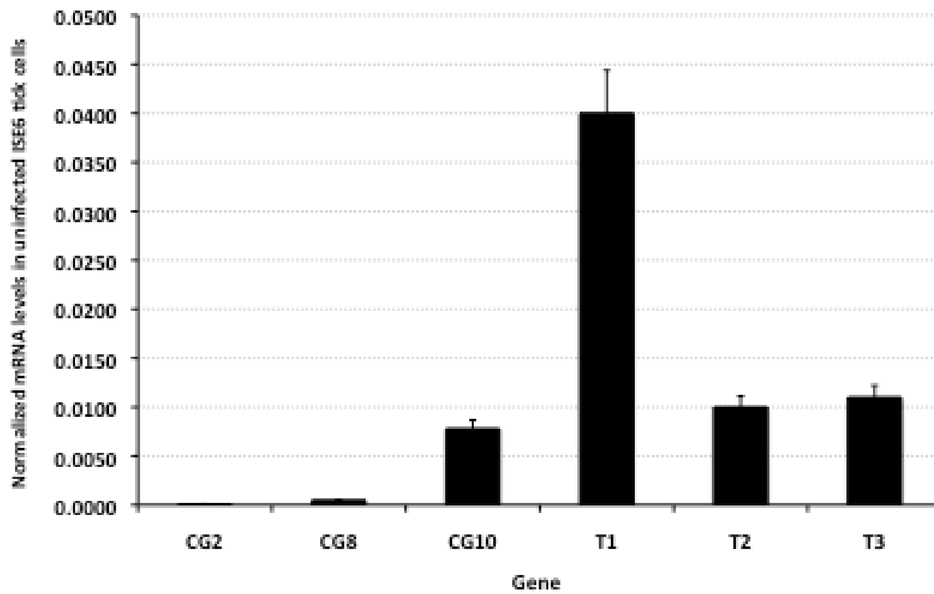


Figure 2. Gene expression in *I. scapularis* ISE6 cells. The CG2, CG8, CG10, T1, T2 and T3 mRNA levels were determined by real-time RT-PCR in ISE6 cells (three independent cultures). Amplification efficiencies were normalized against tick 16S rRNA and normalized mRNA levels were expressed in arbitrary units.

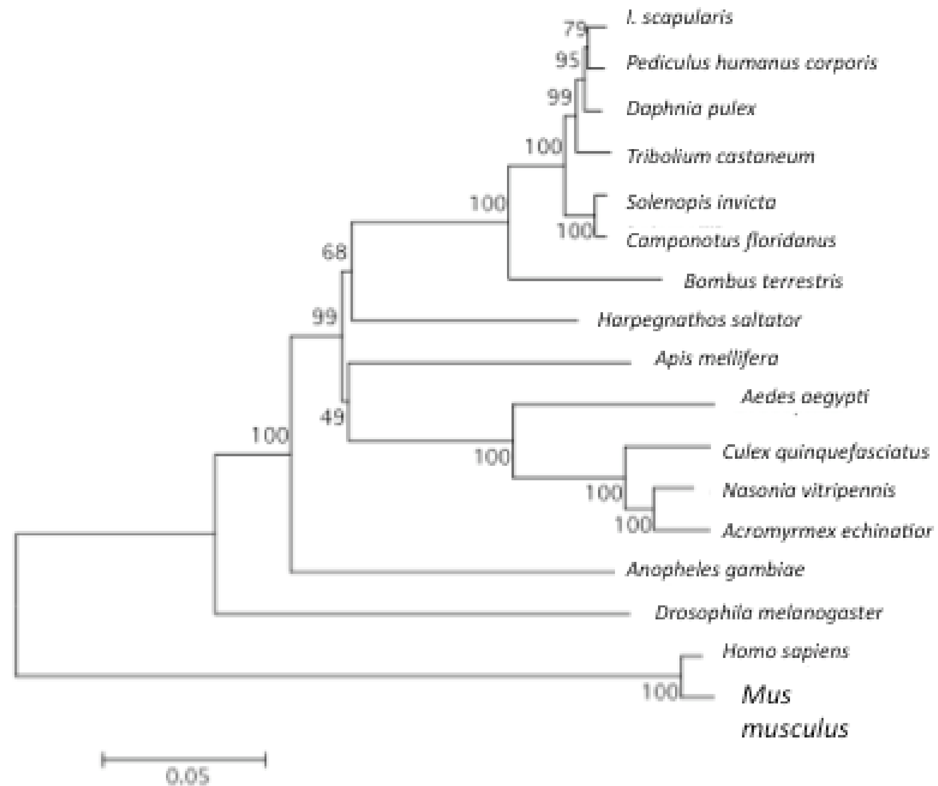


Figure 3. Phylogenetic analysis of spectrin alpha chain or alpha-fodrin (CG8) protein sequences. The evolutionary history was inferred using the Neighbor-Joining method. The optimal tree with the sum of branch length = 1.08474862 is shown. The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (1,000 replicates) are shown next to the branches. The tree is drawn to scale, with branch lengths in the same units as those of the evolutionary distances used to infer the phylogenetic tree. The evolutionary distances were computed using the Poisson correction method and are in the units of the number of amino acid substitutions per site. The analysis involved 17 amino acid sequences. All ambiguous positions were removed for each sequence pair. There were a total of 2,495 positions in the final dataset. Sequences included in the analysis corresponded to *I. scapularis* (XP_002433506),

Pediculus humanus corporis (XP_002430009), *Daphnia pulex* (EFX88672), *Tribolium castaneum* (XP_973750), *Solenopsis invicta* (EFZ23210), *Camponotus floridanus* (EFN61994), *Bombus terrestris* (XP_003394286), *Harpegnathos saltator* (EFN85586), *Apis mellifera* (XP_623691), *Aedes aegypti* (XP_001650465), *Culex quinquefasciatus* (XP_001865112), *Nasonia vitripennis* (XP_001601352), *Acromyrmex echinatior* (EGI62932), *Anopheles gambiae* (XP_316724), *Drosophila melanogaster* (AAA28907), and *Homo sapiens* (NP_001182461) and *Mus musculus* (NP_001171139) as outgroups.

Figure 4. Analysis of voltage-dependent anion-selective channel (VDAC) or mitochondrial porin (T2) protein sequences. (A) Amino acid sequence alignment of tick T2 ortholog sequences in *I. scapularis* (XP_002408065), *R. microplus* (ADT82652) and *A. variegatum* (DAA34069). Asterisks denote amino acids conserved among all sequences analyzed. (B) The evolutionary history was inferred using the Neighbor-Joining method. The optimal tree with the sum of branch length = 1.55282836 is shown. The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (1,000 replicates) are shown next to the branches. The tree is drawn to scale, with branch lengths in the same units as those of the evolutionary distances used to infer the phylogenetic tree. The evolutionary distances were computed using the Poisson correction method and are in the units of the number of amino acid substitutions per site.

The analysis involved 12 amino acid sequences. All ambiguous positions were removed for each sequence pair. There were a total of 282 positions in the final dataset. Sequences included in the analysis corresponded to *I. scapularis* (XP_002408065), *R. microplus* (ADT82652), *A. variegatum* (DAA34069), *Aedes aegypti* (XP_001654143), *Culex quinquefasciatus* (XP_001842637), *Anopheles gambiae* (XP_318947), *Tribolium castaneum* (XP_976150), *Daphnia pulex* (EFX77112), *Drosophila melanogaster* (NP_476813), *Musca autumnalis* (ADT92003), and *Homo sapiens* (CAG33245) and *Mus musculus* (NP_035825) as outgroups.

