EVALUATION OF TARGETRON BASED MUTAGENESIS IN *EHRLICHIA CHAFFEENSIS*

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ABSTRACT

Ehrlichia chaffeensis is an emerging tick-borne rickettsial pathogen that causes infection in people and several vertebrate animals. One of the striking features of *E. chaffeensis* infection is the prolonged persistence in its vertebrate and tick hosts. The mechanism of persistent infection and the reasons for the host immune system failure to clear the infection are not well understood. One hypothesis is that differential gene expression serves as an important adaptive mechanism used *by E. chaffeensis* in support of its continued survival in both tick and vertebrate hosts. One way to test this hypothesis is by performing mutational analysis. However, the methods for introducing mutations in this pathogen have not yet been documented and are challenging, possibly due to its obligate, intraphagosomal growth requirement. Recently, a novel gene mutation method called 'TargeTron Gene Knockout System' that is based on the modified group II intron insertion strategy has been developed. This method appears to be effective in creating mutations in a wide range of gram positive and gram negative bacterial organisms. The group II intron can be programmed for insertion into virtually any desired DNA target with possibly high frequency and specificity. In this study, I focus on creating mutations in *E. chaffeensis* using the TargeTron gene knockout system. I prepared modified group II intron constructs retargeting for insertion into three *E. chaffeensis* genes: Ech_0126 (a transcriptionally silent gene), macrophage-specific expressed gene (p28-Omp 19, Ech_1143) and tick cell-specific expressed gene (p28-Omp 14, Ech_1136). In support of driving the expression of the modified group II introns in *E. chaffeensis*, the pathogen-

specific high-expressing gene promoter (*tuf*) was inserted upstream to the transcription start site. In addition, a chloramphenicol acetyltransferase gene with *E. chaffeensis rpsl* promoter was introduced for use as a selection marker. The constructs were then evaluated by transforming into *E. chaffeensis*. Transformants with mutations, introduced in two of the three genes (Ech_0126 and Ech_1143), were identified by PCR and Southern blot methods. Although the mutants are detectable for up to 48 hours, establishment of stable transformants remains to be challenging. The outcomes of this project will have important implications in defining the pathogenesis of *E. chaffeensis*, particularly to assess the differences in the organism in tick and vertebrate hosts.

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DEDICATION

This dissertation is dedicated to my parents, all my teachers, and to all of my friends who

believed in me, and provided love and support with out which I would have been unable

to complete this project

Chapter I

LITERATURE REVIEW AND SIGNIFICANCE

Vector-Borne Diseases

Vector-borne diseases are the result of pathogen transferring from a vector to a vertebrate host, which caused by arthropod vectors including both insect (especially mosquitoes) and arachnid (ticks and mites). Mosquito-borne diseases include *human malaria, filariasis, yellow fever, dengue fever* and *arthropod-borne viral encephalitis*; tick-borne diseases include *Rocky Mountain spotted fever, tularemia, Colorado tick fever, Q fever* and *relapsing fever*; and mite-borne diseases include *scrub typhus* and *scabies*. In addition, crustacean, including copepods, crabs and prawns, also could transmit pathogens and cause diseases. For example, *vibrio cholerae* can persist in crabs for several weeks and multiply itself rapidly in free-living or refrigerated animals (Nalin *et al.,* 1979). *Vibrio cholerae* can also colonize on the surface of copepods, and through them, it infects fish and human (Colwell *et al,* 1992; Estrada *et al,* 1996). Trematodes also harbor pathogens to serve as invertebrate hosts. For example, flukes host *Neorickettsia* species (Fukuda *et al.,* 1981). In addition, Snails also serve as vector for transmitting disease agents. For example, they serve as intermediate hosts for *Schistosomes* and *Angiostrongglus Cantonensis* and cause eosinophilic meningitis in human (Lv S *et al.,* 2009).

Classification of the vector-borne diseases includes mechanical and biological vectors. Mechanical vectors are the vectors that simply carry pathogen from one host to another. For example, houseflies acquire bacteria on their appendages when they land on cow dung, and transfer the bacteria to humans when such flies land on food consumed by people. In this type of transmission, pathogen does not enter into the body of a

mechanical vector. Contrary to mechanical vectors, biological vectors usually harbor pathogens inside of their body and transmit to new hosts in a very active way, usually through biting. Pathogens develop and multiply themselves inside the body of biological vectors before becoming infective. Biological vectors play critical role in the life cycle of a pathogen. Many blood-feeding arthropods, such as mosquitoes, ticks, fleas and lice, are biological vectors. Some important biological vectors and vector-borne diseases are listed in Table 1.1.

Table 1.1 Biologic vectors and vector-borne diseases

Vector	Scientific name/ group	Disease	Disease pathogen
Insects			
Body louse	*Pediculus humanus corporis*	Epidemic typhus Epidemic relapsing fever	*Rickettsia prowazekii* *Borrelia recurrentis*
Cone nose bugs Reduviid bugs Assassin bugs	*Triatoma* spp *Rhodnius* spp *Panstrongylus* spp	Chagas' Disease	*Trypanosoma cruzi*
Tsetse flies	*Glossina* spp	African Sleeping Sickness	*Trypanosoma brucei rhodesiense* *T. b. gambiense*
Horse flies Sandflies	*Chrysops* spp *Phlebotomus* spp *Lutzomyia* spp	Loiasis Leishmaniasis Sandfly Fever Oroya Fever	*Loa loa* *Leishmania* spp Arbovirus *Bartonella bacilliformis*
Black flies Biting midges	*Simulium* spp *Culicoides* spp	River blindness Filariasis Oropouche Fever	*Onchocerca volvμl us* *Mansonella perstans* *M. ozzardi* Arbovirus

Mosquitoes	*Anopheles* spp	Malaria	*Plasmodium* spp
		Lymphatic filariasis	*Wuchereria bancrofti*
		Onyog'nyong	Arbovirus
	Culex spp	Filariasis	Arbovirus
		Oropouche	Arbovirus
		Ross River	Arbovirus
		VEE.	Arbovirus
		WEE	Arbovirus
		St Louis enceph	Arbovirus
		Jap. B. enceph	Arbovirus
	Aedes spp	Yellow fever	Arbovirus
		Dengue	Arbovirus
		Rift Valley Fever	Arbovirus
		Ross River	Arbovirus
		Chikungunya	Arbovirus
		EEE	Arbovirus
		Lymphaic filariasis	*Brugia* spp
			Wuchereria bancrofti
	Psorophora spp	VEE.	*Wuchereria bancrofti*
		Mayaro	*Wuchereria bancrofti*
	Haemagogus spp	Yellow Fever	*Wuchereria bancrofti*
	Mansonia spp	Lymphatic filariasis	*Brugia* spp
			Wuchereria bancrofti
	Coquillettidia spp	EEE	Arbovirus
	Ochlerotatus spp	Ross River	Arbovirus
Fleas	*Xenopsylla* spp	Bubonic plague	*Yersisia pestis*
		Murine typhus	*Rickettsia typhi*
Mites			
Rat mite	*Leptotrombidium* sp	Scrub typhus	*Rickettsia tsutsugamushi*
Ticks			
Tampan	*Ornithodoros* spp	Endemic Relapsing fever	*Borrelia duttonii*

Hard tick	*Ixodes* spp	Spotted Fevers	*Rickettsia* spp
		Lyme Disease	*Borrelia burgdorferi*
Snake tick	*Aponomma* sp	Flinders Island Spotted Fever	*Rickettsia hone*i
Lone star tick	*Dermacentor* spp	Rocky Mountain Spotted. Fever	*Rickettsia rickettsiae*
Brown tick	*Rhipicephalus* spp	Spotted Fevers	*Rickettsia* spp
Bont tick	*Hyalomma* spp	Congo-Crimean Haemorrhagic Fever	Arbovirus
Bont leg tick	*Amblyomma* spp	Spotted Fevers	
		Ehrlichiosis	*Ehrlichia chaffeensis*
Dog tick	*Haemaphysalis* spp	Spotted Fevers	*Rickettsia* spp

Reference: John M. Goldsmid, Arthropod-borne infections in the tropics: A synopsis

(www.tropmed.org/primer/chapter13.pdf)

Despite the improvement of public health infrastructure in recent years, vector-borne diseases remain a very important issue for human and animal health. It is reported that more than half of total morbidity and mortality in people during the last four centuries resulted from vector-borne diseases (Gubler *et al.,* 1998). The cost associated with vector-borne diseases is very huge because of lacking effective prevention and control. For example, malaria, a serious and potentially fatal disease caused by female mosquitoes of the genus *Anopheles*, causes 300-500 million clinical episodes and 1.2 million deaths each year (WHO, 2009). At the end of 2004, an estimated 3.2 billion people in 107 countries and territories (about 50% of the world population) live in areas at risk of malaria transmission (WHO, 2005). The majority cases of malaria occur in the world's poorest countries, such as sub-Saharan African countries (Harrus *et al.,* 2005). According to the World Health Report 2005, it also serves as one of the leading causes of death of

children in the developing countries (ranked 5th), and young children under the age of five or pregnant women are the most vulnerable group for this disease.

The economic impact of vector-borne diseases, which influence human health, is huge as well. For example, the economic impact of malaria in the developing countries accounts for 2-3% citizen income (Chima *et al.*, 2003; Russel, 2004). The direct costs combined with other indirect costs cause greater impact. For example, the direct mean cost of malaria and other diseases in Nigeria amount to 7% of the household monthly income (Onwujekwe *et al.,* 2000). However, if combined with indirect costs, they are responsible for more than 75% of the household income (Russel, 2004). Lyme disease is another example. Although it is not fatal, the mean annual incidence for this tick-borne disease in the USA during 1993–1996 is 4.73 cases per 100,000 populations. The expected national expenditure over 5 years for therapeutic interventions is about 2.5 billion dollars (Maes *et al.,* 1998). Chagas disease (American *trypanomiasis*) is a gradually decreased disease in South and Central America. In Brazil, an estimated 516 million dollars are invested in the Chagas disease (kissing bug transmitted) control program between 1975 and 1995, of which 78% were spent on vector control. The control program is reported to be cost-effective with each one dollar spent on vector control resulting in 2.01 dollars of savings (Hoetz *et al.,* 2004).

Vector-borne diseases also have enormous economic impact on agricultural animals. In the 1980s, it is estimated that hemoparasitic diseases of cattle could cost an annual global economic loss of 7 billion dollars (Brown, 1997). In the early 1990s, tick borne bovine *babesiosis* and *anaplasmosis* costed an annual economic loss of 875 -1365 million dollars

only in Latin America (James, 1992; Brown, 1997). In Africa, the vector borne diseases are also very severe. For example, tick transmitted heartwater disease is estimated to cost an economic loss of 5.6 million dollars each year in Zimbabwe (Mukhebi *et al.,* 1999).

Introduction of tick and tick-borne diseases

Biology of Ticks

Tick is a common name of small arachnids that belong to the subphylum *Chelicerata*, class *Arachnida* and order *lxodidea* (Sonenshine, 1991). It is an obligate blood sucking arthropods which have nearly 850 tick species (Oliver, 1989; Sonenshine, 1991). According to morphological differences, ticks could be classified as three families: *Ixodidae, Argasidae* and *Nuttalliellidae*. Ticks from family *Ixodidae* usually with tough sclerotized plate on their dorsal body surface, and thus they are called hard tick. Family *Aragasidae* includes five genera and approximately 170 species were identified. Usually, they have a leathery cuticle on the dorsal body surface, and thus they are called soft ticks. Family *Nuttalliellidae* is confined to southern parts of Africa from Tanzania to Namibia and South Africa. This family includes only one species- *Nuttalliella namaqua* (Keirans *et al.,* 1976), which is a morphologically intermediate between the families *Argasidae* and *Ixodidae*. In general, ticks belonging to family *Ixodidae* and family *Argasidae* serve as vectors for transmitting infectious agents to animals and people.

The mouthparts of ticks include three visible components (Figure 1.1): two outside jointed parts are highly mobile palps and between them are paired chelicerae. The chelicerae serve as protector for the center rod-shaped structure named hypostome. It is

the hypostome that has beak-like projection which could enter the skin of the host. Some backward directed projection prevents easy removal of attached tick from the host. In addition, they also secret a cement-like substance from salivary glands and this substance glues the body of the tick with the host. When feeding completed, this substance will dissolve automatically. The difference between family *Ixodidae* (hard ticks) and family *Aragasidae* (soft ticks) is that the mouthparts of hard ticks are visible from above, but the mouthparts of soft ticks are not readily visible if looked from the same position.
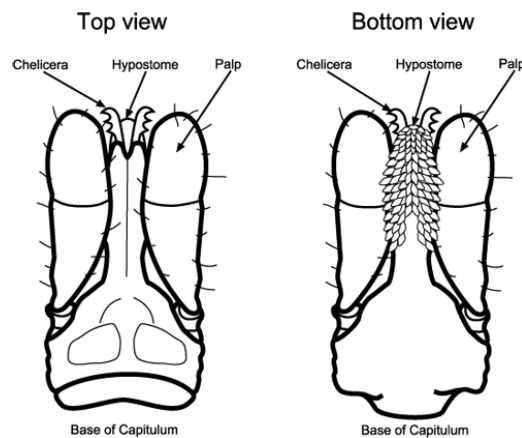
Figure 1.1 Generalized mouthparts of ticks.

(Source: http://extension.entm.purdue.edu/publichealth/images/tick/tick02.gif)

The most important difference between hard ticks and soft ticks lies on their feeding styles and life cycles. Hard ticks usually are slow-feeders. They stay in the body of host for a few days, and take a large volume of blood meal each time, usually about 200-300 times of their unfed body weight. For soft ticks, they usually stay in the body of host for a few minutes or at most a few hours, and take a small volume of blood meal with only 5-10 times of their unfed body weight. Compared to hard ticks, soft ticks are fast feeder.

The life cycle of ticks includes four stages: egg, larva, nymph and adult. Eggs hatch into six legged larva stage, and then larva molt into nymphal stage after taking a blood meals. Hard ticks and soft ticks are very different at this stage. Hard ticks only have one nymphal stage. After nymphal stage, they take another blood meal and develop into the adult stage. After feeding one more time, female hard ticks lay a batch of thousands of eggs and then die. For soft ticks, they molt into a multiple nymphal stages and feed several times during each life stage. Unlike hard ticks, female soft ticks lay multiple small batches of eggs between blood meals and thus the time of completing their whole life cycle is much longer than that of hard ticks, sometimes lasting for more than several years. Also, soft ticks require multiple hosts for developing into their adult stages (Figure 1.3), but hard ticks have less than three hosts all their life (Figure 1.2).
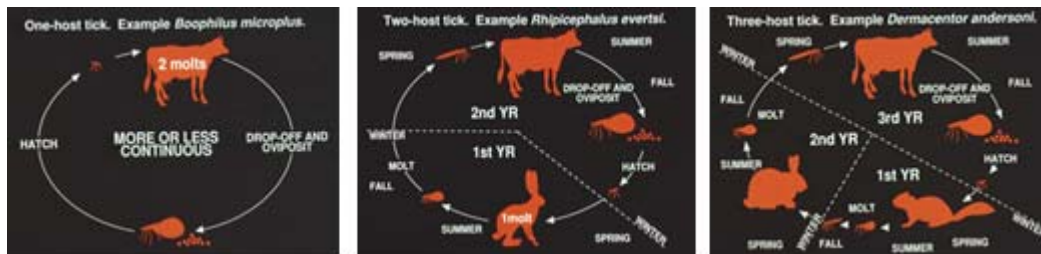
Figure 1.2   One host, two hosts and three hosts of hard ticks.

(Source: http://entomology.ucdavis.edu/faculty/rbkimsey/tickbio.html)
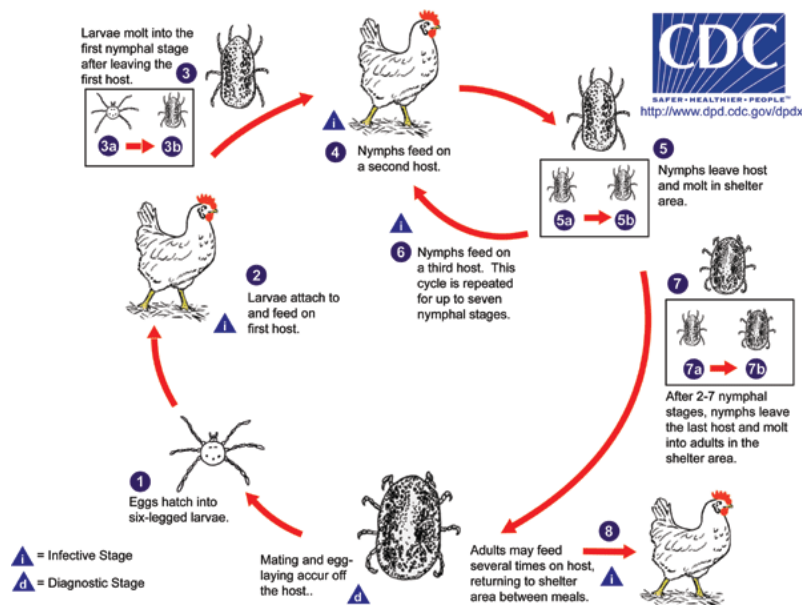
Figure 1.3   Multiple hosts of soft ticks

(Source: http://www.dpd.cdc.gov/DPDx/HTML/Frames/S-Z/Ticks/body_Ticks_argasid_cycle.htm)

Ticks as vectors

Ticks are known as vectors for transmitting a diverse group of infectious agents such as protozoa, bacteria and virus to vertebrate animals. For example, protozoan parasite *Babesia microti* transmitted by *Ixodes dammini* ticks caused infection of human babesiosis in the USA (Rodgers *et al.,* 2007). *Borrelia burgdorferi* transmitted by *Amblyomma americium* ticks caused Lyme diseases to people (CDC, 2001). *Francisella tularensis* transmitted by *Dermacenter* and *Haemophysalis* species ticks caused infection of tularemia in people (Gordon *et al.,* 1983). *Rickettsia rickettsia* transmitted by *Dermacenter* species ticks and cause *Rocky Mountain spotted fever* in the USA (Weber *et al.,* 1991). In addition, *Ehrlichia (E. chaffeensis and E. ewingii)* and *Anaplasma phagocytophilum* transmitted by *Amblyomma* and *Ixodes* ticks caused *Ehrlichiosis* in

people (Dumler *et al.,*1998; Alciati *et al.,* 2001; Anderson *et al.,* 1993; Bakken *et al.,* 2000; Buller *et al.,* 1999; Dumler *et al.,* 1998). Tick-born encephalitis virus (TBEV) transmitted by *Ixodes* ticks caused encephalitis and hemorrhagic fever in people in many parts of the world (Mitzel *et al.,* 2007).

Tick-borne Rickettsiales Diseases

Rickettsiales, transmitted by tick, lice, mites, fleas and flukes, include a group of obligate intracellular bacteria and most pathogen organisms causing fatal and chronic infections in people and vertebrate animals. Tick-borne Rickettsiales diseases are important human and animal diseases. According to the analysis of 16S rRNA, GroESL and several other surface protein gene sequences, they can be divided into two families: *Rickettsiaceae* and *Anaplasmataceae* (Dumler *et al.,* 2001; Fournier *et al.,* 2003).

The family *Rickettsiaceae* includes two genera: *Rickettsia* and *Orientia*. The genus *Rickettsia* has twenty-five species, whereas the genus *Orientia* has only one species- *O. tsutsugamushi* (Dumler *et al.,* 2001). The genus *Rickettsia* can also be subdivided into two groups based on the genotypic and phenotypic similarities: the spotted fever group *rickettsiae* (SFGR) and typhus group *rickettsiae* (TGR) (Raoult *et al.,* 1997; Dumler *et al.,* 2001). SFGR is mainly transmitted by ticks and TGR is transmitted by flea and lice (Walker, 1989; Parola *et al.,* 2001; Raoult *et al.,* 1997; Uchiyama, 2005). Some important diseases of SFGR include *Rocky Mountain spotted fever* (Western hemisphere), *Rickettsialpox* (USA and former Soviet Union), *Boutonneuse fever* (Mediterranean countries, Africa, Southwest Asia and India), *Siberian tick typhus* (Siberia,

Mongolia, northern China), *Australian tick typhus* (Australia), *Oriental spotted fever* (Japan) and *African tick-bite fever* (South Africa). Some important diseases of TGR include *Epidemic, recrudescent* and *sporadic typhus* (worldwide), and *Murine* (endemic) *typhus* (worldwide). The species *O. tsutsugamushi* of the genus *Orientia* is mainly transmitted by mites (Rosenberg, 1997; Watt *et al.,* 2003). A disease named scrub typhus formerly known as *R. tsutsugamushi* has also been reclassified into the genus.

The family *Anaplasmataceae* includes four genera: *Anaplasma, Ehrlichia, Neorickettsia* and *Wolbachia*. The genera *Anaplasma* and *Ehrlichia* are mainly transmitted by *Ixodid* ticks; the genus *Neorickettsia* is mainly transmitted by flukes (Dumler *et al.,* 2001; Rikihisa, 2003; Hechemy *et al.,* 2003; Raoult *et al.,* 1997; Watt *et al.,* 2003); and the genus *Wolbachia* is endosymbionts in arthropods and nematodes (Bandi *et al.,* 1998; Dumler *et al.,* 2001; Jeyaprakash *et al.,* 2000; Werren *et al.,* 2000). Some important tick-borne diseases of the genus *Anaplasma* and *Ehrlichia* in people include: human granulocytic *anaplasmosis, ewingii ehrlichiosis*, and *monocytic ehrlichiosis*, which are caused by *Anaplasma phagocytophilum, Ehrlichia ewingii*, and *Ehrlichia chaffeensis*, respectively. The genus *Neorickettsia* has three species reported pathogenic: *helminthoeca, risticii* and *sennetsu* (Madigan *et al.,* 2000; Rikihisa *et al.,* 1991; Brown *et al.,* 1972). The genus *Wolbachia* is non-pathogenic symbiotic bacteria resided in insects, spiders, mites, ticks and filarial nematodes (Stouthamer *et al.,* 1999). Some of *Rickettsiale* diseases are listed in Table 1.2 and some of well-known tick-borne *Rickettsiales* are discussed below.

Table 1.2   Some of Rickettsiale[a] Diseases

Rickettsia species	Disease	Vectors	Hosts	Geographic distribution
Typhus group:				
Rickettsia prowazekii	Epidemic typhus	Human body	lice Humans	Worldwide
	Recrudescent typhus	None	Humans	Worldwide
		Lice, fleas	Flying squirrels	Eastern USA
R. typhi	Murine typhus	Fleas	Rodents	Worldwide
		Fleas	Opossums	USA
R. felis	Murine typhus like	Fleas	Opossums	USA
Spotted Fever group:				
R. rickettsii	Rocky Mountain spotted fever	Ticks	Small mammals, dogs, rabbits, birds	North & South America
R. conorii	Boutonneuse fever	Ticks	Rodents, dogs	Africa, Southern Europe, India
R. sibirica	North Asia tick typhus	Ticks	Rodents	Eurasia, Asia
R. japonica	Japanese spotted fever	Ticks	Rodents, dogs	Japan
R. australis	Queensland tick typhus	Ticks	Rodents	Australia
R. akari	Rickettsialpox	Mites	House mice, rats	Worldwide
Ehrlichioses group:				
Ehrlichia chaffeensis	Human monocytic ehrlichiosis	Ticks	Humans, deer	USA, Europe

Anaplasma phagocytophilum	Human granulocytic anaplsamosis	Ticks	Humans, deer rodents	USA, Europe
Ehrlichia ewingii	Human ewingii ehrlichiosis	Ticks	Humans, deer	USA, Europe
Neorickettsia sennetsu	Sennetsu ehrlichiosis	Trematode	Humans	Japan, Malaysia

Others:

Orientia tsutsugamushi	Scrub typhus	Mites	Rodents	Asia, Indian subcontinent, Australia

[a]Not listed are *R. helvetica, R. honei,* and *R. slovaca*.

(source: http://www.cdc.gov/ncidod/eid/vol4no2/azad.htm#Table%201)

Rocky Mountain spotted fever

Rocky Mountain spotted fever (RMSF) is the most severe tick-borne rickettsiale disease in the USA, which caused by *Rickettsia rickettsii*. This rickettsiale organism is primarily transmitted by American dog tick (*Dermacentor variabilis*) and Rocky Mountain wood tick (*Dermacentor andersoni*). In southern region of the United States, the main vectors are *Rhipicephalus sanguineus* (brown dog tick) and *Amblyomma cajennense* (CDC, 2010). Humans of all ages are susceptible to RMSF. The pathogen, which spread by blood and the lymphatic system, infects the whole body and causes severe damage to endothelial cells lining the blood vessels of vital organs (Masters *et al.,* 2003). Although this disease is first discovered and named after Rocky Mountain area, recently few cases are reported from that area. About half of the cases occur in the south-Atlantic region of

the United States: Delaware, Maryland, Washington D.C., Virginia, West Virginia, North Carolina, South Carolina, Georgia, and Florida. The highest incidence rates have been found in North Carolina and Oklahoma of the USA (CDC, 2010).

The early clinical signs of RMSF are often nonspecific which may include fever, nausea, vomiting, severe headache, muscle pain, lack of appetite. Usually, the characteristic red, spotted (petechial) rash is not visible until the sixth day or later after the onset of symptoms. Severe cases may also involve infection to the respiratory system, central nervous system, gastrointestinal system, or renal system. The mortality of this disease ranges from 5% to 10% (Chapman *et al.,* 2006), and most of the mortalities are associated with delay in diagnosis (Masters *et al.,* 2003).

Mediterranean spotted fever

Mediterranean spotted fever (MSF), also known as boutonneuse fever, is a tick-borne rickettsiale disease caused by *Rickettsia conorii* and transmitted by the dog tick *Rhipicephalus sanguineus* (Azad *et al.,* 1998). MSF is reported in the Mediterranean countries including southern Europe, northern Africa, and part of Asia (Rovery *et al.,* 2008). Because of increased travel to endemic areas, this disease has also been transmitted to other regions of the world including central Europe and southern and central Africa (Rovery *et al.,* 2008). After infection, the pathogen invades vascular endothelial cells, causing endothelial cells injury and tissue necrosis. Like the other rickettsiale diseases, the symptom is non-specific: fever, severe headache, and maculopapular rash (Sousa *et al.,* 2003; Rovery *et al.,* 2008). If recognized at the initial

stages, MSF can be treated effectively with antibiotics such as chloramphenicol, tetracycline or doxycyline (Rovery *et al.,* 2008).

Japanese spotted fever

Japanese spotted fever, also known as *Oriental spotted fever*, is another rickettsiale disease which transmitted by tick family *Ixodidae* and caused by *Rickettsia japonica*. This disease was first reported in 1984 in Japan and only confined to the region of southwestern Japan, but currently it also has been reported existing in Korea (Ma *et al.,* 2005). Main symptom of the disease includes fever, headache and the appearance of an eschar and rash, and severe cases might involve the damage to the central nervous system (Araki *et al.,* 2002).

North Asian tick typhus

North Asian tick typhus, also called *Siberian Tick Typhus*, is another tick-borne *rickettsiale* disease which caused by the bacteria *Rickettsia sibirica*. Symptoms include a generalized maculopapular rash involving palms and soles, fever, and lymph node enlargement. It occurs in the north, central and east parts of Asia, and because of traveling to endemic area, it was also reported in North America (Lewin *et al.,* 2003). This disease is rarely fatal and easily cured by treating with chloramphenicol.

Flinders Island spotted fever

Flinders Island spotted fever was caused by *Rickettsia honei* and transmitted by *Ixodes holocylus* tick (Stewart, 1991). It was first reported from Flinders Island near Australia in

1991 and isolated later in 1998 (Stewart, 1991; Stenos *et al.,* 1998). The pathogen proliferates within the endothelial cells of small blood vessels and causes vasculitis. The usual symptom of the diseases includes high fever, headache, muscle aches (*myalgia*), slight cough, and arthralgia without joint swelling. Maculopapular rash might occur 5 or 6 days after tick biting. The disease is rarely fatal and can be cured by treatment of tetracyclines.

Australian Spotted Fever

Australian Spotted Fever, also known as *Queensland Tick Typhus*, is caused by *Rickettsia australis* and transmitted by *Ixodes holocyclus* tick (Graves *et al.,* 1993). Like Flinders Island spotted fever, the infectious agent proliferates within the endothelial cells of small blood vessels and induces vasculitis. This disease was first reported in 1946 in north Queensland (Stewart *et al.,* 1991). The symptom involves fever, headache and muscle aches (*myalgia*). Severe cases might also involve stiff neck, nausea, vomiting and mental confusion (Unsworth *et al.,* 2007). This disease is also rarely fatal and more quickly cured with treatment of antibiotics (CDC, 2007).

Ehrlichia ewingii

Ehrlichia ewingii, originally known as the pathogen causing granulocytic ehrlichiosis in canines, was recognized in 1999 as the infectious agent for granulocytic ewingii ehrilichiosis in humans (Buller *et al.,* 1999). The main natural vector of *E. ewingii*- the lone star tick- *Amblyomma americanum* and its recent infection in humans have been reported from Missouri, Oklahoma, and Tennessee (Buller *et al.,* 1999; Paddock *et al.,*

2001). The symptom of the infection includes fever, headache and myalgia, and usually reported in cases that recently be exposed to ticks. The diagnosis of this disease is mainly through isolation of the agent from tissue culture (Childs *et al.,* 1999), and identification by PCR assay with two regions of gene sets serving as primers-16S rRNA genes and *groE* genes (Breitschwerdt *et al.,* 1998; Buller *et al.,* 1999; Sumner *et al.,* 2000). The disease can be effectively cured with antibiotics like doxycyline.

Human Granulocytic Anaplasmosis

Human granulocytic anaplasmosis (HGA), previously known as human granulocytic ehrlichiosis, is caused by a bacteria called *Anaplasma phagocytophilum* and mainly transmitted by deer tick- *Ixodes scapularis.* This disease was first identified in 1990 in a Wisconsin patient who died from a febrile disease after two weeks of tick bite (Chen *et al.,* 1994). Although it is mostly reported from Northeastern and upper Midwestern regions of the United States where the *Ixodes* ticks are highly prevalent (Demma *et al.,* 2006), it also appeared in Pacific western states of the USA (Richter, 1996; Barlough, 1997) and western Europe (Stedink, 1997), where *Ixodes pacificus* (the black-legged tick) and *Ixodes ricinus* (the sheep tick) are prolific. The pathogen targets at white blood cells called neutrophils. Symptom of this disease includes high fever, severe headache, malaise, anorexia, nausea, cough and nonspecific rash. The duration time may only last a couple of days, but may also persist longer if untreated. This disease is also rarely fatal and can be treated effectively with antibiotics such as doxycyline.

Human Monocytic Ehrlichiosis

Human monocytic ehrlichiosis (HME), caused by *Ehrlchia chaffeensis* and transmitted by the lone star tick *Amblyomma americanum*, is one of the most important tick-borne Rickettsiale diseases in the USA. The pathogen is maintained in nature mainly in dual hosts, white-tailed deer (*Odocoileus virginianus*) and the lone star tick *Amblyomma americanum* (Dawson *et al.,* 1994; Lockhart *et al.,* 1995). HME cases were reported from the south-central, southeastern, and mid-Atlantic states of the USA, which corresponds to the geographical distribution of the lone star tick (CDC, 2009). Some cases may also be reported from the outside of the expected regions of the lone star tick, because many travelers serve as vectors to carry the infectious agent outside of the endemic areas (CDC, 2009). The symptoms of this disease include fever, headache, malaise, and muscle aches. Some clinical signs of HME cases are easily to be separated from other diseases like HGA. For example, rash is easier to be found in HME cases (Dumler *et al.,* 2005). Some severe cases of the diseases may involve infection to center nervous system. Diagnosis of human monocytic ehrlichiosis is mainly based on symptom and clinical signs of the disease and confirmatory laboratory test. Some indicatives of human monocytic ehrlichiosis in confirmatory laboratory test include low white blood cell count, low platelet count and elevated liver enzymes (CDC, 2009). Treatment with a tetracycline antibiotics and especially doxycycline has been shown effective for this disease (Hamburg *et al.,* 2008). Much more details about HME will be discussed in the later paragraphs.

Ehrlichia chaffeensis, an emerging human infectious agent

Discovery

Ehrlichia chaffeensis, the causative agent of human monocytic ehrliciosis, is obligatory intracellular tick-transmitted rickettsiales bacteria in the family *Anaplasmataceae* of class *Alpha Proteobacteria*. The disease that the bacteria caused, which is called human monocytic ehrliciosis, was first found in April 1986 by a medical intern in Arkansas scanning the peripheral blood smear of patients (Maeda *et al.,* 1987). Because the description of sustained tick-bitten experience by the patients, it was suspected as Rocky Mountain spotted fever (Fishbein, 1990; Maeda *et at.,* 1987). Then, clinicians and scientists found that this infectious agent actually belongs to the genus *Ehrlichia* (Maeda *et at.,* 1987). It was isolated in cell culture and characterized by molecular techniques at Fort Chaffee, Arkansas (Dawson *et al.,* 1991), and because of that, it was named as *Ehrlichia chaffeensis.*

Background

This obligatory intracellular pathogen principally transmitted by the lone star tick *Amblyomma americanum* (Figure 1.4), and the major vertebrate mammalian host for this organism is the white-tailed deer (Figure 1.5) (Dawson *et al.,* 1994; Lockhart *et al.,* 1997; Parola *et al.,* 2005; Anderson *et al.,* 1993). Other vertebrates including humans, dogs, goats and coyotes may also be susceptible for serving as accidental hosts (Dugan *et al.,* 2000; Yabsley *et al.,* 2004; Dugan *et al.,* 2005; Yu *et al.,* 2008). The life cycle includes propagation of the pathogen in its tick vector and vertebrate hosts. Larval or nymphal ticks get infected by the bacteria while they are feeding the infected vertebrate host and

then they transmit the pathogen to another healthy vertebrate host. Till now, no records have suggested that this pathogen can be transovarially transmitted (Long *et al.,* 2003). HME cases are reported mostly from places where the lone star ticks and white-tailed deer are abundant (from New Jersey to Kansas and Southward) (Standaert *et al.,* 1999; Bakken *et al.,* 1996; Bakken *et al.,* 1998; Dumler *et al.,* 1998; Whitlock *et al.,* 2000; Standaert *et al.,* 2000; Olano *et al.,* 2003; Paddock *et al.,* 2003; McNabb *et al.,* 2008). Recently, the incidence of human monocytic ehrlichiosis in the USA has increased gradually (Figure 1.6) (Paddock *et al.,* 2003; McNabb *et al.,* 2008), with an average of 600 human cases of HME each year (McNabb *et al.,* 2008).

Figure 1.4   *Amblyomma americanum* (Lone star tick) and its distribution.

(source: http://www.cdc.gov/ncidod/dvrd/Ehrlichia/Natural_Hx/Natural_Hx.htm)

Figure 1.5 White-tailed deer.

(source: http://fw.dpnr.gov.vi/education/AndyWebPage/Pictures/Mammals/digiscope029.jpg)
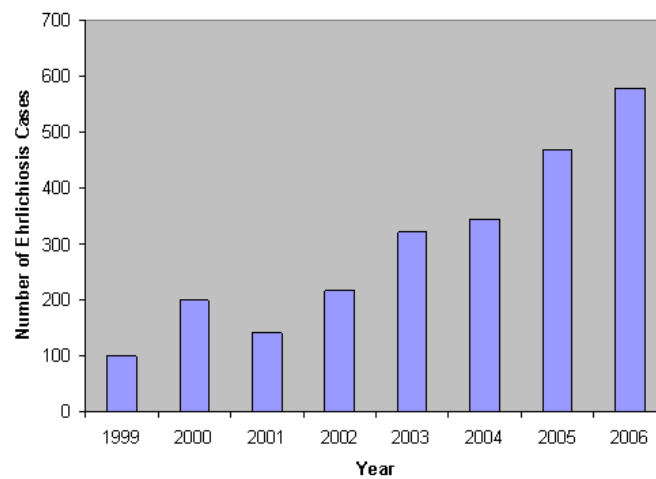
Figure 1.6   Number of Ehrlichiosis cases (caused by *Ehrlichia chaffeensis*) reported to CDC by state

health departments, 1999-2006.

(Source: CDC National Electronic Telecommunications System for Surveillance (NETSS) data)

Clinical signs

The clinical signs usually appear in 1-4 weeks after tick-bite (Parola *et al.,* 2005). The symptom of this disease ranges from asymptomatic to mild flu-like symptoms, and sometimes might deteriorate into severe life threatening situation. The most common symptom includes malaise, fever, headache, muscle aches, chills, nausea and lymphadenopathy (Paddock *et al.,* 2003; Dumler *et al.,* 2007). Sometimes, it might also include cough, pharyngitis, swollen lymph nodes, vomiting, and possible changes in mental status. Unlike the Rocky Mountain spotted fever, only 30-40% of suspected cases reported having a rash associated with infection. Severe cases may include complications such as septic shock-like syndrome, meningitis, organ damage (Paddock *et al.,* 2001; Stone *et al.,* 2004; Dumler, 2005; Dumler *et al.,* 2007). Leucopenia, thrombocytopenia, and an increase in hepatic transaminase levels are usually observable in the laboratory diagnosis (Paddock *et al.,* 2003; Dumler *et al.,* 2007). Although people of all ages are susceptible to the disease, it is reported that immune compromised people and elderly people have greater chance of getting infection (Figure 1.7) (Paddock *et al.,* 2003; Gardner *et al.,* 2003).
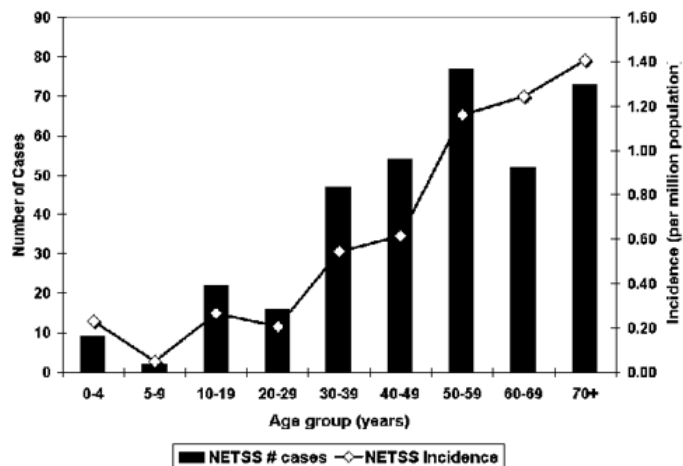
Figure 1.7 Age-specific incidence of Ehrlichiosis (caused by *Ehrlichia chaffeensis*), reported to CDC

2001-2002 through NETSS.

(source: http://www.cdc.gov/ticks/diseases/ehrlichiosis/statistics.html)

Diagnosis and treatment

Because other bacteria or virus also have similar symptom and clinical signs, definitive

diagnosis during early stage of the disease is rather difficult. Thus, accurate description of

tick-bite history and systematic laboratory diagnosis is very helpful to the preliminary

confirmation of this disease (Dumler *et al.,* 2007). Routine laboratory diagnostic methods

for preliminary examination include detecting peripheral blood smear, performing IFA

and PCR. Confirmatory examination usually involves cell culture of the pathogen

(Anderson *et al.,* 1991; Chapman *et al.,* 2006; Dumler *et al.,* 2007; Rikihisa *et al.,* 2007).

For prevention and clearance of the infection, treatment with tetracycline is proven to be

very effective (Paddock *et al.,* 2003). A derivative of tetracycline – doxycyline, which has

been proved having better efficacy, is currently the drug of choice for treating HME

(Dumler *et al.,* 2007).

Molecular biology

Molecular biology of *E. chaffeensis* includes studies on 16S rRNA gene, DO/DeqQ family serine proteases, DsbA-like disulfide bond formation proteins, quinolinate synthetase A (NAD/A), VirB/D, a hypothetical protein (Esp73), fts Z gene, a variable length PCR target gene (VLPT), a homolog of heat shock protein gene (groESL), three glycoproteins(gp47, 120 and 156) and an abundantly expressed p28 kDa outer membrane proteins (p28-Omp) (Yabsley *et al.,* 2003; Yu *et al.,* 1997; Yu *et al.,* 2000; Summer *et al.,* 1997; Summer *et al.,* 1999, Singu *et al.,* 2005; Rikihisa *et al.,* 2007; Yu *et al.,* 1997; Lee *et al.,* 2003; McBride *et al.,* 2002; Rikihisa *et al.,* 2007; Seo *et al.,* 2008). A brief description of several characterized genes is provided below.

16S rRNA

16S rRNA gene sequence has been reported from various *E. chaffeensis* isolates as an identical gene (Wen *et al.,* 1997), and because of it is highly conserved, the 16S rRNA is used for other species differentiation and phylogenetic classification (Anderson *et al.,* 1991; Dumler *et al.,* 2001). For example, PCR assays based on 16S rRNA gene and flowing PCR by RFLP is able to give a rapid discrimination between *A. marginale, A. centrale* and *A. ovis* (Vahid *et al.,* 2009). PCR assays based on 16s rRNA gene and electro spray ionization mass spectrometry can be used to rapidly detect and identify *Ehrlichia* species in blood (Mark *et al.,* 2010).

The 28 kDa outer membrane proteins (p28-Omp)

The 28 kDa outer membrane proteins of *E. chaffeensis* are encoded by a multigene locus containing 22 tandemly arranged paralogous genes (Reddy *et al.,* 1998; Ohasi *et al.,* 1998; Reddy *et al.,* 1999; Yu *et al.,* 2000; Cheng *et al.,* 2003; Singu *et al.,* 2005; Seo *et al.,* 2008). The molecular weights of mature proteins expressed from this multigene locus are predicted to be 28 kDa to 32 kDa, and because of that, it was named as the 28 kDa outer membrane proteins of *E. chaffeensis*. The amino acid sequence identity of these proteins is ranged from 20% to 83%. These arranged paralogous genes are separated by a fragment of 10 to 605 nucleotides non-coding intergenic sequences (Hotopp *et al.,* 2006). Three highly variable regions present in all 22 genes which are significantly different in each gene (Reddy *et al.,* 1998; Li *et al.,* 2001; Li *et al.,* 2002; Winslow *et al.,* 2000). They are hydrophilic in nature and contain the immunogenic B-cell epitopes (Reddy *et al.,* 1998; Winslow *et al.,* 2000; Li *et al.,* 2001; Li *et al.,* 2002).

The p28-Omp locus genes of *E. chaffeensis* isolates also contain considerable variations (Cheng *et al.,* 2003; Miura *et al.,* 2007). According to the sequence variation, *E. chaffeensis* can be classified into three groups (I, II, and III) (Cheng *et al.,* 2003). Ten isolates of *E. chaffeensis* recovered from human patients from various regions in the United States were used for this analysis. Group I contains three isolates: Arkansas, Osceola, and Lithonia. Group II contains five isolates: St. Vincent, Chattanooga, West Paces, Heartland, and Wakulla isolates. Group III contains two isolates: Liberty and Jax (Cheng *et al.,* 2003). In addition, the p28-Omp gene 18 and p28-Omp gene 15 were

found in group I isolates and group III isolates, respectively (Cheng *et al.,* 2003). Variations existing in the genome of different *E. chaffeensis* isolates may have some connection for overcoming host immune responses for continued persistence in its dual hosts. This hypothesis remains to be tested.