Table 1. PCR conditions and sequences of primers used for dsRNA synthesis.

Gene (sample ID)	Forward and reverse primers (5´-3´)*	PCR annealing conditions
Protein hu-li tai shao, Adducin (CG2)	ATGACATGCGGGGCGTGGA ACAAACACCTTGCTGC	57ºC/30s
Spectrin alpha chain, cytoskeletal protein (CG8)	CGCTGGCAACGACATTGAA TTCAGGTCCTTCACCCGGT	57ºC/30s
Beta tubulin (CG10)	TGTGGGAATCAAATCGGTG CTGGTGCACAGACAGGGTG	57ºC/30s
Na+/K+ ATPase, alpha subunit (T1)	TGACCTCAAGCAGGAAGTT CTCTGGTGAGCGTGTCTGA	57ºC/30s
Voltage-dependent anion-selective channel (T2)	ATGGCTCCTCCGTGCTACG AATTGTTGACCTTCGCCCT	57ºC/30s
Fatty acid-binding protein FABP (T3)	ATGGCCTCTGGTCTTCTCG CTATTCATCTCGGTACT	57ºC/30s
Subolesin	ATGGCTTGCGCAACATTAAAG TTATGACAAATAGCTTGGAG	60ºC/30s
Rs86	GGACGCGATAAAGACCAGTAT CACACGGAGCGGCGTAGGCGA	60ºC/30s

*All primers contained T7 promoter sequences (5´-TAATACGACTCACTATAGGGTACT-3´) at the 5´end.

Table 2. PCR conditions and sequences of primers used for real-time RT-PCR.

Gene (sample ID)	Forward and reverse primers (5´-3´)	PCR annealing conditions
Laminin B (CG1)	GCAGCTCGACGCTAAGAAGT TCTGCATCCTTGAGTTGTGC	60 ºC/30s
Protein hu-li tai shao, Adducin (CG2)	GGTAACGGAGCTGCTACTGC AGTGGGTTCACCAGGAAGTG	60 ºC/30s
MCM2; Predicted ATPase involved in replication control (CG3)	AGATGCTGGTGATCCTGGAC CTTGCCGCAGTCATACTTGA	60 ºC/30s
Cell division protein (CG4)	GTACGAAGAGCGCAAACTCC ATGTTCTTGCGGTTGAGTCC	60 ºC/30s
Talin, cytoskeletal associated protein (CG5)	AACAAGGAGCCAAACACTGG CGTTGGAGTCGGAGAAGAAG	60 ºC/30s
Actin beta/gamma (CG6)	AAGGACCTGTACGCCAACAC ACATCTGCTGGAAGGTGGAC	60 ºC/30s
Actin (CG7)	AAAGCAGGTTTTGCTGGAGA GGTTTGAAGCTGCTCTTTGG	60 ºC/30s
Spectrin alpha chain, cytoskeletal protein (CG8)	AGAACCAATACGGCAACCTG AGGTCCGACATGAAGTCGTC	60 ºC/30s
Alpha tubulin (CG9)	AGGAGATTGTGGACCTGGTG GCTTGGACTTCTTGCCGTAG	60 ºC/30s
Beta tubulin (CG10)	CGACTGTCTTCAGGGCTTTC	60 ºC/30s

	AGACAGGGTGGCATTGTAGG	
Beta thymosin (CG11)	GCAGGAGAAGAACCAACTGC CCGAGGTCGGATGAATAGAA	60 ºC/30s
Beta thymosin (CG12)	GGCAAAGGTCAAACTCCAAA GTGCCTTTGACTTCCGTCTC	60 ºC/30s
Na+/K+ ATPase, alpha subunit (T1)	ACGGCCAAGAGTGGACATAC AGCCAAGGCAGTCTCAAAAA	60 ºC/30s
Voltage-dependent anion-selective channel (mt) (T2)	GTCGTGAAACTCGACTGCAA CCGTGTTCCACTTCTCCTTC	60 ºC/30s
Fatty acid-binding protein FABP (T3)	GTCTTCTCGGCAAGTGGAAG AGCAGCGTCGAAGTCTTGAT	60 ºC/30s
Subolesin	GCTTGCGCAACATTAAAGCGAAC TGCTTGTTTGCAGATGCCCATCA	62 ºC/30s

Table 3. Cell growth and transport proteins selected for the characterization of mRNA levels during *A. phagocytophilum* infection of *I. scapularis* ISE6 tick cells.

Protein (sample ID)	**Genbank accession Nos.**	**Biological Process**
Laminin B (CG1)	B7P2Q4 ISCW000339	Cell growth and/or maintenance
Protein hu-li tai shao, Adducin (CG2)	B7P1C8 ISCW000621	Cell growth and/or maintenance
MCM2; Predicted ATPase involved in replication control (CG3)	B7PAS1 ISCW016696	Cell growth and/or maintenance
Cell division protein (CG4)	B7PKQ6 ISCW018707	Cell growth and/or maintenance
Talin, cytoskeletal associated protein (CG5)	B7QM86 ISCW023338	Cell growth and/or maintenance
Actin beta/gamma (CG6)	A4UTU3 ISCW024111	Cell growth and/or maintenance
Actin (CG7)	Q6X4W3 B2YGD3 ISCW022123	Cell growth and/or maintenance
Spectrin alpha chain, cytoskeletal protein (CG8)	B7P1U8 ISCW000012 XP_002433506	Cell growth and/or maintenance
Alpha tubulin (CG9)	Q8WQ4 ISCW015260	Cell growth and/or maintenance

Beta tubulin (CG10)	B7PA92 ISCW017133	Cell growth and/or maintenance
Beta thymosin (CG11)	Q86G66 CO047561	Cell growth and/or maintenance
Na+/K+ ATPase, alpha subunit (T1)	B7P9E4 ISCW002538	Transport
Voltage-dependent anion-selective channel (T2)	B7P5X8 ISCW000781 XP_002408065	Transport
Fatty acid-binding protein FABP (T3)	B7QMW0 ISCW015316	Transport

Table 4. Results of differential expression at the protein (FDR<0.05) and mRNA (P<0.05) levels in infected versus uninfected ISE6 tick cells.

Protein (sample ID)	**Proteomics results**		**RT-PCR results**	
	Early infection	Late infection	Early infection	Late infection
Laminin B (CG1)	-2.53	-5.64	NS	NS
Protein hu-li tai shao, Adducin (CG2)*	NS	-2.17	-100.00	-250.00
MCM2; Predicted ATPase involved in replication control (CG3)	NS	-1.99	NS	NS
Cell division protein (CG4)	NS	-1.72	NS	NS
Talin, cytoskeletal associated protein (CG5)	NS	-1.51	NS	NS
Actin beta/gamma (CG6)	NS	-1.46	NS	NS
Actin (CG7)	+3.27	-1.40	NS	NS
Spectrin alpha chain, cytoskeletal protein (CG8)*	NS	-1.39	-6.25	-10.00
Alpha tubulin (CG9)	NS	-1.37	NS	NS
Beta tubulin (CG10)*	NS	-1.27	NS	-25.00

Beta thymosin (CG11)	NS	+ 4.68	NS	NS
Na+/K+ ATPase, alpha subunit (T1)*	-2.14	-2.38	-3.12	-25.00
Voltage-dependent anion-selective channel (mt) (T2)*	-1.33	-1.40	-50.00	-16.67
Fatty acid-binding protein FABP (T3)*	NS	-1.34	-8.33	-12.50

Asterisks denote genes selected for functional analysis by RNAi based on significant differences in differential expression at the mRNA level. Abbreviations: +, over-expressed in infected cells; -, under-expressed in infected cells; NS, no significant differences between infected and uninfected cells.