E. chaffeensis p28-Omp multigene family in other species of *Ehrlichia* are also found (Gusa *et al.,* 2001; Ohasi *et al.,* 2001; van *et al.,* 2004; Crocquet, 2005; Zhang *et al.,* 2008). For example, in *E. ewingii* and *E. muris,* the p28-Omp multigene locus has 19, and 21 paralogous genes, respectively (van *et al.,* 2004; Crocquet, 2005; Zhang *et al.,* 2008). *E. canis* and *E. ruminantium* p28-multigene locus genus have different names, referred to as p30 Omp genes or MAP l genes (Ohasi *et al.,* 2001; van *et al.,* 2004). However, they are the paralogous of *E. chaffeensis* p28 Omp genes. The p-30 locus of *E. canis* includes 22 tandemly arranged paralogous genes (Ohasi *et al.,* 2001). Map 1/ p30 genes of *E. ruminantium* are 16 tandemly arranged paralogous genes (Brayton *et al.,* 1997; Ohashi *et al.,* 1998).

Another feature of the p28-Omp multigene family is the existence of genes that encode for a putative transcriptional regulator and a *secA* gene (Ohasi *et al.,* 2001; van *et al.,* 2004; Crocquet, 2006; Zhang *et al.,* 2008). The hypothetical transcriptional regulator of *E. ruminantium* is reported being polycistronically transcribed (van *et al.,* 2004). Recent studies from our laboratory also indicated the existence of transcriptional regulator in *E. chaffeensis* (Seo *et al.,* 2008; Sirigireddy *et al.,* 2005). The *secA* protein might be important in the process of translocation of bacterial outer membrane proteins to outer

membrane (Bernstein *et al.,* 2000). The specific role of the putative transcriptional regulator and the *secA* gene in the p28-Omp multigene family of *E. chaffeensis* still needs to be studied.

It is reported that multiple genes of the p28-Omp multigene family are transcriptionally active in *E. chaffeensis* originating from the macrophages (Yu *et al.,* 2000; Ohasi *et al.,* 2001; Long *et al.,* 2002; Unver *et al.,* 2002; Cheng *et al.,* 2003; Seo *et al.,* 2008). However, the major expressed transcriptional protein in vertebrate cells is p28-Omp 19 (Cheng *et al.,* 2003; *Seo et al.,* 2008), and it is also reported that gene 14 of the p28-Omp multigene family is transcriptionally active in *E. chaffeensis* originating from tick cells or infected ticks (Unver *et al.,* 2001; Unver *et al.,* 2002). Similar differential expression for *E. canis* and *E. ruminantium* (Ohasi *et al.,* 2001; Unver *et al.,* 2001; Bekker *et al.,* 2002; van *et al.,* 2004).

Recent proteomic analysis from our laboratory further confirmed the predominant protein expression of p28-Omp gene 19 from vertebrate macrophages and the predominant protein expression of the p28-Omp gene 14 from tick cells (Singu *et al.,* 2005; Singu *et al.,* 2006; Seo *et al.,* 2008). This host-specific predominant expression has also been reported in the p28-Omp locus homologues of *E. canis* (Unver *et al.,* 2001; Felek *et al.,* 2003; Singu *et al.,* 2006). The vertebrate macrophages and tick cells host-specific predominantly expression might be an important mechanism to explain immune evasion and continued persistence of the pathogen in different hosts.

120 kDa surface protein

The 120 kDa surface protein is a potential adhesin of *E. chaffeensis* (Yu *et al.,* 2000). It is one of the major immunoreactive proteins for which antibodies were detected in most patient sera with HME infection and *E. canis* infected dogs (McBride *et al.,* 2000; Yu *et al.,* 1997; Yu *et al.,* 2000). The 120 kDa surface protein gene of Arkansas isolates of *E. chaffeensis* has four identical tandem repeats and each repeats is 240 bp in length. This gene feature shows great divergence between different *Ehrlichia* species, both in the number of the tandem repeats and the amino acid sequence of the repeats (Yu *et al.,* 1997). In addition, all of the major immunoreactive tandem repeat proteins are highly acidic due to a predominance of glutamate/aspirate and also they seems to be serine rich within tandem repeats of the proteins (Doyle *et al.,* 2006; Luo *et al.,* 2008). The 120 kDa surface proteins are differently expressed by dense-cored *E. chaffeensis* and are found on the surface of the organism (Popov *et al.,* 2000). It is reported that these surface proteins interact with important host cell targets and facilitate pathogen survival (Wakeel *et al.,* 2009). However, the specific role of this protein in pathobiology and protective immune response is still undefined.

Variable Length PCR Target gene (VLPT)

VLPT is another immunoreactive protein of *E. chaffeensis* and it can be used for detection and differentiation of *E. chaffeensis* strains (Sumner *et al.,* 1999). The VLPT of Arkansas strain of *E. chaffeensis* is 44 kDa in molecular mass and the open reading frame (ORF) contains four 90 bp imperfect tandem repeats (Sumner *et al.,* 1999). The number

of repeats is also not the same in different isolates of *E. chaffeensis* (Sumner *et al.,* 1999, Cheng *et al.,* 2003). For example, the Wakulla strain has a six-repeat version of the variable-length PCR target gene, whereas the Arkansas and Liberty strains have four repeats (Sumner *et al.,* 1999). Compared to 120 kDa surface proteins, VLPT genes have more tandem repeats which make it more useful for strains differentiation (Yabsley *et al.,* 2003). However, the exact role of VLPT in *E. chaffeensis* pathogenicity still remains to be known.

Heat Shock Gene Operon (groESL)

E. chaffeensis homologs of *Escherichia coli* heat shock gene operon (groESL) contains a stress-inducible promoter, followed by a GroES open reading frame which encodes a protein with molecular mass of 10-20 kDa, and a GroEL open reading frame which encodes a protein with molecular weight of 58-65 kDa (Sumner *et al.,* 1993). GroES and GroEL genes are separated by noncoding sequences and these sequences vary in length in different bacteria (Sumner *et al.,* 1993). GroEL has been called the bacterial common antigen and is related to the HSP 60 family of heat shock proteins (Rikihisa *et al.,* 2007). Heat shock proteins of bacteria serve to protect cells under stressful condition and environment (Lindquist *et al.,* 1988; Jenkins *et al.,* 1988). However, the exact role of GroESL of *E. chaffeensis* is still not well studied.

Immunologic aspects of *Ehrlichia chaffeensis* infection

The possibility of getting infection by infectious agents depends on several factors, such as the age of an animal, or a specific host species, the virulence of a pathogen and the

immune status of the host. Usually, the pathogen intrusion might not indicate the necessary clinical signs of the disease, and the infection level may vary according to the host immune status. For example, the *E. chaffeensis* infection in humans is shown to be more severe in children, elderly and immune compromised people (Safdar *et al.,* 2002; Paddock *et al.,* 2003; Gardner *et al.,* 2003), but very mild in the healthy adults. It is reported that *E. chaffeensis* can persist inside of healthy immune competent people, but it usually appears with no clinical sign (Davidson *et al.,* 2001).

According the status of immune system of host, the responses for a pathogen intrusion may vary from a clinical disease, clearing of clinical signs and possibly even clearing the pathogen (Marshall *et al.,* 2002; Yevich *et al.,* 1995). For better understanding the host response of *E. chaffeensis*, several studies has been carried out since the discovery of the pathogen (Barnewall *et al.,* 1994; Lee *et al.,* 1996; Winslow *et al.,* 1998; Winslow *et al.,* 2000; Winslow *et al.,* 2003; Ganta *et al.,* 2002; Ganta *et al.,* 2004; Ganta *et al.,* 2007; Chapes *et al.,* 2008). Recently, murine models has been proven very useful for illustrating the host immune mechanism against *E. chaffeensis* infection (Winslow *et al.,* 1998; Winslow *et al.,* 2000; Winslow *et al.,* 2003; Ganta *et al.,* 2002; Ganta *et al.,* 2004; Ganta *et al.,* 2007; Chapes *et al.,* 2008).

In the early experiment of using murine models, immunocompetent mice clear the *E. chaffeensis* infection within 10-17 days post infection (Winslow *et al.,* 1998), but immune deficient (SCID) mice who lack T- and B-cells fail to clear the infection of pathogen, and finally SCID mice exhibit severe clinical signs (Winslow *et al.,* 1998). This proves that T-

cells and B-cells play vital role in clearing the infection of *E. chaffeensis* (Winslow *et al.,* 1998; Ganta *et al.,* 2002; Ganta *et al.,* 2004). In another experiment, major histocompatibility complex-II (MHC-II) deficient mice fail to clear the infection of *E. chaffeensis* (Ganta *et al.,* 2002; Ganta *et al.,* 2004), and the persistence of the pathogen in mice last for as long as 92 days (the longest time evaluated) (Ganta *et al.,* 2002; Ganta *et al.,* 2004). Similar results are obtained from toll-like receptor 4 (tlr4) deficient mice and it shows that the persistence of *E. chaffeenis* can last as long as 30 days (Ganta *et al.,* 2002; Ganta *et al.,* 2004). All the data indicate that major histocompatibility complex-II and toll-like recptor 4 are very important factor involving in the host immune response to *E. chaffeensis* infection.

Antibodies play a significant role in clearance of the pathogen. It is reported that SCID mice can be protected from a severe *E. chaffeensis* infection if injected with immune serum from *E. chaffeensis* infected immunocompetent mice (Winslow *et al.,* 2000). The protection time can last for up to 70 days if repeated injected with immune serum (Winslow *et al.,* 2000; Li *et al.,* 2001). Antisera against *E. chaffeensis* are predominantly made against outer membrane expressed proteins (Seo *et al.,* 2008). Although antibodies are known to play critical role in immune response to clear *E. chaffeensis* infection, they alone are not sufficient to clear the pathogen (Seo *et al.,* 2008). In particular, various subsets of T-cells and cytokines are needed for effective clearance of the infection (Li *et al.,* 2001; Winslow *et al.,* 2000).

Some subsets of T-cells have been proven to be important for immune response to *E. chaffeensis* infection (Ganta *et al.,* 2002; Ganta *et al.,* 2004; Bitsaktsis *et al.,* 2004; Bitsaktsis *et al.,* 2006; Nandi *et al.,* 2007; Ismail *et al.,* 2007; Bitsaktsis *et al.,* 2007; Chapes *et al.,* 2008). Helper T-cells deficient MHCII knockout mice can not clear the infection of *E. chaffeensis* for several months (Ganta *et al.,* 2002; Ganta *et al.,* 2004). The time of clearing the pathogen has been delayed for about two weeks in $CD4^+$ helper T-cell deficient mice (Ganta *et al.,* 2002; Ganta *et al.,* 2004). This result indicates that helper T-cells are very important factor for host immune response for *E. chaffeensis*. It is also reported that γδ T-cells are found to be present in peripheral blood of *E. chaffeensis* infected human patients (Caldwell *et al.,* 1996; Caldwell *et al.,* 1996). The result indicates that γδ T-cells may be an important factor for immune response for this pathogen.

Other factors of host immune system might play minimum role for *E. chaffeensis* infection. For example, both $CD4^+$ T-cells deficient mice and $CD4^+$ T-cells deficient MHCII knockout mice exhibit a minimal $CD8^+$ T-cells activity after *E. chaffeensis* infection (Ganta *et al.,* 2004); and β2 microglobulin (β2M, a structural component of MHCI)-knockout mice also exhibit a complete clearance of the infection of *E. chaffeensis* (Chapes *et al.,* 2008). The findings indicate that $CD8^+$ T-cells are a minor factor in host immune clearance for the pathogen. This applies for NKT cells as well. The SCID mice that are deficient for T-cells and B-cells but contain functionally normal NKT cells exhibit persistent infection of *E. chaffeensis* (Winslow *et al.,* 1998). The NKT cells are

functionally normal, but *E. chaffeensis* is persistently existed in mice (Chapes *et al.,* 1993; Ganta *et al.,* 2002; Ganta *et al.,* 2004).

Some immunomodulating agents are involved in the clearance of the infection of *E. chaffeensis*. It is reported that suppression of several proinflammatory cytokine expression, including IL-1α, IL-4, IL-6, IL-12, IL-15, and IL-18 occurs in *E. chaffeensis* infected human monocytic cell lines (THP1 cells) (Zhang *et al.,* 2004). These cytokines are involved in early inflammatory response for the intrusion of the pathogen (Dinarello, 2000). Suppression of these cytokines will facilitate *E. chaffeensis* to escape from being killed by NKT cells and cytotoxic T-cells, and colonize inside the macrophages or monocytes (Dinarello, 2000). Cytokines IL-15 and IL-18 usually serve as activators for CD4^{+} T-cells to produce IFN-γ and activate macrophages mediated killing of the pathogen (Barnewall *et al.,* 1994; Liew *et al.,* 2002; Bitsaktsis *et al.,* 2004). Thus, cytokines are in part a critical factor in immune response for *E. chaffeensis* infection.

The entire immune system works in a network way, and if one subset of T-cells is absent, the other subsets of T-cells will compliment its function. The experiments of intracellular bacteria of *Mycobacterium tuberculosis* and *Francisella tularensis* illustrate this phenomenon. When CD4^{+} T-cells deficient mice are attacked by pathogens, other T-cells such as CD4^{-} T-cells, CD8^{-} T-cells, and natural killer T-cells will compliment for the CD4^{+} T-cell deficiency and work together to help clear the pathogens (Rahemtulla *et al.,* 1994; Cowley *et al.,* 2003; Cowley *et al.,* 2005; Mattner *et al.,* 2005). It is also reported that components of immune system including antibodies, macrophage activation,

involvement of several T-cell subtypes and cytokines work together to clear *E. chaffeensis* infection (Barnewall *et al.,* 1994; Lee *et al.,* 1996; Winslow *et al.,* 1998; Winslow *et al.,* 2000; Ganta *et al.,* 2002; Ganta *et al.,* 2004). Thus, network of different components of immune system serves as the effective solution for the immune system of the host in the clearance of *E. chaffeensis.*

Electromicroscopy

Under electromicroscopy, two morphological forms of *E. chaffeensis* can be observed residing within the intracellular parasitophorous vacuoles (morulae) in macrophages: dense-cored cells (DC) (0.4–0.6 μm) and reticulate cells (RC) (0.4–0.6 μm by 0.7–1.9) (Popov *et al.,* 1995; Zhang *et al.,* 2007) (Figure 1.8). DCs are smaller than RCs and they contain highly dense nucleotide inside; RCs contain uniformly dispersed nucleotide filaments and ribosome (Popov *et al.,* 1995). Some RCs form long projections of the cell wall and these projections protrude the cytoplasmic membrane into the periplasmic space, budding protoplast fragments into the periplasmic space (Popov *et al.,* 1995; Zhang *et al.,* 2007). Both DC and RC forms replicate themselves by binary fission inside the cells. In the beginning of their infectious cycle, usually 24 h after infection, DC usually transform into RC form. In the end of each cycle, the RCs transform back into DCs in the phagosome and they are released from monocytes or macrophages (Zhang *et al.,* 2007). This type of cycle, transforming from dense core to reticulate forms and then back to dense core forms, is very typical for the genera *Ehrlichia* and *Anaplasma* (Ashley *et al.,* 1975; Hodinka *et al.,* 1988; Wolf *et al.,* 2000; Wyrick, 2003). DCs and RCs are also

found in the phagosome of infected tick cells (Ganta *et al.,* 2009). In tick cells, however, both DCs and RCs can exist within the same phagesome (Ganta *et al.,* 2009).
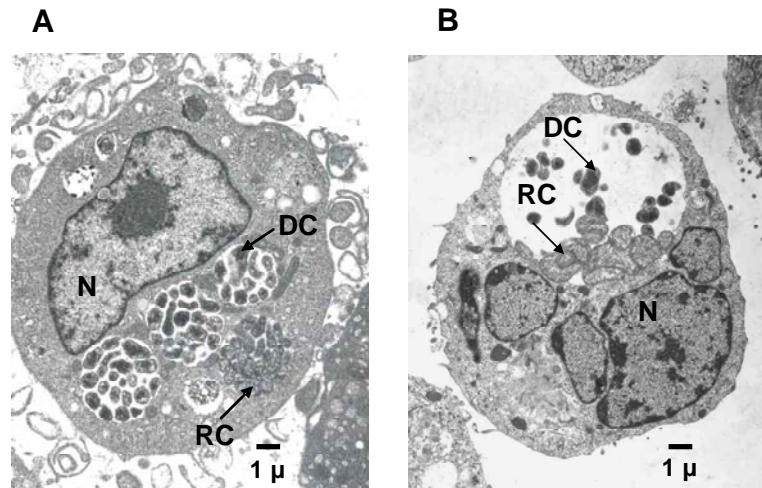
Figure 1.8   Two morphological forms of *E. chaffeensis* (Ganta *et al.,* 2009).

Pathogen persistence

The pathogens of tick-transmitted diseases usually persist in their host for long time and cause severe infection. For example, *Borrelia burgdorferi* causes Lyme diseases in human, dogs and many other animals (CDC, 2010); protozoan parasite *Babesia microti* causes infection of human babesiosis in the USA (CDC, 2010); *Francisella tularensis* causes infection of tularemia in human (CDC, 2010); and *Rickettsia rickettsia* causes Rocky Mountain spotted fever to human (CDC, 2010). For an intracellular bacterium, like *E. chaffeensis*, the critical steps to establish effective infection usually include: attachment, internalization and intracellular multiplication (Rudel *et al.,* 1992; Giron *et al.,* 2002;

Okamoto *et al.,* 2004, Zhang *et al.,* 2004). *E. chaffeensis* lacks the specialized structures to attach and internalize, but it is reported that the 120 kDa surface protein appears to serve as adhesion (Popov *et al.,* 2000). Nonetheless, it is unclear how this pathogen is phagocytosed by the host monocytes or macrophages, and it is also not clear how *E. chaffeensis* can overcome the phagosomal clearance and adapt itself to the host for keeping persistent infection. The mechanism of persistent infection and the reasons for the host immune system failure to clear the infection are not well understood.

One important hypothesis is that differential gene expression may be an important adaptative mechanism used by *E. chaffeensis* in support of its continued survival. Recent studies from our laboratory showed that the protein expression of *E. chaffeensis* from tick and vertebrate host cell environment has significant differences, especially the outer membrane proteins of the p28-Omp multigene family. Proteins of the gene 14 of the p28-Omp locus were found predominantly expressed in tick host, and proteins of gene 19 of the p28-Omp locus were found predominantly expressed in vertebrate host. Much of the fundamental knowledge of the pathogen's persistence and its host specificity can be better understood by performing mutational analysis on the critical genes of the p28-Omp multigene family of *E. chaffeensis*.

Several methods have been documented and proven to be useful in creating effective gene mutations in both gram positive and gram negative bacteria, including in Rickettsial agents. For example, transposon-based mutagenesis has been applied for *Rickettsia* and *Anaplasma* species (Qin *et al.,* 2004; Liu *et al.,* 2007). Homologous recombination based

mutagenesis is extensively used in *Esherichia coli* and many other bacteria (Kowalczykowski *et al.,* 1994; Messerle *et al.,* 1997; Nowosielska *et al.,* 2006). However, the transformation in *E. chaffeensis* is complicated as it is an intracellular obligate pathogen and the host cell free bacteria have shorter life spam (Paddock *et al.,* 2003). Methods like transposon based mutagenesis is usually targeted randomly, thus it is not appropriate to use it as the mutagenesis to knockout specific genes such as the genes of p28-Omp locus of *E. chaffeensis*. Although the homologous recombination based gene knockout system is a method specifically for targeting in one or several gene sites, this gene mutation method is not studied in detail in *E. chaffeensis*.

Creating gene mutation in *E. chaffeensis*

Targetron gene knockout system (mobile group II introns) is a novel gene mutagenesis, which can be programmed to insert into any desired DNA target site with high specificity (Guo *et al.,* 2000; Karberg *et al.,* 2001). Mobile group II introns use a remarkable mobility mechanism mediated by a ribonucleoprotein (RNP) complex which contains the intron-encoded protein (IEP) and the excised intron RNA (Lambowitz *et al,* 1999). Because most of the target specificity comes from base-pairing of the intron RNA to the target DNA sequence, it is possible to reprogram group II introns to insert into desired sites simply by modifying the intron RNA (Guo *et al.,* 1997). This feature combined with their very high insertion frequencies and specificity made it easy to create specific gene mutation on any organism. This method has been utilized successfully for *E.coli, Shigella flexneri, Salmonella typhimurium, Lactococcus lactis, Clostridium perfringens, Staphylococcus aureaus,* and *Francisella tularensis* (Yao *et al.,* 2006; Cui *et al.,* 2007;

John *et al.,* 2007; Rodrigurez *et al.,* 2008). We believe that the Targetron gene knockout system may also be applied to study key genes of *E. chaffeensis* including p28-Omp multilocus genes.

Introduction: Group II intron and Targetrons

Group II intron is a class of intron existing in rRNA, tRNA, mRNA of organelles in fungi, plants, protists, and mRNA in bacteria (Dai *et al.,* 2003). They were first discovered in the organellar mitochondrial and chloroplast genomes of lower eukaryotes and higher plants (Michel *et al.,* 1989). The bacterial group II introns were identified about 17 years ago by PCR screens (Ferat *et al.,* 1993). Then, genome sequencing revealed that group II introns are very common in both gram-negative and gram-positive bacteria (Dai *et al.,* 2003). Later, group II introns have been found in *Methanosarcinales* (Toro *et al.,* 2003; Dai et al., 2003; Rest *et al.,* 2003). Recently, mobile group II introns have been used to develop a new type of gene-targeting method- Targetron Gene Knockout System (Lambowitz *et al.,* 2004).