Table 5. Effect of gene knockdown on *A. phagocytophilum* infection levels in *I. scapularis* ISE6 tick cells.

Sample ID of injected dsRNA	**Ave±SD % gene knockdown with respect to Rs86 control**	***A. phagocytophilum* infection levels**	
		Average±SD normalized *msp4* mRNA levels	**Fold difference with respect to control cells**
CG2	99.3±0.7*	8.8±8.2	2.3±2.0
CG8	74.0±23.8*	1.1±0.2*	0.3±0.1
CG10	78.6±0.0[&]	0.4±0.0	0.1±0.0
T1	0.0±0.0	5.2±2.7	1.3±0.7
T2	95.6±4.5*	16.1±12.1*	4.1±3.0
T3	0.0±0.0	1.5±0.6	0.4±0.2
Subolesin	36.0±0.0*	2058.2±0.2*	527.7±0.1
Rs86 (C-)	---	3.9±2.8	---

The *A. phagocytophilum* infection levels were characterized in ISE6 tick cells after gene knockdown by RNAi. Total RNA and DNA were extracted from infected tick cells after RNAi and analyzed by quantitative RT-PCR or PCR normalizing against 16S rRNA or 16S rDNA to determine gene mRNA levels and *A. phagocytophilum msp4* DNA levels, respectively. Normalized Ct vales were compared between test and negative control (C-) tick cells incubated with the unrelated Rs86 dsRNA by Student's t-test (*$P<0.05$). [&]Only one sample was included in the analysis.

CHAPTER III

DEVELOPMENT OF A SHEEP MODEL FOR STUDYING PATHOGEN/TICK INTERACTIONS OF A HUMAN ISOLATE OF *ANAPLASMA PHAGOCYTOPHILUM* AND *IXODES SCAPULARIS*

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Abstract

Anaplasma phagocytophilum, first identified as a pathogen of ruminants in Europe, has more recently been recognized as an emerging tick-borne pathogen of humans in the U.S. and Europe. *A. phagocytophilum* is transmitted by *Ixodes* spp., but the tick developmental cycle and pathogen/vector interactions have not been fully described. In this research, we report development of a sheep model for studying tick/pathogen interactions of *I. scapularis* and the human NY-18 isolate of *A. phagocytophilum*. *A. phagocytophilum* was propagated in the human promyelocytic cell line, HL-60, and the infected cell cultures were then used to infect sheep by intravenous inoculation. Infections in sheep were confirmed by PCR and an *Anaplasma* competitive ELISA. Clinical signs were not apparent in any of the infected sheep and only limited hematologic and mild serum biochemical abnormalities were identified. While *A. phagocytophilum* morulae were rarely seen in neutrophils, blood film evaluation revealed prominent large granular lymphocytes, occasional plasma cells, and rare macrophages.

Upon necropsy, gross lesions were restricted to the lymphoid system. Mild splenomegaly and lymphadenomegaly with microscopic evidence of lymphoid hyperplasia was observed in all infected sheep. Female *I. scapularis* that were allowed to feed and acquire infection on each of the three experimentally-infected sheep became infected with *A. phagocytophilum* as determined by PCR of guts (80 to 87%) and salivary glands (67 to 100%). Female *I. scapularis* that acquired infection as nymphs on an experimentally-infected sheep transmitted *A. phagocytophilum* to a susceptible sheep, thus confirming transstadial transmission. Sheep proved to be a good host for the production of *I. scapularis* infected with this human isolate of *A. phagocytophilum*, which can be used as a model for future studies of the tick/pathogen interface.

Introduction

Anaplasma phagocytophilum (Rickettsiales: Anaplasmataceae) is a pathogen transmitted by ticks, most notably of the genus *Ixodes*, to a wide range of hosts, including birds, small and large mammals and humans (Goodman 2005; Woldehiwet 2010). This organism is the etiologic agent of a febrile illness of humans (human granulocytic anaplasmosis, HGA), sheep and other ruminants (tick-borne fever, TBF), horses (equine granulocytic anaplasmosis, EGA) and dogs (canine granulocytic anaplasmosis, CGA). While *A. phagocytophilum* is a well established pathogen of small ruminants in Europe (Stuen 2007; Stuen et al. 2009), the pathogen has more recently been shown to cause the emerging tick-borne disease of humans, HGA, in the United States, Europe and Asia (Goodman 2005).

The emergence of HGA has continued to increase in the U.S. A total of 3,637 cases of HGA were reported in the U.S. from 2003 to 2008, with most case reports (834)

occurring in 2007 (Thomas et al. 2009). The incidence of *A. phagocytophilum* infection was recently reported to have increased during this period from 1.4 to 3.0 cases/million persons/year (Dahlgren et al. 2011). *A. phagocytophilum* is vectored by *I. scapularis* in the central and eastern areas of the U.S. Genetic analyses have shown the existence of many strains of *A. phagocytophilum* that can be differentiated between ruminants, horse, dogs and humans (de la Fuente et al. 2005b; Torina et al. 2008; Rar and Golovljova 2011). These and other studies suggest that strains from ruminants may share common characteristics that differ from strains that infect humans (de la Fuente et al. 2005b; Torina et al. 2008; Reichard et al. 2009; Rar and Golovljova 2011).

The recognition of the broad distribution of *A. phagocytophilum* and its emergence as a human tick-borne pathogen has created renewed interest and accelerated research on this organism, particularly on the molecular relationship of the pathogen with its vertebrate and tick hosts (Woldehiwet 2010**).** In this research, we report development of a sheep model that will allow for production of infected *I. scapularis* and for the study of molecular interactions between the tick host and the NY-18 human isolate of *A. phagocytophilum*.

Materials and Methods

Experimental design overview

Four sheep were experimentally infected with *A. phagocytophilum* by intravenous inoculation (iv) of HL-60 cell cultures infected with the human NY-18 isolate of *A. phagocytophilum* (Asanovich et al. 1997; de la Fuente et al. 2006). All sheep were monitored for infection by daily recording of clinical signs, PCR of blood samples, examination of stained blood films and by *Anaplasma* competitive ELISA (cELISA)

serology. After *A. phagocytophilum* infection was confirmed, sheep were infested with *I. scapularis* adults, nymphs or larvae. The first sheep was used to test whether the NY-18 strain of *A. phagocytophilum* was infective for sheep, to determine which tick stages (larvae, nymphs and adults) would feed successfully on sheep and to test whether these ticks could acquire infection from sheep. The next two sheep were experimentally infected with *A. phagocytophilum* and then used to feed female ticks for seven days in order to test their ability to become infected with *A. phagocytophilum*. The fourth sheep was experimentally infected and then infested with female/males pairs and nymphal ticks. The female ticks were used to study the infection dynamics of *A. phagocytophilum* in tick guts and salivary glands during tick feeding by PCR. Nymphs were allowed to acquire infection by feeding to repletion and then the subsequently molted adults were allowed to feed on a susceptible sheep in order to test for transstadial transmission of the pathogen. In addition, the *A. phagocytophilum* infections were documented by PCR in ticks during transmission feeding. In order to study tick infections, ticks guts and a salivary glands were dissected from one half of each tick and tested individually by PCR for *A. phagocytophilum* infection, while the other half of each tick was placed in fixative for future morphology studies. Egg masses produced by replete female ticks, known to be infected with *A. phagocytophilum,* were tested by PCR in order to provide evidence of transovarial transmission. After the completion of tick feeding, all sheep were euthanized and necropsies were performed in order to document pathologic changes that may have resulted from *A. phagocytophilum* infection.

Anaplasma phagocytophilum isolate, propagation in HL-60 cells and infection of sheep

The human NY18 isolate of *A. phagocytophilum* (Asanovich *et al*., 1997; de la Fuente et al. 2006) was propagated in cultures of the human undifferentiated promyelocytic cell line, HL-60. Infected and uninfected cell cultures were maintained at 37°C in RPMI medium as reported previously (de la Fuente et al. 2005a). For inoculation of each sheep, two T-25 flasks of *A. phagocytophilum*-infected HL-60 cells were used (45% infection, as determined by detection of intracellular morulae in stained cytospin cell smears; Hema-3 Stain, Fisher Scientific, Middletown, VA, USA). The cultures were centrifuged and resuspended with serum free RPMI 1640 medium with a final iv dose of 1 X 10^7 cells in 2 ml of cell culture medium.

Ticks

Ixodes scapularis ticks (males, females, nymphs and larvae) were obtained from the laboratory colony maintained at the Oklahoma State University Tick Rearing Facility. Larvae and nymphs were fed on rabbits in order to develop to the nymphal and adult stages, and nymphs and/or adults were fed on sheep in order to acquire *A. phagocytophilum* infection. Off-host ticks were maintained in a 12 hr light: 12 hr dark photoperiod at 22-25 °C and in humidity chambers with a 95% relative humidity (RH).

Sheep

Sheep were purchased at a local livestock auction for use in these studies. Four sheep (001, 165, 102 and 084; Table 1), first determined to be negative for *A. phagocytophilum* by PCR analysis of blood samples, were inoculated with HL-60 cell cultures infected with *A. phagocytophilum* and used for tick feeding experiments. One susceptible sheep, 016, was used as a host for the feeding of female ticks that molted from replete nymphal ticks previously fed on experimentally-infected Sheep 084 (Table

1). Blood and serum samples were collected 3 times per week from all experimentally-infected sheep, and samples collected on Friday were submitted to the clinical pathology laboratory at Oklahoma State University (Antech Diagnostics, Irvine, CA, USA) for analysis. Stained blood films were prepared and examined on blood collection days for the presence of characteristic *A. phagocytophilum* morulae in granulocytes. Serum samples were collected and stored for subsequent testing for *Anaplasma* spp. antibodies using a commercially available *Anaplasma* cELISA (VMRD, Pullman, WA, USA). Use of experimental sheep for this research was done under a protocol approved by the Oklahoma State University, Institutional Care and Use of Animals Committee and according to the regulations of the U.S. Department of Agriculture.

Acquisition feeding of female and nymphal I. scapularis

The stages and numbers of ticks infested on each sheep are listed in Table 1. Female ticks that completed feeding on Sheep 001, 165 and 102 were removed after 7 days, held in the humidity chamber for 4 days and dissected for DNA extraction from guts and salivary glands for PCR studies. From the ticks that were allowed to feed on Sheep 84, the guts and salivary glands were dissected from one group of 20 unfed females, and groups of 20 ticks were removed daily on days 2 through 7 of feeding and the guts and salivary glands dissected for DNA extraction. PCR studies were then done on all samples in order to determine *A. phagocytophilum* infections.

Transstadial transmission studies

Nymphal *I. scapularis* (2,000) were infested on Sheep 084 and allowed to feed to repletion, after which they were held in a humidity chamber. As soon as the nymphs molted to the adult stages, males and females were placed in separate cartons, which were

held in a humidity chamber until they were allowed to feed on susceptible sheep 016 in order to test for transstadial transmission of *A. phagocytophilum*. Groups of 15 female ticks were dissected from unfed ticks (Day 0) and from ticks removed from Sheep 16 on days 2, 3, 4, 5 and 6 of feeding, and the guts and salivary glands were dissected for DNA extraction for PCR studies in order to determine *A. phagocytophilum* infections.

Detection of A. phagocytophilum in sheep and ticks by PCR

DNA was extracted from sheep blood and tick guts, salivary glands and eggs using TriReagent (Sigma, St. Louis, MO, USA) following manufacturer's recommendations. *A. phagocytophilum* infection levels were characterized by *msp4* PCR using the iQ5 thermal cycler (Bio-Rad, Hercules, CA, USA) as described previously (de la Fuente et al. 2006) but using oligonucleotide primers MSP4-L (5'-CCTTGGCTGCAGCACCACCTG-3') and MSP4-R (5'-TGCTGTGGGTCGTGACGCG3') (Busby et al. 2011). PCR reaction products were analyzed by agarose gel electrophoresis to determine positive samples. Correlation analyses were conducted in Microsoft Excel to compare the percent of infected guts and salivary glands at different time intervals after tick infestation.

Serology

Serum was collected three times per week from each experimental sheep before and after inoculation with *A. phagocytophilum* and frozen in 1 ml aliquots. A series of serum samples collected before and after inoculation with *A. phagocytophilum* from each sheep was tested at the Oklahoma Animal Disease Diagnostic Laboratory, Stillwater, OK, using the *Anaplasma* sp. cELISA developed for the detection of antibodies to the major surface protein 5 (MSP5) (Knowles et al. 1996; Torioni de Eschaide et al. 1998). Serum

samples from Sheep 165, 102 and 084 were tested concurrently on the same plate. A positive *A. phagocytophilum* result for the cELISA was >30% percent inhibition.

Clinical pathology and necropsy of experimentally-infected sheep

Blood was collected three times per week from each experimental sheep. Complete blood counts (CBCs) were performed weekly with an automated hematology analyzer (ADVIA 120, Siemens Diagnostics, Tarrytown, NY, USA). Differential leukocyte counts were performed manually on stained blood films. Blood films were prepared from EDTA-anticoagulated blood on each collection day and stained with an aqueous Romanowsky stain using an automated stainer (Aerospray 7120, Wescor Inc., Logan, UT, USA). A minimum of 500 neutrophils were counted at each time point and examined for the presence of morulae. Large granular lymphocytes (LGLs) were enumerated by counting 100 lymphocytes at each time point; lymphocytes containing azurophilic cytoplasmic granules visible at 500X magnification were considered LGLs and expressed as a percentage. Routine clinical biochemical profiles were performed on serum (Olympus AU640, Beckman Coulter, Fullerton, CA, USA).

After completion of tick feeding, sheep were euthanized by a licensed veterinarian and subjected to comprehensive necropsy examination. Representative samples of heart, lung, liver, kidney, spleen, adrenal glands, lymph nodes and brain were fixed in 10% buffered formalin solution. Fixed tissue specimens were routinely processed, embedded in paraffin, sectioned at 5 μm, mounted on glass slides and stained with hematoxylin and eosin (HE) for histopathological examination.