Structures of Group II Introns

Group II introns generally contain 6 domains (Domain I to Domain VI) (Figure 1.9), in which Domain I, Domain V, and Domain VI are involved the splicing reaction, Domain II and Domain III are involved in stabilization of proper folded structures, and Doman IV is involved in folding or catalytic activity. Domain I is the largest and most complex domain, in which the sequences of exon-binding sites 1 and 2 (EBS1 and EBS2) bind specifically to the sequences of intron-binding sites 1 and 2 (IBS1 and IBS2). Domain V is a small hairpin with a bulge in the stem and it contains a loop which is essential for

interaction with Domain I. It is suggested that Domain V is an essential catalytic component of the active ribozyme, and this domain can induce splicing reaction in special conditions (Qin *et al.,* 1998). Domain VI contains a bulging "A" residue which is a branch point to the 5' exon when splicing. Domain IV contains ORF expressing the intron-encoded protein (IEP) gene, and IEP is known to play an essential role in helping *in vivo* folding of the intron RNA into a catalytic active structure (Belfort *et al.,* 1995; Zimmerly *et al.,* 1995). The IEP includes four conserved domains: a N-terminal RT domain, a domain termed 'X' which is a putative RNA-binding domain associated with RNA splicing activity, a C-terminal DNA- binding domain, and a DNA- endonuclease domain. It is believed that the IEP is translated from a 'pre-mRNA' and later processed to the mature protein (Anziano *et al.* 1982; Carignani *et al.* 1983).
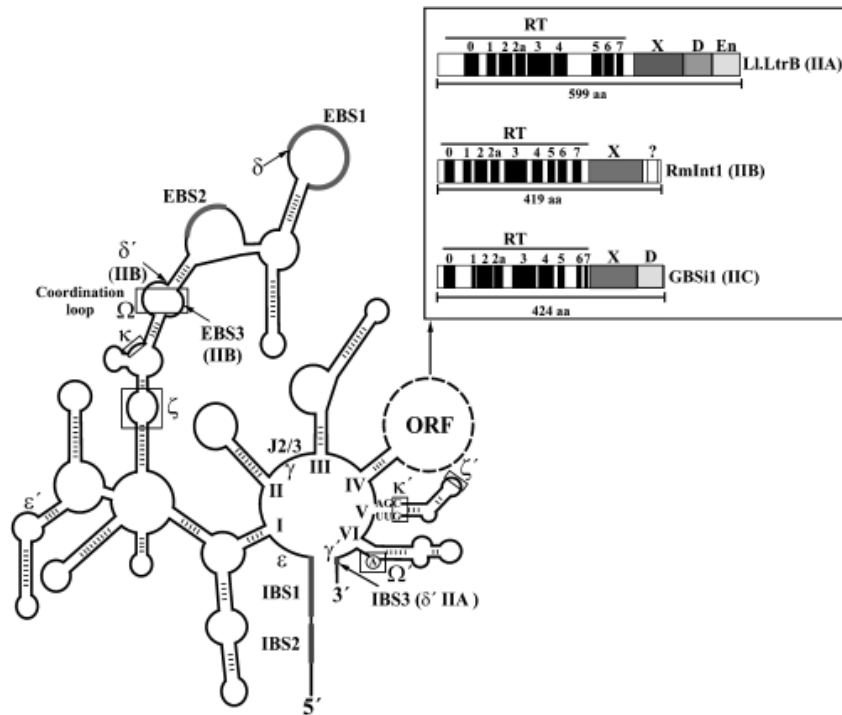

Figure 1.9 Generic secondary structure of group II intron RNA and domain structure of the intron-encoded proteins (IEP) (Zhong *et al.*, 2003).

Retrohoming Mechanism

The mobility of group II introns is first demonstrated for the *S. cerevisiae coxI*-I1 introns (Meunier *et al.,* 1990) and the related *Kluyveromyces lactis coxI*-I1 introns (Skelly *et al.,* 1991). Then, it was reported that an efficient *E.coli* expression system for *Lactococcus lactis* Ll.LtrB intron has been constructed and group II introns could function as mobile genetic elements by inserting directly into target sites in double stranded DNA (Matsuura *et al.,* 1997). This mobility is mediated by the multifunctional IEP, which shows reverse transcriptases (RTs), maturase, and DNA endonuclease activities (En) activities.

Retrohoming occurs through a target DNA-primed reverse transcription mechanism (Figure 1.10) which involves several sequential reactions. First, RNP initiates mobility by recognizing a specific double-stranded DNA target site, with both the IEP and base pairing of the intron RNA contributing to DNA target site recognition. Then, the IEP interacts with a few bases in the distal 5' exon region via major groove and phosphate-backbone interaction, and triggers local DNA unwinding in the same time to facilitate the intron RNA base pairing with the adjacent 14–16 nt local DNA sequence. These base pairing interactions involve three short sequence elements in the DNA target sites: IBS2 and IBS1 and δ′, and they are recognized by three complementary sequence elements: EBS1 and EBS2 and δ in the intron RNA. After binding, the RNA component of the RNP cleaves the sense-strand precisely at the exon junction in the double-stranded DNA, and IEP uses its endonuclease domain (En) to cleave the antisense-strand at position +9 relative to the insertion site. The 3' end of the cleaved antisense-strand is then targeted as

a primer for reverse transcription, generating a cDNA strand that is subsequently incorporated into the recipient DNA primarily by recombination mechanisms (Lambowitz *et al.,* 2004). This mechanism can be used to develop the novel gene mutagenesis- Targetron Gene Knockout System.
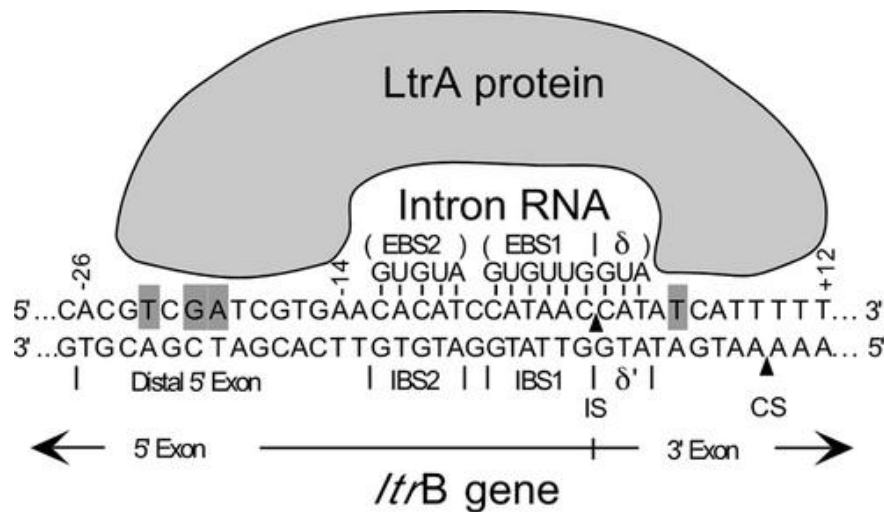
Figure 1.10 Mechanism of DNA target site recognition by the *Ll.LtrB* group II intron (Zhong *et al.*, 2003).

Applications of Targetron Gene Knockout System

Targetron Gene Knockout System is the commercial name of the technology, and it could be used to insert into any desired target DNA and knockout a number of genes. Due to its high efficiency and high specificity, Targetron Gene Knockout System has been successfully applied into gene mutation or modification in plentiful of bacterial species. Also, this technology significantly reduced the time needed for disrupting a gene, compared with conventional homologous recombination methods. For example, *Staphylococcus aureus*, which acquired antibiotic resistance, is a serious and increasing

clinical disease (CDC, 2001; CDC, 2005). In *Staphylococcus aureus, hsa* gene encodes a member of the HU family of bacterial histone-like proteins and *seb* encodes eterotoxin B (Yao *et al.,* 2006). Creating site-specific mutations in this gram-positive bacterium targeting at the *hsa* and *seb* genes by using conventional homologous recombination is rather difficult. However, the *hsa* and *seb* genes were disrupted efficiently with Targetron Gene Knockout System (Yao *et al.,* 2006). In higher organisms, group II introns were designed and selected to insert into HIV1 provirus and the human gene encoding CCR5, an important target site in anti-HIV therapy (Guo *et al.,* 1997). The retargeted intron RNPs retained activity in human cells, inserting into plasmid-borne HIV1 and CCR5 target sites after liposome-mediated transfection (Guo *et al.,* 1997).

Reference:

Alan M. Lambowitz & Steven Zimmerly. 2004. Mobile group II introns. Annu. *Rev. Genet*. **38:**1–35

Alciati, S., E. Belligni, C. S. Del, and A. Pugliese. 2001. Human infections tick-transmitted. Panminerva. Med. **43**:295-304.

Anziano PQ, Hanson DK, Mahler HR & Perlman PS. 1982. Functional domains in introns. Trans-acting and cis-acting regions of intron II of the cob gene. *Cell.* **30:** 925–932

Anderson, B. E., J. E. Dawson, D. C. Jones, and K. H. Wilson. 1991. *Ehrlichia* chaffeensis, a new species associated with human ehrlichiosis. J. Clin. Microbiol. **29**:2838-2842.

Anderson, B. E., K. G. Sims, J. G. Olson, J. E. Childs, J. F. Piesman, C. M. Happ, G. O. Maupin, and B. J. Johnson. 1993. Amblyomma americanum: a potential vector of human ehrlichiosis. Am. J. Trop. Med. Hyg. **49**:239-244.

A.W. Mukhebi, T. Chamboko, C.J. O'Callaghan, T.F. Peter, R.L. Kruska, G.F. Medley, S.M. Mahan and B.D. Perry. 1999. An assessment of the economic impact of heartwater (Cowdria ruminantium infection) and it's control in Zimbabwe, Prev. Vet. Med. **39:** 173–189.

Ashley, C. R., S. J. Richmond, and E. O. Caul. 1975. Identification of the elementary bodies of Chlamydia trachomatis in the electron microscope by an indirect immunoferritin technique. J. Clin. Microbiol. **2**:327-331.

Azad AF, Traub R. 1989. Experimental transmission of murine typhus by Xenopsylla cheopis flea bites. Med Vet Entomol. **3:**429–33.

Azad, A. F. and C. B. Beard. 1998. Rickettsial pathogens and their arthropod vectors. Emerg. Infect. Dis. **4**:179-186.

Bakken, J. S., J. S. Dumler, S. M. Chen, M. R. Eckman, L. L. Van Etta, and D. H. Walker. 1994. Human granulocytic ehrlichiosis in the upper Midwest United States. A new species emerging. JAMA **272:**212–218.

Bakken, J. S., J. Krueth, C. Wilson-Nordskog, R. L. Tilden, k. Asanovich, and J. S. Dumler. 1996. Clinical and laboratory characteristics of human granulocytic ehrlichiosis. JAMA **275**:199-205.

Bakken, J. S., P. Goellner, M. V. Etten, D. Z. Boyle, O. L. Swonger, S. Mattson, J. Krueth, R. L. Tilden, k. Asanovich, J. Walls, and J. S. Dumler. 1998. Seroprevalence of human granulocytic ehrlichiosis among permanent residents of northwestern Wisconsin. Clin. Infect. Dis. **27**:1491-1496.

Bandi, C., T. J. Anderson, C. Genchi, and M. L. Blaxter. 1998. Phylogeny of Wolbachia in filarial nematodes. Proc. Biol. Sci. **265**:2407-2413.

Barnewall, R. E. and Y. Rikihisa. 1994. Abrogation of gamma interferon-induced inhibition of Ehrlichia chaffeensis infection in human monocytes with iron-transferrin. Infect. Immun. **62**:4804-4810.

Belfort M & Perlman PS. 1995. Mechanisms of Intron Mobility. *J. Biol*. Chem. **270:**30237–30240

Bekker, C. P., L. Bell-Sakyi, E. A. Paxton, D. Martinez, A. Bensaid, and F. Jongejan. 2002. Transcriptional analysis of the major antigenic protein 1 multigene family of *Cowdria ruminantium*. Gene. **285**:193-201.

Bernstein, H. D. 2000. The biogenesis and assembly of bacterial membrane proteins. Curr. Opin. Microbiol. **3**:203-209.

Bitsaktsis, C., J. Huntington, and G. Winslow. 2004. Production of IFN-gamma by CD4 T cells is essential for resolving ehrlichia infection. J. Immunol. **172**:6894-6901.

Bitsaktsis, C., B. Nandi, R. Racine, K. C. MacNamara, and G. Winslow. 2007. T-Cell-independent humoral immunity is sufficient for protection against fatal intracellular ehrlichia infection. Infect. Immun. **75**:4933-4941.

Bitsaktsis, C. and G. Winslow. 2006. Fatal Recall Responses Mediated by CD8 T cells during Intracellular Bacterial Challenge Infection. J. Immunol. **177**:4644-4651.

Brayton K A, Fehrsen J, de Villiers E P, van Kleef M, Allsopp B A. 1997. Construction and initial analysis of a representative lambda ZAPII expression library of the intracellular rickettsia Cowdria ruminantium: cloning of map1 and three other Cowdria genes. Vet Parasitol**, 72:**185–199.

Breitschwerdt, E. B., B. C. Hegarty, and S. I. Hancock. 1998. Sequential evaluation of dogs naturally infected with Ehrlichia canis, Ehrlichia chaffeensis, Ehrlichia equi, Ehrlichia ewingii, or Bartonella vinsonii. J. Clin. Microbiol. **36:**2645-2651

Brown, J. L., D. L. Huxsoll, M. Ristic, and P. K. Hildebrandt. 1972. *i*n vitro cultivation of Neorickettsia helminthoeca, the causative agent of salmon poisoning disease. Am. J. Vet. Res **33**:1695-1700.

Buller R. S, Arens M, Hmiel SP, Paddock CD, Sumner JW, Rikhisa Y, et al. 1999. *Ehrlichia ewingii*, a newly recognized agent of human ehrlichiosis. *N Engl J Med*. **341:**148–55 doi: 10.1056/NEJM199907153410303.

Buller, R. S., M. Arens, S. P. Hmiel, C. D. Paddock, J. W. Sumner, Y. Rikihisa, A. Unver, M. Gaudreault-Keener, F. A. Manian, A. M. Liddell, N. Schmulewitz, and G. A. Storch. 1999. Ehrlichia ewingii, a newly recognized agent of human ehrlichiosis. N. Engl. J. Med. **341:**148-155

Buller, R. S., M. Arens, S. P. Hmiel, C. D. Paddock, J. W. Sumner, Y. Rikihisa, A. Unver, M. Gaudreault-Keener,

Buller, R. S., M. Arens, S. P. Hmiel, C. D. Paddock, J. W. Sumner, Y. Rikihisa, Unver, M. Gaudreault-Keener, F. A. Manian, A. M. Liddell, N. Schmulewitz, and G. A. Storch. 1999. Ehrlichia ewingii, a newly recognized agent of human ehrlichiosis. N. Engl. J. Med. **341:**148–155.

Burkot TR, Maupin GO, Schneider BS, Denatale C, Happ CM, Rutherford JS.2001. Use of a sentinel host system to study the questing behavior of Ixodes spinipalpis and its role in the transmission of Borrelia bissettii, human granulocytic ehrlichiosis, and Babesia microti. Am J Trop Med Hyg. **65:**293–9.

Caldwell, C. W., E. D. Everett, G. McDonald, Y. W. Yesus, W. E. Roland, and H. M. Huang. 1996. Apoptosis of gamma/delta T cells in human ehrlichiosis. Am. J. Clin. Pathol. **105**:640-646.

Carignani G, Groudinsky O, Frezza D, Schiavon E, Bergantino E & Slonimski PP. 1983. An mRNA maturase is encoded by the first intron of the mitochondrial gene for subunit I of cytochrome oxidase in S. cerevisiae. *Cell*. **35:** 733–745.

Chapman, A. S., J. S. Bakken, S. M. Folk, C. D. Paddock, K. C. Bloch, A. Krusell, D. J. Sexton, S. C. Buckingham, G. S. Marshall, G. A. Storch, G. A. Dasch, J. H. McQuiston, D. L. Swerdlow, S. J. Dumler, W. L. Nicholson, D. H. Walker, M. E. Eremeeva, and C. A. Ohl. 2006. Diagnosis and management of tickborne rickettsial diseases: Rocky Mountain spotted fever, ehrlichioses, and anaplasmosis--United States: a practical guide for physicians and other health-care and public health professionals. MMWR Recomm. Rep. **55**:1-27.

Chapes, S. K. and Ganta R.R. 2008. Defining the Immune Response to *Ehrlichia species* Using Murine Models. Vet. Parasitol. **158**:344-59.

Chapes, S. K., S. M. Hoynowski, K. M. Woods, J. W. Armstrong, A. A. Beharka, and J. J. Iandolo. 1993. Staphylococcus-mediated T-cell activation and spontaneous natural killer cell activity in the absence of major histocompatibility complex class II molecules. Infect. Immun. **61**:4013-4016.

Chen S-M, Dumler JS, Bakken JS, Walker DH. 1994. Identification of a granulocytotropic Ehrlichia species as the etiologic agent of human disease. J Clin Microbiol. **32:**589–95.

Cheng, C., C. D. Paddock, and G. R. Reddy. 2003. Molecular Heterogeneity of Ehrlichia chaffeensis Isolates Determined by Sequence Analysis of the 28-Kilodalton Outer Membrane Protein Genes and Other Regions of the Genome. Infect. Immun. **71**:187-195.

Childs, J. E., J. W. Sumner, W. L. Nicholson, R. F. Massung, S. M. Standaert, and C. D. . 1999. Outcome of diagnostic tests using samples from patients with culture-proven human monocytic ehrlichiosis: implications for surveillance. J. Clin. Microbiol. **37:**2997-3000

Chima et al., R.I. Chima, C. Goddman and A. Mills. 2003.The economic impact of malaria in Africa: a critical review of the evidence, Health Policy. **63:** 17–36.

Chomel BB, Kasten RW, Floyd-Hawkins K, Chi B, Yamamoto K, Roberts-Wilson J, et al. 1996. Experimental transmission of Bartonella henselae by the cat flea. J Clin Microbiol. **34:**1952–6.

Collins, F.H., and Paskewitz, S.M. 1995. Malaria: current and future prospects for control. Annu. Rev. Entomol. **40:** 195–219.

Colwell RRR and Spira WH. 1992. The ecology of vibrio cholerae, p.107-127. *In* D. Barua, W. B. Greenough, III (ed.), Cholera. Plenum Medical Book Co., New York, NY.

Cowley, S. C. and K. L. Elkins. 2003. Multiple T cell subsets control Francisella tularensis LVS intracellular growth without stimulation through macrophage interferon gamma receptors. J. Exp. Med. **198**:379-389.

Cowley, S. C., E. Hamilton, J. A. Frelinger, J. Su, J. Forman, and K. L. Elkins. 2005. CD4-CD8- T cells control intracellular bacterial infections both in vitro and in vivo. J. Exp. Med. **202**:309-319.

Crocquet-Valdes, P. A., J. W. McBride, H. M. Feng, N. Ismail, M. A. Small, X. J. Yu, and D. H. Walker. 2005. Analysis of ehrlichial p28 gene expression in a murine model of persistent infection. Ann. N. Y. Acad. Sci. **1063**:420-424.

Cui X, Davis G. 2007. Mobile group II intron targeting: applications in prokaryotes and perspectives in eukaryotes. Front Biosci. **12:**4972–4985.

Dai L, Zimmerly S. 2003.ORF-less and reverse-transcriptase-encoding group II introns in archaebacteria, with a pattern of homing into related group II introns ORFs. RNA **9:**14-19

Davidson, W. R., J. M. Lockhart, D. E. Stallknecht, E. W. Howerth, J. E. Dawson, and Y. Rechav. 2001. Persistent *Ehrlichia chaffeensis* infection in white-tailed deer. J Wildl Dis. **37**:538-546.

Dawson, J. E., B. E. Anderson, D. Fishbein, J. Sanchez, C. Goldsmith, K. Wilson, and C. Duntly. 1991. Isolation and characterization of an Ehrlichia sp. from a patient with human ehrlichiosis. J. Clin. Microbiol. **29:**2741–2745

Dawson J.E., Childs JE, Biggie KL, et al. 1994. White-tailed deer as a potential reservoir of *Ehrlichia* spp. *J. Wildl. Dis.* **30:** 162–68

De, S. R., S. D. Nobrega, F. Bacellar, and J. Torgal. 2003. Mediterranean spotted fever in Portugal: risk factors for fatal outcome in 105 hospitalized patients. Ann. N. Y. Acad. Sci. **990**:285-294.

Demma, L. J., R. C. Holman, J. H. McQuiston, J. W. Krebs, and D. L. Swerdlow. 2006. Human monocytic ehrlichiosis and human granulocytic anaplasmosis in the United States, 2001-2002. Ann. N. Y. Acad. Sci. **1078**:118-119.

Dinarello, C. A. 2000. Proinflammatory cytokines. Chest. **118**:503-508.

Doyle, C. K., K. A. Nethery, V. L. Popov, and J. W. McBride. 2006. Differentially expressed and secreted major immunoreactive protein orthologs of *Ehrlichia canis* and *E. chaffeensis* elicit early antibody responses to epitopes on glycosylated tandem repeats. Infect. Immun. **74:**711-720

Dugan, V. G., S. E. Little, D. E. Stallknecht, and A. D. Beall. 2000. Natural infection of domestic goats with Ehrlichia chaffeensis. J. Clin. Microbiol. **38**:448-449.

Dugan, V. G., J. K. Gaydos, D. E. Stallknecht, S. E. Little, A. D. Beall, D. G. Mead, C. C. Hurd, and W. R. Davidson. 2005. Detection of Ehrlichia spp. in raccoons (Procyon lotor) from Georgia. Vector. Borne. Zoonotic. Dis. **5**:162-171.

Dumler J. S., A. F. Barbet, C. P. Bekker, G. A. Dasch, G. H. Palmer, S. C. Ray, Y. Rikihisa, and F. R. Rurangirwa. 2001. Reorganization of genera in the families Rickettsiaceae and Anaplasmataceae in the order Rickettsiales: unification of some species of Ehrlichia with Anaplasma, Cowdria with Ehrlichia and Ehrlichia with Neorickettsia, descriptions of six new species combinations and designation of Ehrlichia equi and 'HGE agent' as subjective synonyms of Ehrlichia phagocytophila. Int. J. Syst. Evol. Microbiol. **51:**2145–2165.

Dumler, J. S. and J. S. Bakken. 1998. Human ehrlichioses: newly recognized infections transmitted by ticks. Annu. Rev. Med. **49**:201-213.

Dumler J.S., Choi KS, Garcia-Garcia JC, et al. 2005. "Human granulocytic anaplasmosis and Anaplasma phagocytophilum". Emerging infectious diseases **11:** 1828–34.

Dumler, J. S., J. E. Madigan, N. Pusterla, and J. S. Bakken. 2007. Ehrlichioses in humans: epidemiology, clinical presentation, diagnosis, and treatment. Clin. Infect. Dis. **45 Suppl 1**:S45-S51

Duncan Brown. 1997. Dynamic and impact of tick-borne diseases of cattle. Trop. Anim. Health Prod. **29:** 15–35.

Dzik A. J. 1983. Snails, schistosomiasis, and irrigation in the tropics. Public Health. **97:**214-7.

E. Maes, P. Lecomte and N. Ray. 1998. A cost-of-illness study of Lyme disease in the United States, Clin. Ther.. **20:** 993-1008

F. A. Manian, A. M. Liddell, N. Schmulewitz, and G. A. Storch. 1999. Ehrlichia ewingii, a newly recognized agent of human ehrlichiosis. N. Engl. J. Med. **341:**148-155

Fishbein, D. B. 1990. Human ehrlichiosis in the United States, p. 100–111. In J. C. Williams and I. Kakoma (ed.), Ehrlichiosis. Kluwer Academic Publishers, Amsterdam, The Netherlands.

Felek, S., R. Greene, and Y. Rikihisa. 2003. Transcriptional analysis of p30 major outer membrane protein genes of Ehrlichia canis in naturally infected ticks and sequence analysis of p30-10 of E canis from diverse geographic regions. J. Clin. Microbiol. **41**:886-888.

Ferat JL, Le Gouar M & Michel F. 2003. A group II intron has invaded the genus Azotobacter and inserted within the termination codon of the essential groEL gene. Mol Microbiol. **49:** 1407–1423.

Fournier, P. E., J. P. Durand, J. M. Rolain, J. L. Camicas, H. Tolou, and D. Raoult. 2003. Detection of Astrakhan fever rickettsia from ticks in Kosovo. Ann. N. Y. Acad. Sci. **990**:158-161.

Fukuda T, Yamamoto S. 1981. Neorickettsia-like organism isolated from metacercaria of a fluke, Stellantchasmus falcatus. Jpn J Med Sci Biol. **34:**103-7.

Ganta, R. R., M. J. Wilkerson, C. Cheng, A. M. Rokey, and S. K. Chapes. 2002. Persistent Ehrlichia chaffeensis infection occurs in the absence of functional major histocompatibility complex class II genes. Infect. Immun. **70**:380-388.

Ganta, R. R., C. Cheng, M. J. Wilkerson, and S. K. Chapes. 2004. Delayed clearance of Ehrlichia chaffeensis infection in CD4+ T-cell knockout mice. Infect. Immun. **72**:159-167.

Ganta, R. R., C. Cheng, E. C. Miller, B. L. McGuire, L. Peddireddi, K. R. Sirigireddy, and S. K. Chapes. 2007. Differential clearance and immune responses to tick cell-derived versus macrophage culture-derived Ehrlichia chaffeensis in mice. Infect. Immun. **75**:135-145.

Gardner, S. L., R. C. Holman, J. W. Krebs, R. Berkelman, and J. E. Childs. 2003. National surveillance for the human ehrlichioses in the United States, 1997-2001, and proposed methods for evaluation of data quality. Ann. N. Y. Acad. Sci. **990:**80-9.

Ge, Y. and Y. Rikihisa. 2007. Surface-exposed proteins of Ehrlichia chaffeensis. Infect. Immun. **75**:3833-3841.

Giron, J. A., A. G. Torres, E. Freer, and J. B. Kaper. 2002. The flagella of enteropathogenic Escherichia coli mediate adherence to epithelial cells. Mol. Microbiol. **44**:361-379

Gordon, J. R., B. G. McLaughlin, and S. Nitiuthai. 1983. Tularaemia transmitted by ticks (Dermacentor andersoni) in Saskatchewan. Can. J. Comp Med. **47**:408-411.

Graves, S. R., L. Stewart, J. Stenos, R. S. Stewart, E. Schmidt, S. Hudson, J. Banks, Z. Huang, and B. Dwyer. 1993. Spotted fever group rickettsial infection in south-eastern Australia: isolation of rickettsiae. Comp Immunol. Microbiol. Infect. Dis. **16**:223-233.

Gross L. 1995. How the plague bacillus and its transmission through fleas were discovered: reminiscences from my years at the Pasteur Institute in Paris. PNAS. **92:**7609–7611.

Gubler DJ. 1998. Resurgent vector-borne diseases as a global health problem. **4:**442-50

Guo, H., Zimmerly, S., Perlman, P.S. & Lambowitz, A.M. 1997. Group II intron endonucleases use both RNA and protein subunits for recognition of specific sequences in double-stranded DNA. *EMBO J.* **16**, 6835–6848.

Guo,H., Karberg,M., Long,M., Jones,J.P.,3rd, Sullenger,B. and Lambowitz,A.M. 2000. Group II introns designed to insert into therapeutically relevant DNA target sites in human cells. *Science*, **289**, 452–457.

Gusa, A. A., R. S. Buller, G. A. Storch, M. M. Huycke, L. J. Machado, L. N. Slater, S. L. Stockham, and R. F. Massung. 2001. Identification of a p28 gene in Ehrlichia ewingii: evaluation of gene for use as a target for a species-specific PCR diagnostic assay. J. Clin. Microbiol. **39**:3871-3876.

Hamburg BJ, Storch GA, Micek ST, Kollef MH. 2008. "The importance of early treatment with doxycycline in human ehrlichiosis". Medicine **87:** 53–60

Hechemy, K. E., T. vsic-Zupanc, J. E. Childs, and D. A. Raoult. 2003. Rickettsiology: present and future directions: preface. Ann. N. Y. Acad. Sci. **990:xvii-xx.**:xvii-xvxx.

Hotopp, J. C., M. Lin, R. Madupu, J. Crabtree, S. V. Angiuoli, J. Eisen, R. Seshadri, Q. Ren, M. Wu, T. R. Utterback, S. Smith, M. Lewis, H. Khouri, C. Zhang, H. Niu, Q. Lin, N. Ohashi, N. Zhi, W. Nelson, L. M. Brinkac, R. J. Dodson, M. J. Rosovitz, J. Sundaram, S. C. Daugherty, T. Davidsen, A. S. Durkin, M. Gwinn, D. H. Haft, J. D. Selengut, S. A. Sullivan, N. Zafar, L. Zhou, F. Benahmed, H. Forberger, R. Halpin, S. Mulligan, J. Robinson, O. White, Y. Rikihisa, and H. Tettelin. 2006. Comparative genomics of emerging human ehrlichiosis agents. PLoS. Genet. **2**:e21.

Hodinka, R. L., C. H. Davis, J. Choong, and P. B. Wyrick. 1988. ultrastructural study of endocytosis of Chlamydia trachomatis by McCoy cells. Infect Immun. **56**:1456-1463.

Ismail, N., E. C. Crossley, H. L. Stevenson, and D. H. Walker. 2007. Relative importance of T-cell subsets in monocytotropic ehrlichiosis: a novel effector mechanism involved in Ehrlichia-induced immunopathology in murine ehrlichiosis. Infect. Immun. **75**:4608-4620.

Jeyaprakash, A. and M. A. Hoy. 2000. Long PCR improves Wolbachia DNA amplification: wsp sequences found in 76% of sixty-three arthropod species. Insect Mol. Biol. **9**:393-405.

Jenkins, D. E., J. E. Schultz, and A. Matin. 1988. Starvation-induced cross protection against heat or H2O2 challenge in Escherichia coli. J. Bacteriol. **170**:3910-3914.

John T. Heap, Oliver J. Pennington, Stephen T. Cartman, Glen P. Carter and Nigel P. Minton. 2007. The ClosTron: A universal gene knock-out system for the genus Clostridium. *Journal of Microbiological Methods*, **70:** 452-464.

Jui-shan Ma. 2005. Janpanese Spotted Fever in Korea. Journal of clinical microbiology.4306-4307. Doi:10.1128/JCM.43.8.4306-4307.2005

Jun Yao, Jin Zhong, Yuan Fang, Edward Geisinger, Richard P. Novick and Alan M. Lambowitz. 2006. Use of targetrons to disrupt essential and nonessential genes in *Staphylococcus aureus* reveals temperature sensitivity of Ll.LtrB group II intron splicing. RNA **12:**1271-1281.

Karberg,M., Guo,H., Zhong,J., Coon,R., Perutka,J. and Lambowitz,A.M. 2001. Group II introns as controllable gene targeting vectors for genetic manipulation of bacteria. *Nature Biotechnol.*, **19**, 1162–1167

Keirans, J. E. C. M. C. H. H. a. E. R. E. 1976. Discovery of Nuttalliella namaqua Bedford (Acarina: Ixodoidea: Nuttalliellidae) in Tanzania and re-description of the female based on scanning electron microscopy. Ann. Entomol. Soc. Am. **69**:926-932.

Lambowitz, A.M., Caprara, M.G., Zimmerly, S. & Perlman, P.S. 1999. Group I and group II ribozymes as RNPs: clues to the past and guides to the future. In *The RNA world*, Edn. 2. (eds Gesteland, R.F., Cech, T.R. & Atkins, J.F.) 451–484(Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY).

Lee, E. H. and Y. Rikihisa. 1996. Absence of tumor necrosis factor alpha, interleukin-6 (IL-6), and granulocyte-macrophage colony-stimulating factor expression but presence of IL-1beta, IL-8, and IL-10 expression in human monocytes exposed to viable or killed Ehrlichia chaffeensis. Infect. Immun. **64**:4211-4219.

Lee, K. N., I. Padmalayam, B. Baumstark, S. L. Baker, and R. F. Massung. 2003. Characterization of the ftsZ gene from Ehrlichia chaffeensis, Anaplasma phagocytophilum, and Rickettsia rickettsii, and use as a differential PCR target. DNA Cell Biol. **22**:179-186.

Li, J. S., F. Chu, A. Reilly, and G. M. Winslow. 2002. Antibodies highly effective in SCID mice during infection by the intracellular bacterium Ehrlichia chaffeensis are of picomolar affinity and exhibit preferential epitope and isotype utilization. J. Immunol. **169**:1419-1425.

Li, J. S., E. Yager, A. Reilly, C. Freeman, G. R. Reddy, Reilly A.A., F. K. Chu, and G. M. Winslow. 2001. Outer membrane protein specific monoclonal antibodies protect SCID mice from fatal infection by the obligate intracellular bacterial pathogen *Ehrlichia chaffeensis*. J Immunol. **166**:1855.

Liew, F. Y. and I. B. McInnes. 2002. Role of interleukin 15 and interleukin 18 in inflammatory response. Ann. Rheum. Dis. **61 Suppl 2**:ii100-ii102.

Lindquist, S. and E. A. Craig. 1988. The heat-shock proteins. Annu Rev Genet. **22**:631-677.

Lockhart JM, Davidson WR, Dawson JE, Stallknecht DE. 1995. Temporal association of *Amblyomma americanum* with the presence of *Ehrlichia chaffeensis* reactive antibodies in white-tailed deer. *J. Wildl. Dis.* **31:**119–24

Lockhart, J. M., W. R. Davidson, D. E. Stallknecht, J. E. Dawson, and E. W. Howerth. 1997. Isolation of Ehrlichia chaffeensis from wild white-tailed deer (Odocoileus virginianus) confirms their role as natural reservoir hosts. J. Clin. Microbiol. **35**:1681-1686.

Long, S. W., X. Zhang, J. Zhang, R. P. Ruble, P. Teel, and X. J. Yu. 2003. Evaluation of transovarial transmission and transmissibility of Ehrlichia chaffeensis (Rickettsiales: Anaplasmataceae) in Amblyomma americanum (Acari: Ixodidae). J. Med. Entomol. **40**:1000-1004.

Long, S. W., X. F. Zhang, H. Qi, S. Standaert, D. H. Walker, and X. J. Yu. 2002. Antigenic variation of Ehrlichia chaffeensis resulting from differential expression of the 28-kilodalton protein gene family. Infect. Immun. **70**:1824-1831.

Luo, T., X. Zhang, A. Wakeel, V. L. Popov, and J. W. McBride. 2008. A variable-length PCR target protein of *Ehrlichia chaffeensis* contains major species-specific antibody epitopes in acidic serine-rich tandem repeats. Infect. Immun. **76:**1572-1580.

Lv S, Zhang Y, Liu H-X, Hu L, Yang K, et al. 2009. Invasive Snails and an Emerging Infectious Disease: Results from the First National Survey on Angiostrongylus cantonensis in China. PLoS Negl Trop Dis **3(2):** e368. doi:10.1371/journal.pntd.0000368

Madigan, J. E., N. Pusterla, E. Johnson, J. S. Chae, J. B. Pusterla, E. DeRock, and S. P. Lawler. 2000. Transmission of *Ehrlichia* risticii, the agent of Potomac horse fever, using naturally infected aquatic insects and helminth vectors: preliminary report. Equine Vet. J. **32**:275-279.

Maeda, K., N. Markowitz, R. C. Hawley, M. Ristic, D. Cox, and J. E. McDade. 1987. Human infection with Ehrlichia canis, a leukocytic rickettsia. N. Engl. J. Med. **316:**853–856.

Manabu Araki, Katsuya Takatsuka,Junichiro Kawamura, and Yuko Kanno. 2002. Japanese Spotted Fever Involving the Central Nervous System: Two Case Reports and a Literature Review. 40.10:3874-3876. DOI: 10.1128/JCM.40.10.3874-3876.2002

Mark W. Eshoo, Chris D. Crowder, Haijing Li, Heather E. Matthews, Shufang Meng, Susan E. Sefers, Rangarajan Sampath, Charles W. Stratton, Lawrence B. Blyn, David J. Ecker, and Yi-Wei Tang. 2010. Detection and Identification of Ehrlichia Species in Blood by Use of PCR and Electrospray Ionization Mass Spectrometry. Journal of Clinical Microbiology. **48:** 472-478. doi:10.1128/JCM.01669-09.

Marshall, G. S., R. F. Jacobs, G. E. Schutze, H. Paxton, S. C. Buckingham, J. P. DeVincenzo, M. A. Jackson, J. San, V, S. M. Standaert, and C. R. Woods. 2002. Ehrlichia chaffeensis seroprevalence among children in the southeast and south-central regions of the United States. Arch. Pediatr. Adolesc. Med. **156**:166-170.

Masters, E. J., G. S. Olson, S. J. Weiner, and C. D. Paddock. 2003. Rocky Mountain spotted fever: a clinician's dilemma. Arch. Intern. Med. **163**:769-774.

Mattner, J., K. L. Debord, N. Ismail, R. D. Goff, C. Cantu, III, D. Zhou, P. Saint-Mezard, V. Wang, Y. Gao, N. Yin, K. Hoebe, O. Schneewind, D. Walker, B. Beutler, L. Teyton, P. B. Savage, and A. Bendelac. 2005. Exogenous and endogenous glycolipid antigens activate NKT cells during microbial infections. Nature. **434**:525-529.

McBride, J. W., X. J. Yu, and D. H. Walker. 2000. Glycosylation of homologous immunodominant proteins of Ehrlichia chaffeensis and Ehrlichia canis. Infect. Immun**. 68:**13-18.

McBride, J. W., L. M. Ndip, V. L. Popov, and D. H. Walker. 2002. Identification and functional analysis of an immunoreactive DsbA-like thio-disulfide oxidoreductase of Ehrlichia spp. Infect. Immun. **70**:2700-2703.

McNabb, S. J., R. A. Jajosky, P. A. Hall-Baker, D. A. Adams, P. Sharp, C. Worshams, W. J. Anderson, A. J. Javier, G. J. Jones, D. A. Nitschke, A. Rey, and M. S. Wodajo. 2008. Summary of notifiable diseases--United States, 2006. MMWR Morb. Mortal. Wkly. Rep. **55**:1-92.

Matsuura M, Saldanha R, Ma H, Wank H, Yang J, et al. 1997. A bacterial group II intron encoding reverse transcriptase, maturase, and DNA endonuclease activities: biochemical demonstration of maturase activity and insertion of newgenetic information within the intron. Genes Dev. 11:2910–24

Meunier B, Tian G-L, Macadre C, Slonimski PP, Lazowska J. 1990. Group II introns transpose in yeast mitochondria. In *Structure Function and Biogenesis of Energy Transfer Systems*, ed. E Quagliariello, S Papa, F Palmieri, C Saccone, pp. 169–74. Amsterdam: Elsevier

Michel F, Umesomo K & Ozeki H. 1989. Comparative and functional anatomy of group II catalytic introns – a review. *Gene* **82:** 5–30.

Mitzel, D. N., J. B. Wolfinbarger, R. D. Long, M. Masnick, S. M. Best, and M. E. Bloom. 2007. Tick-borne flavivirus infection in *i*xodes scapularis larvae: development of a novel method for synchronous viral infection of ticks. Virology **365**:410-418.

Miura, K. and Y. Rikihisa. 2007. Virulence potential of Ehrlichia chaffeensis strains of distinct genome sequences. Infect Immun. **75**:3604-3613.

Murphy GL, Ewing SA, Whitworth LC, Fox JC, Kocan AA . 1998. A molecular and serologic survey of *Ehrlichia canis, E. chaffeensis*, and *E. ewingii* in dogs and ticks from Oklahoma. *Vet Parasitol*. **79:**325–39 doi: 10.1016/S0304-4017(98)00179-4

Nalin DR. Daya V, Reid A, et al. 1979. Adsorption and growth of Vibrio cholerae on chitin. Infect Immun. **25:**768-770.

Nandi, B., K. Hogle, N. Vitko, and G. M. Winslow. 2007. CD4 T-cell epitopes associated with protective immunity induced following vaccination of mice with an ehrlichial variable outer membrane protein. Infect. Immun. **75**:5453-5459.

Ohashi N, Unver A, Zhi N, Rikihisa Y. 1998. Cloning and characterization of multigenes encoding the immunodominant 30-kilodalton major outer membrane proteins of Ehrlichia canis and application of the recombinant protein for serodiagnosis. J Clin Microbiol. **36:**2671–2680.

Ohashi, N., N. Zhi, Q. Lin, and Y. Rikihisa. 2002. Characterization and transcriptional analysis of gene clusters for a type IV secretion machinery in human granulocytic and monocytic ehrlichiosis agents. Infect. Immun. **70**:2128-2138.

Ohashi, N., Y. Rikihisa, and A. Unver. 2001. Analysis of Transcriptionally Active Gene Clusters of Major Outer Membrane Protein Multigene Family in Ehrlichia canis and *E. chaffeensis*. Infect. Immun. **69**:2083-2091.

Olano, J. P., W. Hogrefe, B. Seaton, and D. H. Walker. 2003. Clinical manifestations, epidemiology, and laboratory diagnosis of human monocytotropic ehrlichiosis in a commercial laboratory setting. Clin. Diagn. Lab Immunol. **10**:891-896.

Oliver, J. H. Jr. 1989. Biology and systematics of ticks (Acari: Ixodida). Annu . Rev. Ecol. Syst. **20**:397-430.

Onwujekwe et al., 2000 O. Onwujekwe, R. Chima and P. Okonkwo. 2000. Economic burden of malaria illness on households versus that of all other illness episodes: a study in five malaria holo-endemic Nigerian communities, Health Policy. **54:** 143–159.

Paddock CD, Folk SM, Shore GM, Machado LJ, Huycke MM, Slater LN, et al. 2001.Infections with *Ehrlichia chaffeensis* and *Ehrlichia ewingii* in persons coinfected with human immunodeficiency virus. *Clin Infect Dis***. 33:**1586–94 doi: 10.1086/323981.

Paddock, C. D. and J. E. Childs.2003. Ehrlichia chaffeensis: a prototypical emerging pathogen. Clin. Microbiol. Rev. **16**:37-64.

Pancholi P, Kolbert CP, Mitchell PD, Reed KD Jr, Dumler JS, Bakken JS. 1995. Ixodes dammini as a potential vector of human granulocytic ehrlichiosis. J Infect Dis. **172:**1007–12.

Parola, P., B. Davoust, and D. Raoult. 2005. Tick- and flea-borne rickettsial emerging zoonoses. Vet. Res. **36**:469-492

P.J. Hoetz, J.H.F. Reme, P. Buss, G. Alleyne, C. Morel and J.G. Breman. 2004. Combating tropical infectious diseases: report of the disease control priorities in developing countries project, Clin. Infect. Dis. **38:** 871–878.

Popov, V. L., X. Yu, and D. H. Walker. 2000. The 120 kDa outer membrane protein of Ehrlichia chaffeensis: preferential expression on dense-core cells and gene expression in Escherichia coli associated with attachment and entry. Microb. Pathog**. 28**:71-80.

Qin PZ, Pyle AM.1998. The architectural organization and mechanistic function of group II intron structural elements. Curr. Opin. Struct. Biol. **8:**301-8

Rahemtulla, A., T. M. Kundig, A. Narendran, M. F. Bachmann, M. Julius, C. J. Paige, P. S. Ohashi, R. M. Zinkernagel, and T. W. Mak. 1994. Class II major histocompatibility complex-restricted T cell function in CD4-deficient mice. Eur. J. Immunol. **24**:2213-2218

Raoult, D., P. E. Fournier, F. Fenollar, M. Jensenius, T. Prioe, J. J. de Pina, G. Caruso, N. Jones, H. Laferl, J. E. Rosenblatt, and T. J. Marrie. 2001. Rickettsia africae, a tick-borne pathogen in travelers to sub-Saharan Africa. N. Engl. J. Med. **344**:1504-1510

Raoult, D. and V. Roux. 1997. Rickettsioses as paradigms of new or emerging infectious diseases. Clin. Microbiol. Rev. **10**:694-719.