Results

Tick infestation and recovery in sheep

Differences were observed between tick stages recovered from infested sheep (Table 2). Larvae were not recovered from sheep 001 and therefore this tick stage was not used in subsequent studies. The recovery of nymphal ticks was similar for Sheep 001 and 084, being 16% and 18%, respectively. Molting success of nymphs recovered from Sheep 084 was 69% (Table 2). Female ticks fed successfully on all sheep with a recovery rate ranging from 75% to 100%. Male ticks were not observed to feed on the sheep but were found to be paired and mating with the females. Males that were not paired with the females later in feeding were not recovered. Males were, however, included with each infestation to insure that the females would feed and engorge successfully. Because the males were not feeding and acquiring a bloodmeal, they were not dissected or tested for infection with *A. phagocytophilum.*

A. phagocytophilum infection in experimentally-inoculated sheep.

All sheep experimentally inoculated with *A. phagocytophilum*-infected HL-60 cell cultures became infected as determined by PCR. The prepatent periods, based on PCR results, for Sheep 001, 165, 102 and 084 were 14, 21, 14, and 10 days, respectively (Average±SD prepatent period, 15±6 days) (Table 3). Seroconversion was observed in all three sheep inoculated with *A. phagocytophilum* (Table 3), but the cELISA did not prove to be a consistent diagnostic tool for detection of *A. phagocytophilum* infection.

Acquisition of A. phagocytophilum by female ticks feeding on experimentally-infected sheep

Female ticks acquired *A. phagocytophilum* infection from Sheep 165 and 102 as determined by PCR analysis of individual tick guts and salivary glands (Table 4). Infections in tick guts were found in 85% and 87% of the ticks, respectively, while the salivary glands of 100% of the ticks from both sheep were infected with *A. phagocytophilum.*

A. phagocytophilum infection in ticks during 7 days of feeding on sheep 084.

High percentages of female ticks were found to be infected with *A. phagocytophilum* by PCR after 2 through 7 days of feeding on experimentally-infected Sheep 084 (Table 5). PCR results on individual ticks revealed that 65-100% of the tick guts and 65-90% of salivary glands became infected with *A. phagocytophilum* (Table 5). The percent of infected guts peaked after 4 days of feeding, reaching 100% infected ticks (Fig. 1A). However, the highest percentage of infected salivary glands (90%) was obtained after 5 days of feeding and stayed at that level until day 7 (Fig. 1A). When the infection rates were compared between guts and salivary glands, a positive correlation was observed (Fig. 1B).

Transstadial transmission of A. phagocytophilum and infections in female ticks infected as nymphs.

Transstadial transmission of *A. phagocytophilum* was demonstrated when ticks infected as nymphs on Sheep 084 were allowed to feed after molting on Sheep 016. Sheep 016 became infected with *A. phagocytophilum* 14 days after the ticks were applied. In the females that were infected as nymphs, 27% and 0% of the guts and salivary glands, respectively in unfed females were infected with *A. phagocytophilum* (Table 6). The infection rate increased to 80% and 33% for guts and salivary glands, respectively, after 5

days of feeding (Table 6). The percentage of infected guts and salivary glands peaked after 5 days of feeding and then decreased on day 6 to 40% and 13%, respectively (Fig. 1C). As with the ticks from Sheep 084, when the infection rates were compared between guts and salivary glands, a positive correlation was observed (Fig. 1D).

Transovarial transmission of A. phagocytophilum

Egg masses from two groups of replete females, infected either as nymphs or adults on Sheep 084, were negative for *A. phagocytophilum* by PCR.

Clinical pathology findings

Limited hematologic abnormalities were identified. CBC results remained largely within reference intervals except for mild lymphopenia (Sheep 102), slight eosinophilia (Sheep 084), and mild neutrophilia (Sheep 016). Blood film evaluation revealed prominent large granular lymphocytes comprising up to 30% of total lymphocytes, occasional reactive lymphocytes and plasma cells, and rare macrophages. Rare neutrophils contained clustered granular basophilic inclusions consistent with small morulae (Fig. 2). Clinical chemistry abnormalities were mild, consisting of hypocholesterolemia, hypoalbuminemia, decreased creatine kinase activity, and increased activity of alkaline phosphatase and gamma-glumatyltransferase. In some cases these values were slightly outside reference intervals prior to inoculation with *A. phagocytophilum*, and thus unlikely related to infection.

Necropsy findings

Relevant gross changes seen at necropsy were similar among the five sheep (001, 165, 102, 084 and 016) and were mild and restricted to the lymphoid system. The spleen of each sheep was mildly enlarged with conspicuous lymphoid follicles visible on cut

surface. Sublumbar and prefemoral lymph nodes were mildly enlarged with maintenance of distinct corticomedullary architecture. In each of the sheep gross lesions, interpreted to be background changes unrelated to *A. phagocytophilum* infection, were occasionally seen and included small numbers of *Haemonchus contortus* within the abomasums of all sheep, scattered pulmonary and hepatic abscesses in Sheep 084 and a lymph node abscess in Sheep 016. Microscopic examination of lymphoid tissues revealed mild to moderate lymphoid hyperplasia with germinal center formation in spleen and lymph nodes of each sheep. Minimal and patchy neutrophilic pneumonitis was also seen in two of the sheep 102 and 165. Two sheep (001 and 165) had moderate numbers of eosinophils in medullary sinuses of lymph nodes and three sheep (001, 016 and 084) has small to moderate numbers of eosinophils present in perisinusoidal regions of the adrenal medullas.

Discussion

Emergence of HGA in the U.S. has resulted in accelerated research on the etiologic agent, *A. phagocytophilum*. Our research is focused on the tick/pathogen interface because ticks are a necessary host in the pathogen life cycle and tick feeding is the mechanism by which *A. phagocytophilum* is transmitted to humans. The goal of this research was to develop an animal model for infection of ticks with a human isolate of *A. phagocytophilum* that would allow us to infect ticks for studies of tick/pathogen interactions. Sheep were chosen for this research because they were known to be susceptible to infection with *A. phagocytophilum*; TBF, caused by *A. phagocytophilum*, is a common disease of sheep in Europe (Stuen 2007). Furthermore, sheep are easily

restrained for tick feeding and their body size can accommodate tick infestations and multiple tick feeding cells as required for our experiments.

Infection of sheep with the NY-18 isolate was difficult to assess. Clinical signs were not apparent in any of the sheep, and clinical pathology findings were minimal and uniform among the sheep. The only pathologic changes observed were indicative of general immune stimulation. Demonstration of morulae in circulating neutrophils, reported as a reliable method for detection of *A. phagocytophilum* in sheep with TBF (Giudice et al. 2011), was a rare event in the sheep in this study of a human isolate. In the entire study involving five sheep, only a few infected neutrophils were found that contained small *A. phagocytophilum* morulae.

Many variants of *A. phagocytophilum* have been reported which differ in host preferences, host responses and tick vectors, and variants from ruminants were recently found to differ from human ones (de la Fuente et al. 2005b; 2005c; Torina et al. 2008; Rar and Golovljova 2011). For example, the Ap-V1 variant was shown to be infective for goats and deer but was not associated with human disease or infective for hamsters, mice and gerbils (Massung et al. 2006; Reichard et al. 2009). Furthermore, *I. scapularis* ticks that were allowed to feed on deer inoculated with the Ap-V1 or the NY-18 isolates only became infected with the Ap-V1 variant (Reichard et al. 2009). Therefore, it was not surprising that infection and diagnostic parameters of the human NY-18 isolate in sheep differed from those reported in sheep with TBF (Stuen 2007; Giudice et al. 2011) and in mice infected with the same human *A. phagocytophilum* NY-18 isolate (Blas-Machado et al. 2007).

Nonetheless, sheep proved to be a good host for the feeding of *I. scapularis* nymphs and females, both relevant tick stages for the characterization of tick/host and tick/pathogen interfaces. Sheep were easily adaptable to the temporary restraint required for tick feeding and their size accommodated multiple tick feeding cells. Larvae did not feed successfully on sheep and, while nymphs fed to repletion on sheep, the return was low, approximately 18%. *I. scapularis* nymphs required special handling and precise placement on sheep, and the return of nymphs in future studies could likely be enhanced by improved infestation techniques. While female ticks fed successfully at a rate 75 to 100%, male ticks did not feed but rather paired rapidly with the females and remained engaged until the last day of feeding. Therefore, while male ticks may acquire infection as nymphs, they would not likely be involved in transstadial transmission in sheep.

Experimentally-infected sheep used in these studies proved to be an excellent host for production of ticks infected with human NY-18 *A. phagocytophilum* isolate. Both tick guts and salivary glands were shown to become infected in female ticks after they fed and acquired infection on the experimentally-infected sheep, demonstrating a tick infection rate of 85-87% and 100% for guts and salivary glands, respectively. Furthermore, positive correlations were shown between infection rates in guts and salivary glands over the tick feeding period in female ticks infected either as nymphs or adults. The uniform initiation of feeding in these ticks appears to have contributed to the synchronized multiplication of *A. phagocytophilum* in tick guts and salivary glands.

Transstadial transmission of *A. phagocytophilum* was demonstrated by ticks that acquired infection as nymphs and transmitted as adults. However, evidence of transovarial transmission was not found in this study because the eggs masses from

infected, replete nymphs were PCR negative. However, subsequently hatched larvae from these egg masses were not fed on susceptible sheep to test for transovarial transmission.

The lack of circulating neutrophils infected with *A. phagocytophilum* morulae in these sheep suggested that a fixed tissue cell to which the ticks would be exposed and ingest during feeding may be a host cell for this human isolate of *A. phagocytophilum*. Certainly the high rate of tick infections does not correlate with the minimal parasitemia observed in circulating neutrophils. A recent study demonstrated that murine bone marrow-derived mast cells (BMMCs) could serve as a host cell for *A. phagoctyphilum* and that infection of BMMCs inhibited mast cell activation (Ojogun et al. 2011). Further studies are needed to determine whether a fixed tissue cell, such as mast cells, harbor *A. phagocytophilum* in sheep.

Conclusions

Sheep became infected with the human NY-18 isolate of *A. phagocytophilum* and served as a host for infection of *I. scapularis* ticks. PCR consistently demonstrated infection in sheep but serologic diagnosis by use of the *Anaplasma* cELISA was not consistently useful as a diagnostic tool. Distinct morulae in circulating neutrophils were small, singular and rarely seen. Hematology and biochemical abnormalities were mild. Necropsy lesions were minimal and included mild splenomegaly and lymph node enlargement, which demonstrated evidence of systemic immune stimulation.

Nevertheless, sheep proved to be a good host for some tick stages. While larvae and males did not feed on sheep, a small return of nymphs and most of the females fed to repletion. While clinical signs of disease were not apparent in the infected sheep, tick infection rates in guts and salivary glands were quite high. The absence of infected

circulating neutrophils suggests that a tissue based cell may likely be the source of infection for ticks. Mast cells may be a good candidate because they have recently been shown to be a host cell for *A. phagocytophilum*. Transstadial transmission was demonstrated by female ticks that were infected as nymphs. Lack of demonstration of *A. phagocytophilum* in egg masses by PCR from infected females suggested that transovarial transmission was not likely to occur.

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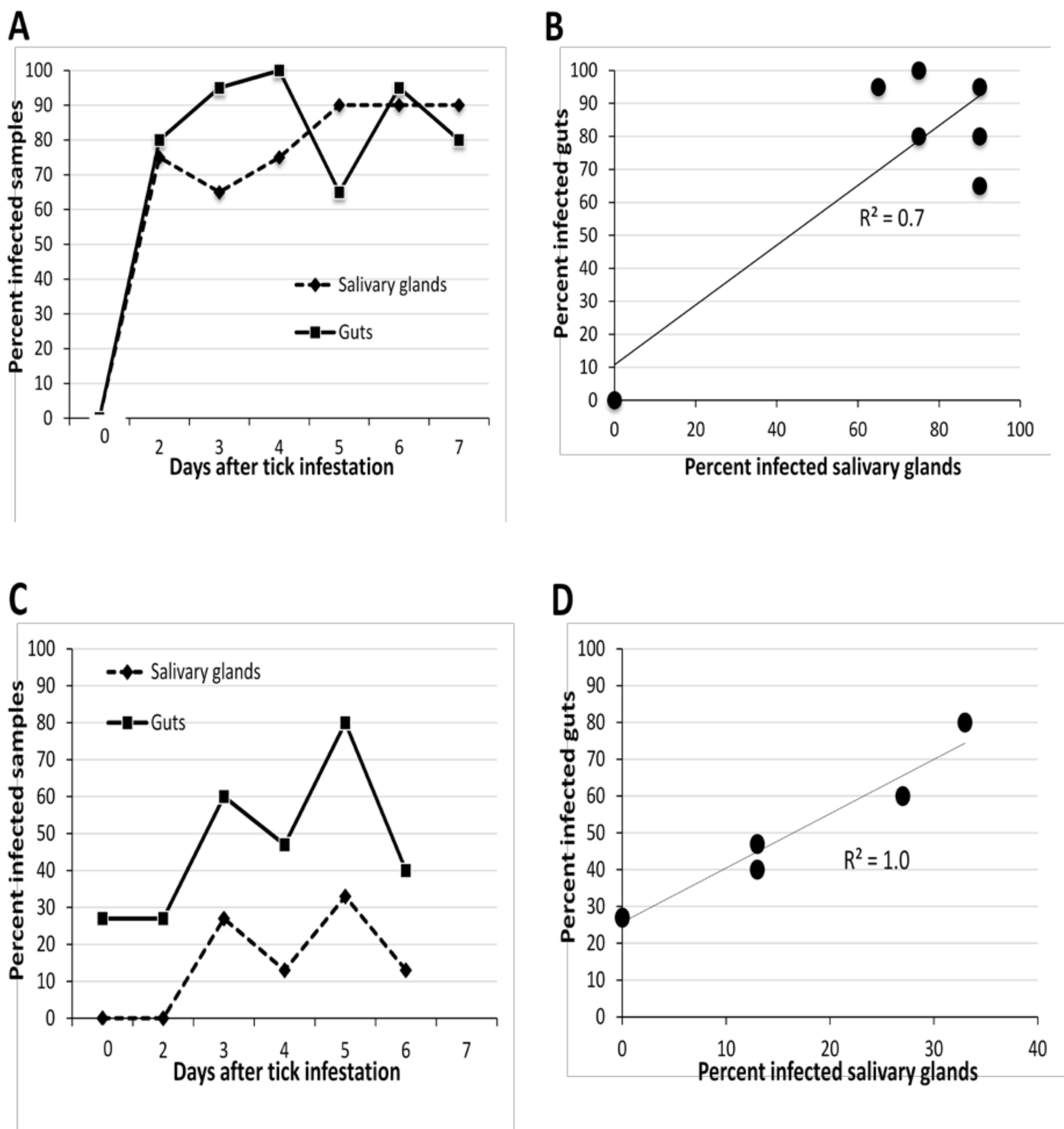

Figure 1. Infection of ticks during feeding on *A. phagocytophilum*-infected sheep. The infection of individual ticks was determined by PCR in guts and salivary glands from female ticks infected as adults (A, B; N=20) or nymphs (C, D; N=15) during feeding on sheep 084. Correlation analyses were conducted in

Microsoft Excel to compare the percent of infected guts and salivary glands at different time intervals after tick infestation (B, D).