Reddy, G.R., C.R. Sulsona, R.H. Harrison, S.M. Mahan, M.J. Burridge, and A.F. Barbet. 1996. Sequence heterogeneity of the major antigenic protein 1 genes from Cowdria ruminantium isolates from different geographical areas. Clin.Diag.Lab.Immunol. **3:**417-422.

Reddy, G. R. and C. P. Streck. 1999. Variability in the 28-kDa surface antigen protein multigene locus of isolates of the emerging disease agent *Ehrlichia chaffeensis* suggests that it plays a role in immune evasion. Molecular Cell Biology Research Communications **1**:167-175.

Reddy, G. R., C. R. Sulsona, A. F. Barbet, S. M. Mahan, M. J. Burridge, and A. R. Alleman. 1998. Molecular characterization of a 28 kDa surface antigen gene family of the tribe Ehrlichiae. Biochem. Biophys. Res. Commun. **247**:636-643.

Reed W. 1992. Recent researches concerning the aetiology, propagation, and prevention of yellow fever, by the United States Army Commission. J Hyg. **2:** 102–119.

Rest JS & Mindell DP. 2003. Retroids in archaea: phylogeny and lateral origins. Mol Biol Evol. **20:** 1134–1142.

Richter PJ, Kimsey RB, Madigan JE, Barlough JE, Dumler JS, Brooks DL. 1996. Ixodes pacificus (Acari: Ixodidae) as a vector of Ehrlichia equi (Rickettsiales: Ehrlichieae). J Med Entomol. **33:**1–5.

Richter PJ, Kimsey RB, Madigan JE, Barlough JE, Dumler JS, Brooks DL. 1996. Ixodes pacificus as a vector of Ehrlichia equi. J Med Entomol. **33:**1–5

Rikihisa, Y. 2003. Mechanisms to create a safe haven by members of the family Anaplasmataceae. Ann. N. Y. Acad. Sci. **990**:548-555

Rikihisa, Y. 1991. The tribe *Ehrlichieae* and *Ehrlichia*l diseases. Clin. Microbiol. Rev. **4**:286-308.

Rodgers, S. E. and T. N. Mather. 2007. Human Babesia microti incidence and *i*xodes scapul aris distribution, Rhode *i*sland, 1998-2004. Emerg. *i*nfect. Dis. **13**:633-635.

Roman Reddy Ganta, Lalitha Peddireddi, Gwi-Moon Seo, Sarah Elizabeth DeDonder, Chuanmin Cheng, and Stephen Keith Chapes. 2009. Molecular characterization of Ehrlichia interactions with tick cells and macrophages. Frontiers in Bioscience. **14:**3259-3273.

Rosenberg, R. 1997. Drug-resistant scrub typhus: Paradigm and paradox. Parasitol. Today. **13**:131-132.

Rodriguez, S. A., J. J. Yu, G. Davis, B. P. Arulanandam, and K. E. Klose. 2008. Targeted inactivation of Francisella tularensis genes by group II introns. Appl. Environ. Microbiol. **74**:2619-2626

Rovery, C., P. Brouqui, and D. Raoult. 2008. Questions on Mediterranean spotted fever a century after its discovery. Emerg. Infect. Dis. **14**:1360-1367.

Rudel, T., J. P. van Putten, C. P. Gibbs, R. Haas, and T. F. Meyer. 1992. Interaction of two variable proteins (PilE and PilC) required for pilus-mediated adherence of Neisseria gonorrhoeae to human epithelial cells. Mol. Microbiol. **6**:3439-3450.

Russel, 2004 S. Russel. 2004. The economic burden of illness for households in developing countries: a review of studies focusing on malaria, tuberculosis, and human immunodeficiency virus/acquired immunodeficiency syndrome, Am. J. Trop. Med. Hyg. **71:** 147–155.

S. Harrus and G. Baneth. 2005. Drivers for the emergence and re-emergence of vector-borne protozoal and bacterial diseases. Parasitic Zoonoses. **35 (11,12):**1153-1332.

S. Montenegro-James. 1992. Prevalence and control of babesiosis in the Americas, Mem. Inst. Oswaldo Cruz. **87:** 27–36.

Safdar, N., R. B. Love, and D. G. Maki. 2002. Severe *Ehrlichia chaffeensis* infection in a lung transplant recipient: a review of ehrlichiosis in the immunocompromised patient. Emerg Infect Dis. **8**:320-323.

Santos, A. S., Alexandre, N., Sousa, R., Nuncio, M. S., Bacellar, F., Dumler, J. S. 2009. Serological and molecular survey of Anaplasma species infection in dogs with suspected tickborne disease in Portugal. *Vet Rec.* **164:** 168-171.

Seo, G.-M., C. Cheng, J. Tomich, and R. R. Ganta. 2008. Total, membrane, and immunogenic proteomes of macrophage- and tick cell-derived *Ehrlichia chaffeensis* evaluated by LC-MS/MS and MALDI-TOF methods, *In* .

Singu, V., H. Liu, C. Cheng, and R. R. Ganta. 2005. Ehrlichia chaffeensis expresses macrophage- and tick cell-specific 28-kilodalton outer membrane proteins. Infect. Immun. **73**:79-87.

Singu, V., L. Peddireddi, K. R. Sirigireddy, C. Cheng, U. G. Munderloh, and R. R. Ganta. 2006. Unique Macrophage and Tick Cell-specific Protein Expression from the p28/p30 Omp Multigene Locus in *Ehrlichia* Species. Cell Microbiol. **8**:1475-87.

Sirigireddy, K. R. and R. R. Ganta. 2005. Multiplex detection of ehrlichia and anaplasma species pathogens in peripheral blood by real-time reverse transcriptase-polymerase chain reaction. J. Mol. Diagn. **7**:308-316.

Sonenshine, D. E. 1991. Biology of Ticks, Volume 1 . Oxford University Press.

Stafford, K. C., III, R. F. Massung, L. A. Magnarelli, J. W. Ijdo, and J. F. Anderson. 1999. Infection with agents of human granulocytic ehrlichiosis, lyme disease, and babesiosis in wild white-footed mice (Peromyscus leucopus) in Connecticut. J. Clin. Microbiol. **37**:2887-2892.

Standaert, S. M., T. Yu, M. A. Scott, J. E. Childs, C. D. Paddock, W. L. Nicholson, J. J. Singleton, and M. J. Blaser. 2000. Primary isolation of *Ehrlichia chaffeensis* from patients with febrile illnesses: clinical and molecular characteristics. J. Infect. Dis. **181**:1082-1088

Stenos, J., V. Roux, D. Walker, and D. Raoult. 1998. Rickettsia honei sp. nov., the aetiological agent of Flinders Island spotted fever in Australia. Int. J. Syst. Bacteriol. **4**:1399-1404.

Stewart, R. S. 1991. Flinders Island spotted fever: a newly recognised endemic focus of tick typhus in Bass Strait. Part 1. Clinical and epidemiological features. Med. J. Aust. **154**:94-99.

Stone, J. H., K. Dierberg, G. Aram, and J. S. Dumler. 2004. Human monocytic ehrlichiosis. JAMA **292**:2263-2270.

Stouthamer, R., J. A. Breeuwer, and G. D. Hurst. 1999. Wolbachia pipientis: microbial manipulator of arthropod reproduction. Annu. Rev. Microbiol. **53**:71-102.

Sumner, J. W., G. A. Storch, R. S. Buller, A. M. Liddell, S. L. Stockham, Y. Rikihisa, S. Messenger, and C. D. Paddock. 2000. PCR amplification and phylogenetic analysis of groESL operon sequences from Ehrlichia ewingii and Ehrlichia muris. J. Clin. Microbiol. **38:**2746-2749.

Sumner, J. W., J. E. Childs, and C. D. Paddock. 1999. Molecular cloning and characterization of the *Ehrlichia chaffeensis* variable-length PCR target: an antigen-expressing gene that exhibits interstrain variation. J Clin. Microbiol. **37**:1447-1453.

Sumner, J. W., K. G. Sims, D. C. Jones, and B. E. Anderson. 1993. *Ehrlichia chaffeensis* expresses an immunoreactive protein homologous to the *Escherichia coli* GoEL protein. Infect. Immun. **61**:3536-3539.

Sumner, J. W., W. L. Nicholson, and R. F. Massung. 1997. PCR amplification and comparison of nucleotide sequences from the groESL heat shock operon of *Ehrlichia* species. J Clin. Microbiol. **35**:2087-2092.

Teresa Estrada-Garcia and Eric D. Mintz. 1996. Cholera: Foodborne Transmission and Its Prevention. European Journal of Epidemiology. **12:** 461-469.

Toro N. 2003. Bacteria and archaea group II introns: additional mobile elements in the environment. Environ Microbiol **5:**143–151

Uchiyama, T. 2005. Growth of typhus group and spotted fever group rickettsiae in insect cells. Ann. N. Y. Acad. Sci. **1063**:215-221.

United States Patent 7556816. 2009. *Ehrlichia chaffeensis* 28 kDa outer membrane protein multigene family.

Unsworth, N. B., J. Stenos, S. R. Graves, A. G. Faa, G. E. Cox, J. R. Dyer, C. S. Boutlis, A. M. Lane, M. D. Shaw, J. Robson, and M. D. Nissen. 2007. Flinders Island spotted fever rickettsioses caused by "marmionii" strain of Rickettsia honei, Eastern Australia. Emerg. Infect. Dis. **13**:566-573.

Unver, A., M. Perez, N. Orellana, H. Huang, and Y. Rikihisa. 2001. Molecular and antigenic comparison of Ehrlichia canis isolates from dogs, ticks, and a human in Venezuela. J. Clin. Microbiol. **39**:2788-2793.

Unver, A., Y. Rikihisa, R. W. Stich, N. Ohashi, and S. Felek. 2002. The omp-1 major outer membrane multigene family of Ehrlichia chaffeensis is differentially expressed in canine and tick hosts. Infect. Immun. **70**:4701-4704.

Van Vliet A H, Jongejan F, van Kleef M, van der Zeijst B A. 1994. Molecular cloning, sequence analysis, and expression of the gene encoding the immunodominant 32-kilodalton protein of Cowdria ruminantium. Infect Immun. **62:**1451–1456.

van, H. H., N. E. Collins, K. A. Brayton, C. Rademeyer, and B. A. Allsopp. 2004. Characterization of a major outer membrane protein multigene family in *Ehrlichia ruminantium*. Gene **330:159-68.**:159-168.

Velasco-Villa A, Messenger SL, Orciari LA, Niezgoda M, Blanton JD, Fukagawa C, Rupprecht CE. 2008. New Rabies Virus Variant in Mexican Immigrant. Emerg Infect Dis. **12:**1906-1908

Vobis M, D'Haese J, Mehlhorn H, Mencke N. 2005. Experimental quantification of the feline leukaemia virus in the cat flea (Ctenocephalides felis) and its faeces. Parasitological Research. **97:** 5102-5106

Vahid Noaman and Parviz Shayan. 2009. A new PCR-RFLP method for detection of Anaplasma marginale based on 16S rRNA. Veterinary Research Communications. **34:** 43-50 doi: 10.1007/s11259-009-9331-3

Walker, D. H. 1989. Rickettsioses of the spotted fever group around the world. J. Dermatol. **16**:169-177.

Wakeel, A., J. A. Kuriakose, and J. W. McBride. 2009. An *Ehrlichia chaffeensis* tandem repeat protein interacts with multiple host targets involved in cell signaling, transcriptional regulation, and vesicle trafficking. Infect. Immun. **77:**1734-1745.

Watt, G. and P. Parola. 2003. Scrub typhus and tropical rickettsioses. Curr. Opin. Infect. Dis. **16**:429-436.

Weber, D. J. and D. H. Walker. 1991. Rocky Mountain spotted fever. *i*nfect. Dis. Clin. North Am. **5**:19-35.

Wen, B., Y. Rikihisa, J. M. Mott, R. Greene, H. Y. Kim, N. Zhi, G. C. Couto, A. Unver, and R. Bartsch. 1997. Comparison of nested PCR with immunofluorescent-antibody assay for detection of Ehrlichia canis infection in dogs treated with doxycycline. J. Clin. Microbiol. **35**:1852-1855.

Werren, J. H. and D. M. Windsor. 2000. Wolbachia infection frequencies in insects: evidence of a global equilibrium? Proc. Biol. Sci. **267**:1277-1285.

Whitlock, J. E., Q. Q. Fang, L. A. Durden, and J. H. Oliver, Jr. 2000. Prevalence of Ehrlichia chaffeensis (Rickettsiales: Rickettsiaceae) in Amblyomma americanum (Acari: Ixodidae) from the Georgia coast and Barrier Islands. J. Med. Entomol. **37**:276-280.

Winslow, G. M., E. Yager, K. Shilo, E. Volk, A. Reilly, and F. K. Chu. 2000. Antibody-mediated elimination of the obligate intracellular bacterial pathogen *Ehrlichia chaffeensis* during active infection. Infect. Immun. **68**:2187-2195.

Winslow, G. M., E. Yager, K. Shilo, D. N. Collins, and F. K. Chu. 1998. Infection of the Laboratory Mouse with the Intracellular Pathogen *Ehrlichia chaffeensis*. Infect. Immun. **66**:3892-3899.

Winslow, G. M., E. Yager, and J. S. Li. 2003. Mechanisms of humoral immunity during Ehrlichia chaffeensis infection. Ann. N. Y. Acad. Sci. **990**:435-443.

Wolf, K., E. Fischer, and T. Hackstadt. 2000. Ultrastructural analysis of developmental events in Chlamydia pneumoniae-infected cells. Infect. Immun. **68**:2379-2385.

World Health Organization (WHO). 2009. Health and Environment Linkages Initiative - HELI: Priority environment and health risks

World, 2002 World Health Organization (WHO). 2002. world health report: reducing risks, promoting healthy life. Geneva, Switzerland: WHO, 192–197.

Yabsley, M. J., S. E. Little, E. J. Sims, V. G. Dugan, D. E. Stallknecht, and W. R. Davidson. 2003. Molecular variation in the Variable-length PCR target and 120-kilodalton antigen genes of *Ehrlichia chaffeensis* from white-tailed deer (*Odocoileus virginianus*). *J. Clin. Microbiol.* **41:** 5202–5206

Yabsley, M. J., T. M. Norton, M. R. Powell, and W. R. Davidson. 2004. Molecular and serologic evidence of tick-borne Ehrlichiae in three species of lemurs from St. Catherines Island, Georgia, USA. J. Zoo. Wildl. Med. **35**:503-509.

Yao, J., J, Zhong, Y. Fang, E. Geisinger, R. P. Novick & A. M. Lambowitz. 2006. Use of targetrons to disrupt essential and non-essential genes in *Staphaphylococcus aureus* reveals temperature sensitivity of group II intron splicing. *RNA*, **12:** 1271-81

Yevich, S. J., J. L. Sanchez, R. F. DeFraites, C. C. Rives, J. E. Dawson, I. J. Uhaa, B. J. Johnson, and D. B. Fishbein. 1995. Seroepidemiology of infections due to spotted fever group rickettsiae and Ehrlichia species in military personnel exposed in areas of the United States where such infections are endemic. J. Infect. Dis. **171**:1266-1273.

Yu, X. J., P. Crocquet-Valdes, and D. H. Walker. 1997. Cloning and sequencing of the gene for a 120-kDa immunodominant protein of Ehrlichia chaffeensis. Gene **184:**149-154

Yu X.J., Crocquet-Valdes P, Walker DH. 1997. Cloning and sequencing of the gene for a 120-kDa immunodominant protein of Ehrlichia chaffeensis. Gene. **184(2):**149-54

Yu X.J., McBride JW, Diaz CM, Walker DH. 2000. Molecular cloning and characterization of the 20-kilodalton protein gene of Ehrlichia canis and application of the recombinant 120-kilodalton protein for serodiagnosis of canine ehrlichiosis. J Clin Microbiol. **38(1):**369-74.

Yu XJ, P. A. Crocquet-Valdes, L. C. Cullman, and D. H. Walker. 1996. The recombinant 120-kilodalton protein of Ehrlichia chaffeensis, a potential diagnostic tool. J. Clin. Microbiol. **34:**2853–2855.

Yu, X. J., J. W. McBride, C. M. Diaz, and D. H. Walker. 2000. Molecular cloning and characterization of the 120-kilodalton protein gene of Ehrlichia canis and application of the recombinant 120-kilodalton protein for serodiagnosis of canine ehrlichiosis. J. Clin. Microbiol. **38:**369-374.

Yu, D. H., Y. H. Li, J. S. Yoon, J. H. Lee, M. J. Lee, I. J. Yu, J. S. Chae, and J. H. Park. 2008. Ehrlichia chaffeensis infection in dogs in South Korea. Vector. Borne. Zoonotic. Dis. **8**:355-358.

Zhang, J. Z., V. L. Popov, S. Gao, D. H. Walker, and X. J. Yu. 2007. The developmental cycle of *Ehrlichia chaffeensis* in vertebrate cells. Cell. Microbiol. **9:**610-618.

Zhang, C., Q. Xiong, T. Kikuchi, and Y. Rikihisa. 2008. Identification of 19 polymorphic major outer membrane protein genes and their immunogenic peptides in Ehrlichia ewingii for use in a serodiagnostic assay. Clin. Vaccine Immunol. **15**:402-411.

Zhang, P., B. B. Chomel, M. K. Schau, J. S. Goo, S. Droz, K. L. Kelminon, S. S. George, N. W. Lerche, and J. E. Koehler. 2004. A family of variably expressed outer-membrane proteins (Vomp) mediates adhesion and autoaggregation in Bartonella quintana. Proc. Natl. Acad. Sci. U. S. A **101**:13630-13635.

Zimmerly S, Guo H, Eskes R, Yang J, Perlman PS & Lambowitz AM. 1995. A Group II Intron RNA Is a Catalytic Component of a DNA Endonuclease Involved in Intron Mobility. *Cell.* **83:** 529–538

Chapter II

Scope of Thesis

Ehrlichia chaffeensis, an intracellular tick-transmitted rickettsial, is the agent of an infectious disease termed human monocytic ehrlichiosis (HME). The infection results in symptoms that vary from asymptomatic infection to severe life threatening disease with a mortality of about 3% (Paddock *et al.,* 2003). Recently, the incidence of HME in the USA has increased (Paddock *et al.,* 2003; McNabb *et al.,* 2008) with an average of 600 reported cases each year (McNabb *et al.,* 2008). The estimated cases may be significantly higher by at least four times more than the reported cases (CDC, 2007). One of the striking features of this disease causing agent is its dual host life cycle and prolonged persistence in both tick and vertebrate hosts. The mechanism of this persistent infection and the reasons for the host immune system failure to clear the infection is not clear.

There may be several ways by which the pathogen could persist. One important hypothesis is that differential gene expression may be an important adaptative mechanism used by *E. chaffeensis* in support of its continued survival in its dual hosts. Recent studies from our laboratory also support this hypothesis. Protematic analysis showed that the proteins of *E. chaffeensis* from tick and vertebrate host cell environment differed significantly. The differentially expressed proteins include many outer member proteins. An outer membrane protein from the p28-Omp mutigene locus gene p28-Omp 14 was found to be predominantly expressed in tick host and protein of the p28-Omp 19 gene was found to be the major expressed protein in vertebrate host environment. The differential expression was also confirmed in the bacteria under *in vivo* conditions in ticks and vertebrate hosts. Much of the fundamental knowledge of the pathogen's persistence

and its host specificity may be better understood by performing mutational analysis on the critical genes of *E. chaffeensis*.

Studies to date on *E. chaffeensis* lead to considerable progress in identifying the differential expression of many proteins in a host cell specific manner. The recent studies also aided in understanding the host immune response needed for clearing the pathogen in a vertebrate host. In particular, studies suggest that *E. chaffeensis* can be cured rapidly if it originates from vertebrate macrophages, but the clearance may be delayed if the organism replicates in tick cell environment. These observations, together with host cell-specific differential expression, suggest that the altered pathogen gene expression in tick cell environment may provide an opportunity to persist in a vertebrate host. Similarly, the pathogen originating from vertebrate macrophages may persist in ticks. This may be important strategy for the pathogen's continued life cycle in vertebrate and tick hosts. The above stated hypothesis is exciting, however, at this time we do not have molecular tools to test it. Importantly, there are no established methods to create mutations in *E. chaffeensis*, assessing the functional significance of differentially expressed protein.

Several methods have been documented and proven to be useful in creating effective gene mutations in both gram positive and gram negative bacteria, including in Rickettsial agents. For example, transposon based mutagenesis has been applied for *Rickettsia* and *Anaplasma* species (Qin *et al.,* 2004; Liu *et al.,* 2007). Homologous recombination based mutagenesis is extensively used in *Esherichia coli* and many other bacteria (Kowalczykowski *et al.,* 1994; Messerle *et al.,* 1997; Nowosielska *et al.,* 2006).

Nonetheless, these approaches have not been tested for *E. chaffeensis*. One of the challenging with *E. chaffeensis* is its obligate parasitic life and the pathogen has a limited survival outside a host cell. The focus of this study is to fill in this gap of knowledge by evaluating mutational methods. We opted to evaluate a relatively recently established method, 'TargeTron Gene Knockout System' for creating mutations in *E. chaffeensis*. This method utilizes the target specific insertions using modified group II intron. This method was effective in creating mutations in a wide range of gram positive and gram negative bacterial organisms. The justification for using this method is that the Group II intron can be programmed for insertion into virtually any desired DNA target with possibly high frequency and specificity. We focused this study with the objective to create mutations in *E. chaffeensis* at three genomic regions.