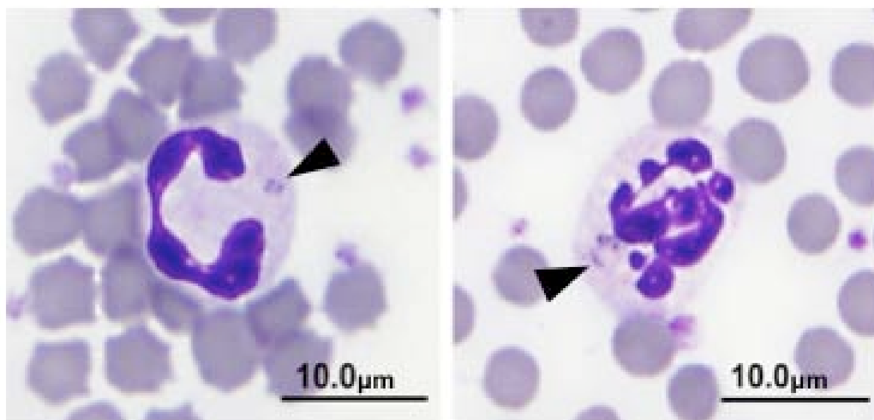

Figure 2. Morulae of *A. phagocytophilum* (arrowheads) in peripheral blood neutrophils from Sheep 016 (left) and Sheep 102 (right) (Aqueous Romanowsky stain).

Table 1. Experimental sheep, source of *A. phagocytophilum* for infection and tick stages and numbers that were allowed to feed on the sheep.

Sheep No.	Source of *A. phagocytophilum* infection	Tick stage infested
001	iv inoculation of infected HL-60 cell culture	100 male-female pairs, 200 nymphs 500 larvae
165	iv inoculation of infected HL-60 cell culture	50 male-female pairs
102	iv inoculation of infected HL-60 cell culture	50 male-female pairs
084	iv inoculation of infected HL-60 cell culture	200 male-female pairs, 2,000 nymphs
016	Females infected as nymphs on Sheep 084	90 male-females pairs

Table 2. Infestation and recovery of ticks from experimental sheep.

Sheep	Day of tick infestation after inoculation with *A. phagocytophilum*	Number of tick stages recovered after feeding
001	11	0/500 larvae (0%) 32/200 nymphs (16%) 75/100 females (75%) 0/100 males (0%)*
165	29	31/40 females (87%) 0/40 males (0%)*
102	18	31/40 females (77%) 0/40 males (0%)*
084	41	174/200 female ticks (87%)* 363/2,000 nymphs (18%)**
016	Not inoculated	90/90 females (100%)*

* Male ticks did not feed but were attached to the females.

** Replete nymphs molted into 120 females (48%) and 130 males (52%).

Table 3. *A. phagocytophilum* infections in sheep.

Sheep No.	Prepatent period* (days post inoculation)	cELISA positive (days post inoculation)
Sheep 165	21	11, 13 and 15
Sheep 102	14	14
Sheep 084	10	10 and 17

*The prepatent period was calculated as the number of days from the time of inoculation of sheep with *A. phagocytophilum* infected cell cultures to the first day that blood samples were found to be *A. phagocytophilum* positive by PCR.

Table 4. Infection of female ticks that fed for 7 days and acquired infection on sheep 165 and 102.

Sheep No.	PCR-positive guts	PCR-positive salivary glands
165	17/20 (85%)	20/20 (100%)
102	13/15 (87%)	11/11 (100%)

Table 5. Infection of female ticks that fed for varying periods and acquired infection on sheep 084.

Tick Tissue	PCR-positive samples						
	Day 0 (unfed)	Day 2	Day 3	Day 4	Day 5	Day 6	Day 7
Guts	0/20 (0%)	16/20 (80%)	19/20 (95%)	20/20 (100%)	13/20 (65%)	19/20 (95%)	16/20 (80%)
Salivary glands	0/20 (0%)	15/20 (75%)	13/20% (65%)	15/20 (75%)	18/20 (90%)	18/20 (90%)	18/20 (90%)

Table 6. Infection of female ticks that acquired infection as nymphs on sheep 084 and subsequently molted adults were allowed to feed for varying periods on sheep 016.

Tick Tissue	PCR-positive samples					
	Day 0 (Unfed)	Day 2	Day 3	Day 4	Day 5	Day 6
Guts	4/15 (27%)	4/15 (27%)	9/15 (60%)	7/15 (47%)	12/15 (80%)	6/15 (40%)
Salivary glands	0/15 (0%)	0/15 (0%)	4/15 (27%)	2/15 (13%)	5/15 (33%)	2/15 (13%)

CHAPTER IV

SUMMARY

Anaplasma phagocytophilum, a tick-borne pathogen vectored by ticks of the genus *Ixodes*, was first described in Europe as the cause tick-borne fever (TBF) in sheep. Granulocytes are the host cell for *A. phagocytophilum,* where the pathogen multiples within a parasitophorous vacuole that allow for evasion of the immune system. This pathogen differs from the other *Anaplasma* sp. which are host specific for ruminants because of its wide host range, including rodents, birds, cats, deer, horses, cattle, sheep, dogs and humans. Recently, *A. phagocytophilum* was found to be the cause of human granulocytic anaplasmosis (HGA), an emerging tick-borne disease in the United States and Europe, and the variants of *A. phagocytophilum* identified in humans were shown to vary from those that cause disease in ruminants. Recognition of the broad host range of *A. phagocytophilum* and its emergence as a human pathogen has created renewed interest in research on this pathogen, particularly in the molecular relationship of the pathogen with both vertebrate and tick host cells (Woldehiwet 2010).

Ticks are the biological vector for the *A. phagocytophilum*, and are necessary for transmission of the pathogen among humans and reservoir hosts. While the developmental cycle of *A. phag*ocytophilum in ticks has not been fully described, the AP-1 variant has been identified in tick gut cells. Recent research has contributed to our understanding of these tick cell/pathogen interactions, but continued studies are needed

on the molecular interactions at the tick-pathogen interface in order to identify the tick genes that are required for tick vector competency and pathogen transmission.

The focus of this research was two-fold: (1) to characterize the gene expression in culture tick cells in response to infection with *A. phagocytophilum* infection in order to identify tick genes involved in infection and multiplication of *A. phagocytophilum* and (2) to develop a sheep model for characterization of a human isolate of *A. phagocytophilum* thus providing a host on which to infect ticks for future tick-pathogen interaction studies of this human isolate. The overall hypothesis for the research in our laboratory is that tick cell proteins differentially expressed in response to pathogen infection would include those involved in pathogen infection, multiplication and transmission, as well as being involved in the tick protective response against pathogen infection.

In the first part of this research, the characterization of *I. scapularis* proteins differentially expressed by tick cells in response to *A. phagocytophilum* infection, was done by a combination of proteomic and gene silencing studies in the ISE6 tick cell line that was originally derived from embryos of *I. scapularis*. After infection of the cultured cells, the proteomic analysis was done using protein one-step in-gel digestion, peptide iTRAQ labeling, IEF fractionation, LC-MS/MS analysis and peptide identification. Protein identification was carried out as described previously (Lopez-Ferrer et al. 2004) using SEQUEST algorithum (Bioworks 3.2 package, Thermo Finnigan), allowing optional (Methionine oxidation) and fixed modifications (Cysteine carboxamidomethylation, Lysine and N-terminal modification of +114.1020 Da). The alphaproteobacteria Swissprot database was used to identify and discard *Anaplasma* and

possible symbiotic bacterial sequences from further analyses. Analysis of protein ontology for differentially expressed proteins demonstrated that biological processes such as cell growth, protein and nucleic acid metabolism and transport were affected during early and late infections. Significant differences were observed between under- and over-represented proteins in early and late infections for cell growth and transport biological processes and between the early and late infections for cell growth. The transport biological processes were under-represented in both early and late infections. Proteins in cell growth and transport biological processes were selected for the characterization of mRNA levels during *A. phagocytophilum* infection in *I. scapularis* ISE6 tick cell culture systems.

Real time RT-PCR was subsequently used to analyze the mRNA levels of genes encoding for the proteins in cell growth and transport in early and late-infected cells and were compared with the proteomic results. Based on this analysis, six genes, CG2, CG8, CG10, T1, T2, T3, were selected for the analysis of mRNA levels in ISE6 cells and for gene silencing by RNA interference (RNAi). The expression of four of the six genes (CG2, CG8, T2 and subolesin) was found to be significantly reduced in cultured tick cells after RNAi. Of these genes RNAi of CG8, T2 and subolesin resulted in a significant difference in the *A. phagocytophilum* infection levels. This research represents the first report of the analysis of global protein changes in tick cells in response to *A. phagocytophilum* infection. Genes confirmed to be differentially expressed at both the protein and mRNA levels with predicted function provided evidence that these genes are involved in pathogen infection/multiplication and tick response to infection.

In the second part of this research focused on development of a sheep model of a human isolate of *A. phagocytophilum*, four sheep were experimentally-infected with *A. phagocytophilum* by intravenous inoculation of HL-60 cell cultures infected with the human NY-18 isolate of *A. phagocytophilum.* After the sheep were confirmed to be infected by PCR, the sheep were infested with *I. scapularis* adults and/or nymphal and larvae. Ticks that were allowed to feed on the infected sheep also became infected with *A. phagocytophilum,* and PCR of tick guts and salivary glands demonstrated that 65 to 100% of the female ticks were infected. Transstadial transmission was demonstrated when the susceptible sheep fed on by female ticks that were infected as nymphs was shown to become PCR positive for *A. phagocytophilum*. Infection rates of ticks used to demonstrate transstadial transmission tick ranged from 65 to 100 %.

In summary, the results of the cell culture studies resulted in characterization of genes differentially expressed in response to *Anaplasma phagocytophilum.* The genes CG8, T2 and subolesin were found to impact *A. phagocytophilum* infection levels. The sheep model developed herein provided a model system for study of the human *A. phagocytophilum* strain. Although clinical signs were not observed in any of the experimentally- infected sheep, the sheep proved to a good host for infection for the *I. scapularis* nymphs and adults. Characterization of the molecular interactions at the vector-pathogen interface is needed to identify candidate antigens for development of vaccines for the control of tick infestations and prevention of pathogen infection/transmission.

VITA

Ann Taylor Busby

Candidate for the Degree of

Master of Science

Thesis: STUDIES OF A HUMAN ISOLATE OF *ANAPLASMA PHAGOCYTOPHILUM* IN CULTURED TICK CELLS, TICKS, AND SHEEP.

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Education: Completed the requirements for the Master of Science in Veterinary Biomedical Science at Oklahoma State University, Stillwater, Oklahoma in December, 2011. Completed the requirements for the Bachelor of Science in Animal Science at Oklahoma State University, Stillwater, Oklahoma in December, 2006.

Experience: Employeed by Oklahoma State University, Undergraduate Admissions Office, January 2008-December 2009; employeed by Oklahoma State University, Graduate college, December 2009-August 2011; Oklahoma State University, College of Engineering, Architecture and Technology, graduate assistant, September to present

Name: Ann Taylor Busby Date of Degree: December, 2011

Institution: Oklahoma State University Location: Stillwater, Oklahoma

Title of Study: STUDIES OF A HUMAN ISOLATE OF *ANAPLASMA PHAGOCYTOPHILUM* IN CULTURED TICK CELLS, TICKS, AND SHEEP.

Pages in Study: 140 Candidate for the Degree of Master of Science

Major Field: Veterinary Biomedical Science

Scope and Method of Study: The proteome of the *Ixodes scapularis*-derived cell line, ISE6, was characterized after infection with the tick-borne pathogen, *Anaplasma phagocytophilum*. Proteomics were used to identify proteins differentially expressed in cultured tick cells in response to *Anaplasma phagocytophilum* and these results were correlated with mRNA levels by real-time RT-PCR. RNA interference (RNAi) was then used to characterize the function of genes found to be differentially expressed in the infected cells. In the second portion of the research a sheep/tick/*A. phagocytophilum* model was developed in order to study tick/pathogen interactions of the human NY-18 isolate of *A. phagocytophilum* and *I. scapularis. A. phagocytophilum* was propagated in the human promyelocytic cell line, HL-60, and the infected cell cultures were then used as an inoculum for infection of sheep by intravenous inoculation. After the sheep were shown by PCR to be infected, ticks were allowed to feed on the sheep and acquire *A. phagocytophilum* infection. Tick infection dynamics were then characterized by PCR.

Findings and Conclusions: The proteomic analysis of cultured ISE6 cells after *A. phagocytophilum* infection demonstrated that expression of proteins involved in cell growth and transport was significantly reduced, thus reflecting the effect of pathogen multiplication on these cell processes. New genes were identified, including spectrin alpha chain or alpha-fodrin and Voltage-dependent anion-selective channel or mitochondrial porin, to be involved in tick cell infection/multiplication of *A. phagocytophilum,* thus contributing to our understanding of the molecular events at the tick-pathogen interface. In the sheep model study, female *I. scapularis* acquired infection on each of the three experimentally-infected sheep as determined by PCR of tick guts (80 to 87%) and salivary glands (67 to 100%). Transstadial transmission was confirmed 14 days after female *I. scapularis*, infected as nymphs, transmitted *A. phagocytophilum* to a susceptible one. The results from this research demonstrated that sheep are a good host for infection of *I. scapularis* ticks with the human NY-18 isolate of *A. phagocytophilum.* The results of this research have contributed to our understanding of the tick/*A. phagocytophilum* interface and will likely contribute towards the development of novel tick control measures.

ADVISER'S APPROVAL: Katherine M. Kocan