Materials and methods

1 Target site selection and primer ordering

The sequences of Ech_1136 (p28-Omp gene 14), Ech_1143 (p28-Omp gene 19) and Ech_0126 (p28-Omp gene 126) of *E. chaffeensis* downloaded from GenBank were used in preparing primers for retargeting group II intron with the aid of the TargeTron Design Site software (www.sigmaaldrich.com/targetron). The primer sets (IBS, EBS2, and EBS1d) that are likely to be most effective in the insertion mutations were ordered from Sigma Aldrich Company.

2 PCR for Group II intron re-targeting

2.1 Primer preparation

The IBS and EBS1d primers were diluted to 100 μM, and the EBS2 primer was diluted to 20 μM, in nuclease free water. These three primers (one set each for each gene retargeting) were mixed with the fourth universal primer, EBS universal primer, to prepare a mix for overlapping PCR as show in Figure 2.1:

Primer Mix

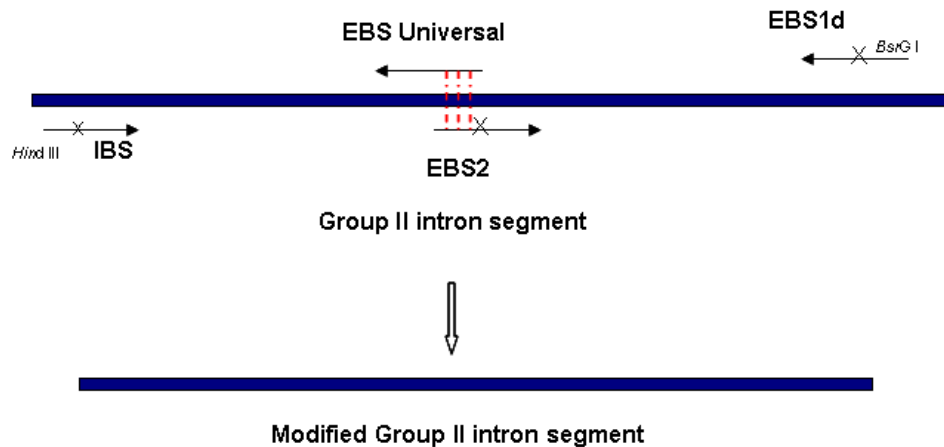|	
2 μl	IBS primer (100 μM)
2 μl	EBS1d primer (100 μM)
2 μl	EBS2 primer (20 μM)
2 μl	EBS universal primer (20 μM)
12 μl	Nuclease free water
20 μl	Total volume

Figure 2.1   Overlapping PCR for group II intron retargeting.

2.2 PCR reaction set up and thermorcycling

Amplification reactions were performed using the four primer mixes specific for each gene target and group II intron PCR template (Sigma, St. Louis, MO) by preparing the mixes and temperature cycles outlined below.

PCR reaction mixture

23 μl	water (molecular biology reagent)
1 μl	Four-primer mix (Lac Z control or specific primers for your target site)
1 μl	Intron PCR template
25 μl	JumpStart REDTaq Readymix
50 μl	Total volume

Temperature cycles

Initial denaturation	94 ℃ for 30 sec
30 cycles	
Denaturation	94℃ for 15 sec
Annealing	55℃ for 30 sec
Extension	72℃, 30 sec
Final extension	72℃, 2 min
Soak	4℃

2.3 Agarose gel electrophoresis

The PCR products were resolved in a 0.9 % agarose gel in 1X TAE buffer (40 mM Tris-acetate, 1 mM EDTA; final pH 8.0) containing 0.1 μg/ml of ethidium bromide by subjecting to 120 V of electricity for 1.5 h. The presence of 0.35 kb amplicons were identified with the aid of resolved molecular reagent markers. Agarose gel preparation and electrophoresis were performed by following instruction outlined in the volume 1 of the third edition of Molecular Cloning (Joseph *et al.,* 2001).

2.4 Phenol purification of the PCR products and restriction enzyme digestions

PCR products were purified by following phenol:chloroform:isoamylalcohol and ethanol precipitation method (Joseph *et al.,* 2001). Briefly, to 40 μl of PCR products, sodium acetate was added to a final concentration of 0.3 M. Two hundred μl of phenol (pH 8.0) was mixed to the PCR product and salt mix, vortexed and spun at 15,000 *g* for 5 min and aqueous layer was transferred to a clean 1.5 ml micro centrifuge tube. The organic extraction step was repeated two more times: one with phenol:chloroform:isoamylalcohol (25:24:1) and one with chloroform:isoamylalcohol (24:1). To the final resolved aqueous

layer, 500 µl of 100% ethanol was added and vortexed to mix the contents, and incubated at -20°C for 15 min followed by centrifugation at 15,000 *g* for 15 min. The supernatant was discarded and the DNA pellet was rinsed with 0.5 ml of 70% ethanol. The final pellet was air dried, resuspended in 20 µl TE buffer (pH 8.0) and stored at -20°C until use. Purified PCR products were digested with *Hin*d III and *Bsr*G I (Note: These restriction sites were created by inserting the R.E. recognition sequence in the 5' end of IBS, and 3' end of EBS1d primers, respectively) under the conditions outlined in the table below.

2 µl	10X N.E. Buffer 2
8 µl	purified PCR products (about 200 ng)
1 µl	*Bsr*G I
1 µl	*Hin*d III
0.2 µl	BSA (100 µg/ ml)
8 µl	Nuclease free water
20 µl	Final volume

The reaction was incubated for 30 min at 37℃, 30 min at 60℃ and 10 min at 80℃, sequentially.

One hundred ng of pACD4K-C plasmid DNA obtained from Sigma Aldrich company was also digested with with *Hin*d III and *Bsr*G I under conditions outlined in the table below.

2 μl	10X N.E. Buffer 2
5 μl	pACD4K-C plasmid DNA (100 ng)
1 μl	*Bsr*G I
1 μl	*Hin*d III
0.2 μl	BSA (100 μg/ ml)
11 μl	Nuclease free water
20 μl	Final volume

The reaction was incubated for 30 min at 37℃, 30 min at 60℃ and 10 min at 80℃, sequentially.

3 Construction of pACD4K-C- gene 14/ gene 19/ gene 126 vector

3.1 Ligation reactions

The R.E. digested pACD4K-C plasmid DNA was resolved on a 0.9 % agarose gel (Joseph *et al.,* 2001) and 6.9 kb fragment of a gel was isolated and purified by phenol:chloroform:isoamylalcohol and precipitation method (as described previously). Final purified DNA was resuspended in TE buffer (10 mM Tris and 1 mM EDTA at pH 8.0) to the final concentration of 20 ng/ml. *Hin*d III and *Bsr*G I digested PCR products was also purified by phenol:chloroform:isoamylalcohol and precipitation method. Subsequently, the PCR DNA and isolated pACD4K-C plasmid DNA segment were used in the ligation reaction outlined below.

2 µl	pACD4K-C linear Vector (40 ng)
1 µl	*Bsr*G I/ *Hin*d III-digested intron PCR product (10 ng)
1 µl	10X T4 DNA liagase buffer
1 µl	T4 DNA liagase (Promega, Madison)
5 µl	Nuclease free water
10 µl	Total volume

Ligate at 16 ℃ for overnight

3.2 Transformation in *E. coli* and selection of positive clones

Two hundred µl of chemical competent cells of *E. coli* are mixed with 50 µl of 100 mM CaCl₂ and 49 µl of sterile water. One µl of ligation products were added to this suspension and mixed well by gentle tapping at the bottom of the tube. The contents were incubated on ice for 15 min, followed by a heat shock at 42°C for 2 min. The cells were incubated at room temperature for 10 min, added 1 ml of LB medium, and incubated at 37°C in a shaker incubator for 1 h. Two hundred µl of culture were transferred onto LB agar plates containing 25 mg/ml chloramphenicol, and also including 30 µl each of 20 mg/ml X-Gal and 100 mM IPTG spreaded onto the plates. The plates were incubated overnight in a 37°C incubator. White colonies were randomly selected and screened for the presence of retargeted insert containing pACD4K-C plasmids.

3.3 Extraction of Plasmid DNA

Plasmid DNAs were extracted by boiling preparation method (Joseph *et al.,* 2001) from white colony cultures grown in LB medium with chloramphenicol (25 µg/ml). Briefly, 1.5 ml of overnight grown bacterial cultures was transferred into micro-centrifuge tubes

and centrifuged 12,000 *g* for 5 min. The supernatant was aspirated carefully and cell pellet was resuspended in 0.4 ml plasmid lysis buffer (10mM Tris-HCl pH 8.0, 0.1 M NaCl, 1mM EDTA, 5% v/v Triton X-100). Twenty five µl of freshly prepared lysozyme (10 mg/ml) in 10 mM Tris-HCl buffer (pH 8.0) was added. The contents of the tube were vortexed for few sec to mix, placed in a boiling water bath exactly for 40 sec, and centrifuged at 12,000 *g* for 15 min at 4°C. The pellet containing cell debris was removed with the help of a tooth pick. Four hundred and twenty µl of cold (-20°C) 100% isopropanol was added to the supernatant and mixed by vortexing, incubated at room temperature for 5 min and centrifuged at 12,000 *g* for 15 min to recover plasmid DNA. Supernatant was discarded and the DNA pellet was rinsed with 1 ml of 70% ethanol and dried in a speed-vac system (Labconco Centrivap Concentrator, Kansas City, MO) typically for about 5 min. Final pellet was resuspended in 100 µl of TE buffer and contaminating bacterial RNA was removed by treating with 1 µl of RNase A (1 mg/ml) at 37°C for 5 min. The quantity and quality of the plasmid DNA were checked by agarose gel electrophoresis.

3.4 Confirmation by Restriction Enzyme reaction

The extracted plasmids (5 µl each) were digested with *Bsr*G I and *Hin*d III enzyme similar to the method outlined previously. The clones with the correct inserts were selected for sequencing analysis to verify mutation in the modified group II intron segments.

3.5 Confirmation by Sequencing

The final constructs of pACD4K-C-Ech_1136 (p28-Omp gene 14), pACD4K-C-Ech_1143 (p28-Omp gene 19) and pACD4K-C-Ech_0126 (p28-Omp gene 126) (all three constructs are also called pACD4K-C-gene 14/gene 19/gene 126) were sequenced by gene analytic system, using T7 primer (forward primer) and one gene specific primer (IBS or EBS1d). The protocols for sequencing analysis were followed as described in the CEQ 2000 Dye Terminator Cycle Sequencing with Quick Start Kit manual (Beckman Coulter, CA). Sequences were manually evaluated to confirm mutations specific for targeting genes _1136 (gene 14), Ech_1143 (gene 19) and Ech_0126 (gene 126).

4 Cloning the *tuf* promoter into a promoterless vector, pBlue-TOPO

4.1 Cloning *tuf* promoter

RRG725 (*tuf*-Forward primer) and RRG726 (*tuf*-Reverse primer) were designed based on the *E. chaffensis*, Arkansas whole genome sequence information obtained from Genbank (accession CP000236). The primers were obtained from Intergrated DNA technologies (Coralville, Iowa). These primers and *E. chaffeensis* genomic DNA were used to amplify '*tuf*' promoter region. Details of the PCR experiment were described below.

PCR reaction mixture

2 ml	HiFi PCR buffer (10 X)
0.5 μl	d NTP (10 mM)
1 μl	MgSO4 (50 mM)
0.5 μl	RRG 725 (10 μM)
0.5 μl	RRG 726 (10 μM)
0.2 μl	HiFi polymerase
1 μl	*E. chaffeensis* (7000) diluted
14.3 μl	Nuclease free water
20 μl	Total volume

Temperature cycles

Initial denaturation	94℃ for 2 min
40 cycles	
Denaturation	94℃ for 30 sec
Annealing	51℃ for 30 sec
Extension	68℃ for 40 sec
Final extension	68℃ for 10 min
Soak	4℃

Predicted size PCR products (0.37 kb) were confirmed after resolving on a 0.9% agarose gel. Subsequently, two μl of the PCR product was used to ligate into the linear pBlue TOPO vector and recombinant plasmids containing the inserts were selected by following protocols outlined by the manufacturer (Invitrogen, CA).

4.2 Evaluation of *tuf* promoter activity by β-galactosidase assay

β-galactosidase assay was performed by following instruction manual of β-galactosidase assay kit (Agilent Technologies, CA). Breifly, *E. coli* transformants containing the recombinant plasmids (pBlue-TOPO-*tuf*) were cultured overnight in 3 ml LB medium containing 50 µg/ml ampicillin, and centrifuge at 250 *g* for 5 min to remove the growth medium. One ml 1 X phoshate buffered saline (PBS) was added into each tube and centrifuged at 250 *g* for 5 min. The pellet was resuspended in 100 µl 1 X lysis buffer, and the sample was frozen on dry ice and thawed in a 37℃ water bath to promote bacteria lysis. This step of freezing and thawing was repeated twice. Then, the sample was centrifuged at 12000 *g* for 5 min in 4℃, and the supernatant was transferred into a microtiter plate. Samples were diluted at 1:100 and 10 µl of each was transferred into the wells containing 50 µl 1 X cleavage buffer with β-mercaptoethanol and 17 µl o-Nitrophenyl-β-D-galactosidase (ONPG). The plates were incubated in 37℃ for 30 min. The reaction was stopped by adding 125 µl stop buffer. The absorbance at 420 nM and the specific activity of *tuf* promoter (as assessed by the production of β-galactosidase per milligram of protein) were measured.

N moles ONPG hydrolyzed = (OD420) (1.92 x 105 nl) / (4500 nl/ nmole-cm) (1cm)

Specific activity = n moles ONPG hydrolyzed / mg/ protein

5 Construction of pACD4K-C-gene 14/gene19/gene 126-*tuf* vector

PBlue-TOPO-*tuf* and pACD4K-C-gene 14/gene 19/gene 126 were digested with Hind III, the desired fragments were isolated, phenol purified and then ligated using T4 DNA ligase at 4℃ overnight. One µl of the ligation mixture was transformed into XL1-Blue

strain of *E. coli* chemically prepared competent cells by heat shock method as described above. Transformed cultures were plated onto LB plate containing chloramphenicol (25 μg/ml) and incubated at 37℃ overnight. The colonies were screened for the insertion and correct orientation by plasmid DNA isolation, followed by *Afl* II and *Hin*d III digestion and by sequence analysis. Methods for restriction enzyme digestion, ligation, and cloning and sequence analysis were similar to the methods previously described in this chapter. The constructs with the correct inserts and orientations were saved in -80℃ for future use.

6 Construction of pACD4K-C-gene 14/19/126-*tuf-rpsl*-CAT

The pACD4K-C-gene14/19/126-*tuf* plasmids were used to insert antibiotic cassette, chloramphenicol acetyltransferase (CAT) during expression using a transcrpitionally active *E. chaffeensis* promoter, *rpsl*. The DNA segment of *rpsl*-CAT was already prepared and is available in our laboratory as a recombinant plasmid. Primers were designed to amplify *rpsl*-CAT DNA segment. Both the primers included a *Mlu*I site at the 5' ends to facilitate cloning into Targetron constructs. PCR products with *Mlu*I digested fragment were prepared and used for ligation with *Mlu*I digested plasmids of pACD4K-C-gene14/19/126-*tuf*, T4 DNA ligations were performed as described previously. One μl of the ligation mixture was transformed into XL1-Blue chemical competent cells. The positive clones were screened and *rpsl*-CAT insert containing plasmids were verified by *Mlu*I digestion of plasmid DNA. The presence of inserts was also confirmed by plasmid DNA sequence analysis. The final constructs were used for preparing large quantity of plasmid DNAs by using Qiagen plasmid DNA maxi kit (Qiagen Inc. CA) and saved at -80℃ for future use.

7 Evaluating the transcription from Group II intron in *Escherichia coli*

7.1 Performing RNA isolation from *E. coli*

PACD4K-C-gene14/19/126-*tuf-rpsl*-CAT plasmids culturing *E. coli* cultures were grown in 5 ml of LB media containing chlorephenicol (25 µg/ ml) for almost 14 h at 37℃. Total RNA from the cultures were isolated using Tri-reagent method (Sigma Chemical Corporation, St. Louis, MO). Briefly, 1.5 ml of culture was harvested by centrifuging at 12,000 *g* for 5 min at 4℃. To the cell pellet, 1ml of tri-reagent was added and vortexed to mix. The lysates were incubated at room temperature for 5 min. Two hundred µl of chloroform was added to the cell suspension, mixed by vortexing and incubated at room temperature for 10 min. Subsequently, the samples were centrifuged at 12,000 x g for 15 min at 4℃. The top aqueous layer containing RNA was transferred into a clean micro centrifuge tube, mixed with 0.5 ml of cold isopropanol (-20℃) and incubated at room temperature for 10 min. The contents are centrifuged at 12,000 *g* for 15 min at 4℃. The RNA pellet recovered was rinsed with 70% ethanol and then resuspended in 100 µl of nuclease free water. To prevent the degradation of RNA, 40 µl of RNase inhibitor (Ambion Corporation, Austin, TX) was added and stored at -80℃ until use.

7.2 RNA analysis

The total RNA was treated with RQ1 RNase-free DNase enzyme. Gel rig including combs and gloves were treated by washing with DEPC treated H_2O. Agarose gel was made by mixing 0.6 g agarose in 28.8 ml water and microwaving for 30 sec. When it cooled down to 60℃, 8 ml of 36% formaldehyal and 4 ml of 10 X MOPS were added

into agarose gel. RNA samples were prepared by adding 10 µl formaldehyde loading dye, heating at 65 ℃ for 15 min and chilling on ice. RNA running buffer was prepared by adding 25 µl of 10 X MOPS into 225 µl of DEPC. All of the samples were loaded onto gel and run at 70 V for 1 h.

7.3 RT-PCR analysis

Two PCR and one RT-PCR were performed using 10 µM Primers (IBS and EBS1d). RT-PCR for extracted RNA was designed to confirm the transcription of pACD4K-C-gene 14/19/126-*tuf*-*rpsl*-CAT in *E. coli.* PCR for extracted RNA was designed as the negative control for the transcription, and PCR for plasmid DNA was designed as the positive control for the transcription. In addition, another negative control was set up by using RNase free water as the templates. The RT-PCR reaction was performed as follows: reverse transcription at 48℃ for 1 h; initiation at 94℃ for 30 sec; and 25 cycles for (denaturation at 94℃ for 15 sec, annealing at 55℃ for 30 sec and extension at 72℃ for 30 sec); then elongation at 72℃ for 2 min, and final soaking at 4℃.

8 Preparation of *Ehrlichia chaffeenis* for transformation experiments

8.1 *In vitro* cultivation of *E. chaffeensis*:

The Arkansas isolate of *E. chaffeensis* was cultivated *in vitro* in the canine macrophage cell line DH82 at 37°C, by following the protocols established before (Chen *et al.,* 1994; Munderloh *et al.,* 1999).

8.2 Purification of *E. chaffeensis*:

E. chaffeensis infected macrophage cultures were harvested when the infectivity reached to 80-90 % in nearly 100% confluent flasks. Infection was assessed by microscopic examination of polychromatic stained cytospin slides. About 25 ml of infected culture was collected into a 50 ml sterile falcon tube and centrifuged at 15,000 *g* for 15 min. The supernatant was discarded and the pellet is resuspended in 10 ml of 0.25 M ice cold sucrose solution. The cells were lysed by adding 1 mm diameter glass beads and vortexing twice for 30 sec. The lysed cell suspension was transferred to a new sterile tube free of glass beads and centrifuged at 100 *g* for 10 min. The supernatant was carefully collected and transferred to a new tube and centrifuged at 15,000 *g* for 15 min to collect cell-free *Ehrlichia* organism. At the end of centrifugation, supernatant was discarded and the cell-free bacteria pellet was resuspended in 10 ml of 0.25 M ice cold sucrose. The centrifugation step was repeated one more time and the final *E. chaffeensis* organism pellet was resuspended in 0.6-1 ml of 0.25 M sucrose solution.

8.3 Preparation of plasmid DNA for electroporation

Grow 100 ml of pACD4K-C gene 14/19/126-*tuf* -*rpsl*-CAT plasmids containing *E. coli* in LB media with chloramphenicol (25 µg/ml) for about 16 h. The cultures were used to isolate plasmid DNA by following the protocols outlined by EndoFree Plasmid Purification Kit (QIANGEN, CA). Briefly, the culture was centrifuged at 6,000 *g* for 15 min at 4℃, and then the supernatant was discarded. The pellet was resuspended in 10 ml buffer P1 (QIANGEN, CA), and mixed thoroughly with 10 ml buffer P2 (QIANGEN, CA), followed by incubating at room temperature 24℃ for 5 min. During the incubation, the QIAfilter Cartridge was prepared (screw the cap onto the outlet nozzle of the

QIAfilter Maxi Catridge and place the QIAfilter Cartridge in a convenient tube). Ten ml chilled buffer P3 (QIANGEN, CA) was added to the lysate, and mixed immediately and thoroughly by vigorously inverting for 6 times (do not incubate the lysate on ice). The lysate was poured into the barrel of the QIAfilter Cartridge, and incubated at room temperature for 10 min. The cap from the QIAfilter Catridge outlet nozzle was removed. The plunger was gently inserted into the QIAfilter Maxi Cartridge and the cell lysate was filtered into a 50 ml tube. Two point five ml buffer ER (QIANGEN, CA) was added to the filtered lysate, mixed by inverting the tube approximately 10 times, and incubated on ice for 30 min. A QIAGEN-tip 500 was equilibrated by applying 10 ml buffer QBT (QIANGEN, CA), and allowing the column empty by gravity flow. The filtered lysate was applied to the QIAGEN-tip. The QIAGEN-tip was washed with 30 ml buffer QC (QIANGEN, CA) for 2 times. Plasmid DNA was eluted with 15 ml buffer QN (QIANGEN, CA) and precipitated by adding 10.5 ml room-temperature isopropanol, followed by mixing and centrifuging immediately at 15,000 g for 30 min at 4℃. The supernatant was carefully decanted. DNA pellet was washed with 5 ml of endotoxin-free room-temperature 70% ethanol (40 ml of 96-100% ethanol was added to the endotoxin-free water supplied with the kit) and centrifuged at 15,000 g for 10 min. The supernatant was carefully decanted without disturbing the pellet. The pellet was air-dried for 10 min, and the DNA was redissolved in 100 μl endotoxin-free buffer TE (QIANGEN, CA). The purified plasmid was stored at -80℃ for later use.

9 Transformation with *E. chaffeensis* in DH 82 macrophage cells

E. chaffeensis cultures were grown in macrophage cell line, DH 82, and when infection reached to > 90%, cultures were harvested by spinning at 15, 000 *g* for 15 min at 4℃. To the pellet, 10 ml of ice-cold 0.25 M sucrose solution was added and vortexed to resuspend the culture. The host cells were broken by passing through 27.5 gauge bended needles for 4 times, and spinning at 200 *g* for 10 min at 4℃ to pellet the cell debris. The supernatant was filtered through 2.7 µm filter and spun at 15,000 *g* for 15 min at 4℃ to collect the cell free bacteria. The pellet was washed twice with ice cold sucrose solution (0.25 M) and the final pellet was resuspended in 0.6-1 ml of 0.25 M ice-cold sucrose. The final sucrose solution varied depending on the bacteria volume and 100 µl each of the purified *E. chaffeensis* suspension was used for transformation expressions. One µl plasmid DNA (20 µg/ml) of pACD4K-C-gene 14/19/126-*tuf-rpsl*-CAT and 100 µl of cell free bacteria was mixed in an electroporate cuvette for use in electroporation. The electroporation was performed twice at 1800 V. To serve as a control, 100 µl of cell-free bacteria were also electroporated by giving double shock at 1800 V. The electroporated cells were transferred into T 25 cm^2 flask of DH82 cells having 80% confluency (5 ml culture volume). The electroporate cuvettes were washed with 400 µl of MEM medium, and then transferred the contents to the respective culture flask. The culture flasks were incubated at 37℃ for overnight. Fraction of cultures from each flask (1.5 ml each) were harvested at different times post transformation (6 h, 12 h, 24 h, 48 h and 6 days) for use in DNA isolation and analysis. To the remaining culture, media is replaced every three or four days and *E. chaffeensis* infection was monitored for up to two months. The culture flasks with 2 µg/ml chloramphenicol were starting from the day six following transformation.

10 Methods for monitoring transformants

Total genomic DNAs of recovered cultures were isolated using QIAamp DNA Mini Kit (QIAGEN, CA). PCR analysis was performed by using primers spanning the expected insertion regions, one targeted to group II intron mutated genes and the other targeted to *E. chaffeensis* genome. The presence of genetically modified *E. chaffeensis* was also assessed by microscopic analyzing polychromatic stained culture slides and evaluating the growth or lack of growth of the mutants. Any culture that appeared to be positive was further evaluated by molecular techniques.

The PCR products were then resolved on a 0.9% agarose gel by subjecting to 40 V for 6 h and the resolved DNA was transferred to a nylon membrane. The prehybridization solution stock was prepared at final concentration of (6 X SSC, 10 mm pH 6.8 NaPO$_4$ buffer, 1 mm EDTA pH 8.0, 10 X Denhard's, 100 μg/ml ssDNA, and 0.5% SDS). The membrane was prehybridized for 2 h at 68℃. The ^{32}P-labeled DNA probes for the group II intron was synthesized by using Primer-It II Random primer labeling kit (Stratagene, CA). After 14 h of hybridization at 68 ℃, the membrane was washed once with 6 X SSC, 0.1% SDS followed by one wash at 2 X SSC, 0.1 % SDS, one wash at 1 X SSC, 0.1% SDS, and a final wash at 0.1 X SSC, 0.1% SDS. The nylon membrane was then exposed to an X-ray film at -70℃ by using an intensifying screen. The X-ray film was developed and presence of group II intron segments within *E. chaffeensis* genome was assessed from the autoradiograph.

Reference:

Cui X, Davis G. 2007. Mobile group II intron targeting: applications in prokaryotes and perspectives in eukaryotes. Front Biosci. **12:**4972–4985

Guo,H., Karberg,M., Long,M., Jones,J.P.,3rd, Sullenger,B. and Lambowitz,A.M. 2000. Group II introns designed to insert into therapeutically relevant DNA target sites in human cells. *Science*. **289**: 452–457

Guo, H., Zimmerly, S., Perlman, P.S. & Lambowitz, A.M. 1997. Group II intron endonucleases use both RNA and protein subunits for recognition of specific sequences in double-stranded DNA. *EMBO J.* **16**, 6835–6848.

Karberg,M., Guo,H., Zhong,J., Coon,R., Perutka,J. and Lambowitz,A.M. 2001. Group II introns as controllable gene targeting vectors for genetic manipul ation of bacteria. *Nature Biotechnol.* **19**: 1162–1167

John T. Heap, Oliver J. Pennington, Stephen T. Cartman, Glen P. Carter and Nigel P. Minton. 2007. The ClosTron: A universal gene knock-out system for the genus Clostridium. *Journal of Microbiological Methods*. **70 (3):** 452-464.

Jun Yao, Jin Zhong, Yuan Fang, Edward Geisinger, Richard P. Novick and Alan M. Lambowitz. 2006. Use of targetrons to disrupt essential and nonessential genes in *Staphylococcus aureus* reveals temperature sensitivity of Ll.LtrB group II intron splicing. RNA **12:**1271-1281.

Kowalczykowski, S.C., Dixon, D.A., Eggleston, A.K., Lauder, S.D., and Rehrauer, W.M. 1994. Biochemistry of homologous recombination in *Escherichia coli.* Microbiol. Rev. **58:** 401–465.

Lambowitz, A.M., Caprara, M.G., Zimmerly, S. & Perlman, P.S. 1999. Group I and group II ribozymes as RNPs: clues to the past and guides to the future. In *The RNA world*, Edn. 2. (eds Gesteland, R.F., Cech, T.R. & Atkins, J.F.) 451–484(Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY).

Liu Z. M., Tucker A. M., Driskell L. O., Wood D. O. 2007 Mariner-based transposon mutagenesis of Rickettsia prowazekii. Appl. Environ. Microbiol. **73:** 6644–6649

McNabb, S. J., R. A. Jajosky, P. A. Hall-Baker, D. A. Adams, P. Sharp, C. Worshams, W. J. Anderson, A. J. Javier, G. J. Jones, D. A. Nitschke, A. Rey, and M. S. Wodajo. 2008. Summary of notifiable diseases--United States, 2006. MMWR Morb. Mortal. Wkly. Rep. **55**:1-92.

Messerle M, Crnkovic I, Hammerschmidt W, Ziegler H, Koszinowski UH. 1997. Cloning and mutagenesis of a herpesvirus genome as an infectious bacterial artificial chromosome. Proc. Natl Acad. Sci. USA. **94:**14759–14763.

Nowosielska, A., Smith, S.A., Engelward, B.P., and Marinus, M.G. 2006. Homologous recombination prevents methylation-induced toxicity in *Escherichia coli.* Nucleic Acids Res. **34:** 2258–2268

Paddock CD, Childs J. E. 2003. *Ehrlichia chaffeensis*: a prototypical emerging pathogen. *Clin Microbiol Rev 16:* 37–64

Rodriguez, S. A., J. J. Yu, G. Davis, B. P. Arulanandam, and K. E. Klose. 2008. Targeted inactivation of *Francisella tularensis* genes by group II introns. Appl. Environ. Microbiol. **74:**2619-2626.

Qin A Tucker AM, Hines A, Wood DO. 2004. Transposon mutagenesis of the obligate intracellular pathogen Rickettsia prowazekii. Appl Environ Microbiol.**70:**2816-2822

Chapter III

Evaluation of Targetron Based Mutagensis in *Ehrlichia chaffeensis*

ABSTRACT

Ehrlichia chaffeensis is an emerging tick-borne rickettsial pathogen that causes infection in people and several vertebrate animals. One of the striking features of *E. chaffeensis* infection is the prolonged persistence in its vertebrate and tick hosts. The mechanisms of persistent infection and the reasons for the host immune system failure to clear the infection are not well understood. One hypothesis is that differential gene expression serves as an important adaptive mechanism used *by E. chaffeensis* in support of its continued survival in both tick and vertebrate hosts. One way to test this hypothesis is by performing mutational analysis. The methods for introducing mutations in this pathogen have not yet been documented and are challenging, possibly due to its obligate, intraphagosomal growth requirement. A novel gene mutation method, called 'TargeTron Gene Knockout System' that is based on the modified group II intron insertion strategy has been developed recently, are used to create mutations in a wide range of gram positive and gram negative bacterial organisms. The objective of this study is to create mutations in *E. chaffeensis* using the TargeTron gene knockout system. Three *E. chaffeensis* genes: Ech_0126 (a transcriptionally silent gene), macrophage-specific expressed gene (p28-Omp 19) and tick cell-specific expressed gene (p28-Omp 14) were evaluated for mutational analysis. Group II intron was modified for retargeting to these genes and its expression in E. chaffeensis is accomplished using the pathogen-specific high-expressing gene promoter (*tuf*) inserted upstream to the transcription starting site. In addition, a chloramphenicol acetyltransferase gene with *E. chaffeensis rpsl* promoter (another transcriptionally active, high expression gene promoter) was introduced for use

as an antibiotic selection marker. The constructs were evaluated by transforming into *E. chaffeensis*. Transformants with mutations introduced in two of the three genes (Ech_0126 and p28-Omp 19) were identified by PCR and Southern blot methods for up to 48 h. This is the first report which demonstrates the creation of mutations in *E. chaffeensis*. Establishment of stable transformants, however, requires additional modifications to the constructs. The outcomes of this study will have important implications in defining the pathogenesis of *E. chaffeensis* and other closely related bacteria to assess the functional significance of host specific differential expression in the organisms in tick and vertebrate hosts.

Introduction

E. chaffeensis, an intracellular tick-transmitted rickettsial, is the agent of an infectious disease termed human monocytic ehrlichiosis (HME). Despite the increased incidence of HME in the USA (Paddock *et al.,* 2003; McNabb *et al.,* 2008), efficient methods to control the illness remain to be established.

Recent studies demonstrate that the pathogen gene expression is significantly different when it replicates in tick cell compared to its growth in vertebrate macrophages (Ohusi *et al.,* 1998; Unver *et al.,* 2002; Long *et al.,* 2002; Singu *et al.,* 2005; Seo *et al.,* 2008). Studies which evaluated host immune response in the mouse model also suggested that the tick cell-derived *E. chaffeensis* persist pathogen (Ganta *et al.,* 2002; Ganta *et al.,* 2004; Ganta *et al.,* 2007). The delayed clearance in the murine host is also associated with altered host immune response (Ganta *et al.,* 2002; Ganta *et al.,* 2004; Ganta *et al.,* 2007). The evaluation of differential expressed *E. chaffeensis* proteins will be useful in creating mutation.

'TargeTron Gene Knockout System' that is based on the modified group II intron insertion strategy has been utilized for creating mutations in several gram negative and gram positive bacteria. In this study, we evaluated the feasibility of creating mutations in *E. chaffeensis* using Group II intron based strategy. Three *E. chaffeensis* genes were assessed and the results from this study and their implication were discussed.

Result

1 Construction of plasmids for retargeting group II intron for inserting mutations in *E. chaffeensis* genome

1.1 PCR for intron re-targeting in genes of *E. chaffeensis*:

Genomic sequences of *E. chaffeensis* genes, Ech_1136 (p28-Omp gene 14), Ech_1143 (p28-Omp gene 19) and Ech_0126 were uploaded to Sigma-Aldrich company website (www.sigma-aldrich.com/targetron), and three primers (IBS, EBS-1d, EBS-2) for each gene were designed and synthesized commercially for each gene-specific retargeting (Table 3.1) (Gene-specific retargeting regions were identified in italics and bold text in the primers). The PCR amplification was performed using group II intron as template using each gene-specific retargeted primer. Retargeted PCR products of 0.35 kb for each gene were identified in the ethidium bromide stained agarose gel (Figure 3.1).
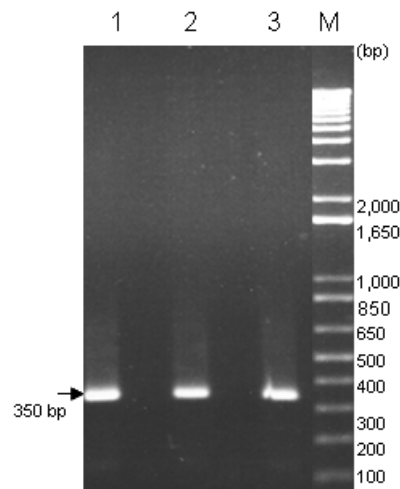
Figure 3.1 PCR for intron retargeting in gene 14, gene 19 and gene 126 of *E. chaffeensis.* Lanes 1, 2, 3 are Ech_1136 (p28-Omp gene 14), Ech_1143 (p28-Omp gene 19) and Ech_0126, respectively.

1.2 Ligation and recombination plasmid construction

1.2.1 Restriction enzyme digestion

To engineer the retargeted PCR products into group II intron cassette, PCR products and pACD4K-C plasmids (Sigma, St. Louis, MO) were digested separately with *Hin*d III and *BsrG* I restriction enzymes. The plasmid segment of 6.9 kb containing the active group II intron fragment, but lacking the *lacZα* segment of 0.76 kb was isolated and ligated with RE.digested retargeted PCR products for genes Ech_1136, Ech_1143 and Ech_0126 using T4 DNA ligase. The ligated products were transformed into *E.coli* strain, XL1 Blue, plated on agar plates containing chloramphenicol (25 µg/ml), white colonies were picked and screened for the presence of the retargeted inserts.
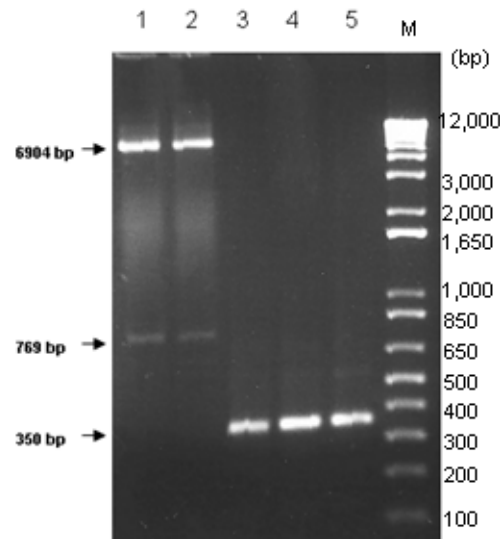
Figure 3.2   Double digestion of the retargeted PCR products and pACD4K-C plasmids with *Hin*d III and *BsrG* I. Lanes 1 and 2 are pACD4K-C plasmids; lanes 3, 4, and 5 are retargeted PCR product for Ech_1136, Ech_1143, and Ech_0126; M represents marker in base pairs.

1.22 Confirmation of constructed plasmids pACD4K-C-gene 14/19/126 by restriction enzyme digestion. The plasmid DNAs were isolated from the liquid cultures of transformants by boiling preparation method and assessed for the presence of specific inserts size (Figure 3.3). Fragments of about 350 bp *Hin*d III and *Bsr*G I digested products were identified in several clones.
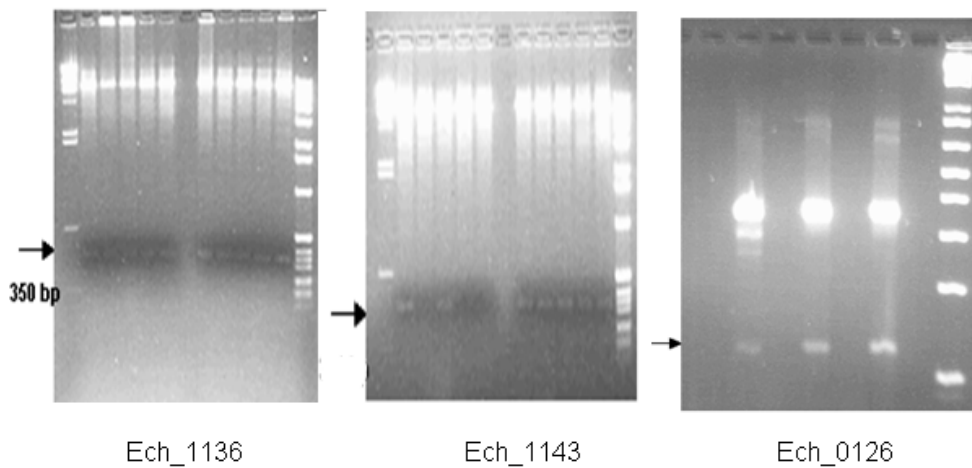

Figure 3.3   Double digestion of constructed plasmids pACD4K-C- gene 14/ 19/126 with *Hin*d III and *Bsr*G I (the arrow indicates the 350 bp size fragments).

1.3 Verification of modified intron

Three clones from each gene were randomly selected and the sequence modifications in pACD4K-C-gene 14/gene 19/gene 126 plasmids were determined by CEQ-8000 gene analytic system for DNA sequencing. These modified plasmid containing *E. coli* cultures were stored in -80℃ as glycerol stocks for subsequent use in isolating plasmid DNAs.

1.4 Insertion of *E. chaffeensis* promoter

To drive the expression of modified group II introns in *E. chaffeensis*, we identified a strong transcriptionally active *E. chaffeensis* gene promoter, *tuf*. This promoter activity was primarily determined by our group (Unpublished data). To clone the promoter region of *tuf*, two primers were designed (RRG 725 and RRG 726) (Table 3.1) and used in a PCR to amplify from *E. chaffeensis* genome. To facilitate cloning upstream to modified group II introns in pACD4K-C plasmids, *Hin*d III restriction sites sequence were engineered in the primers. PCR products were evaluated in 0.9 % agarose gel by subjecting to electrophoresis at 120 V for 1 h. Predicted fragment of 374 bp was detected in the gel under UV light (Figure 3.4).
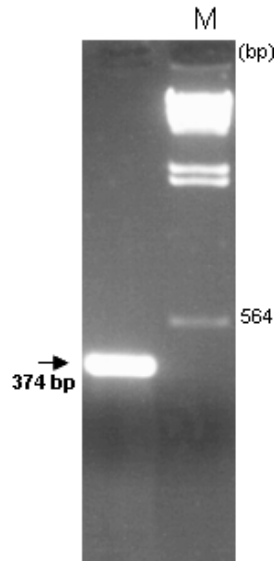
Figure 3.4   PCR for *tuf* promoter.

E. chaffeensis tuf promoter PCR products were cloned into pBlue-TOPO vector by following the protocol described by Invitrogen pBlue-TOPO TA Expression Kit (Invitrogen, CA). The pBlue-TOPO plasmid contains a promoterless *LacZ* gene placed down stream to multiple cloning site regions. If the promoter inserted (such as the *tuf*) is active, β-galactosidase proteins will be made in *E.coli* cultures containing the

recombinant plasmid. *Tuf* promoter inserted pBlue-TOPO plasmids were assessed for transcriptional activity by β-galactosidase assay. The results showed that the transcriptional activities of plasmids containing *tuf* promoter are 11.58 and 15.3 times higher (Figure 3.5; lanes 2 and 3) than the control (pBlue-TOPO vector without inserts, lane 1), suggesting that *tuf* promoter of *E. chaffeensis* is a strong promoter during the expression of β-galactosidase in *E. coli.*
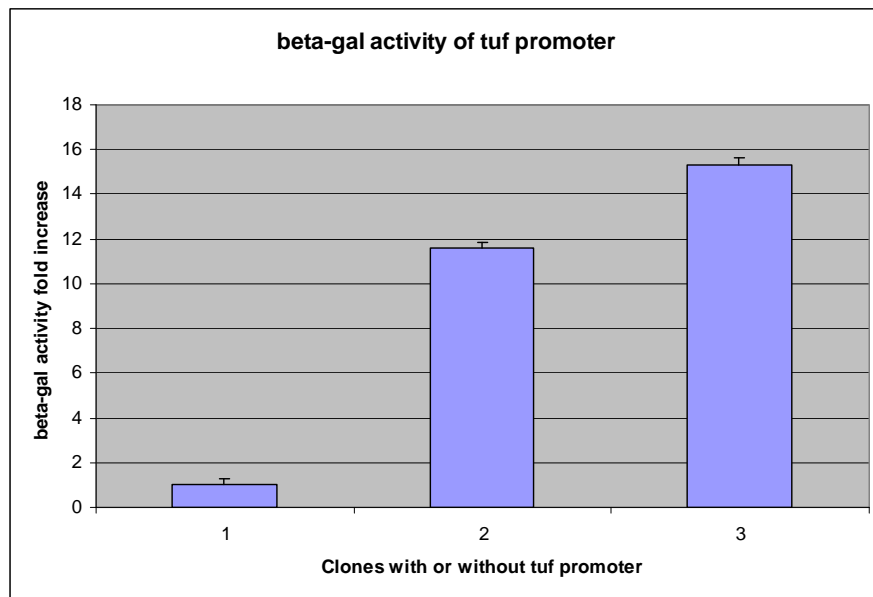

beta-gal activity of tuf promoter

Figure 3.5   β-galactosidase activity of *tuf* promoter. Lane 1, lysates of *E.coli* containing pBlue-TOPO plasmid without inserts; lanes 2 and 3 contained *E. coli* lysates prepared from two randomly selected clones of pBlue-TOPO vector with *tuf* promoter.

1.4.1 Restriction enzyme digestion

The plasmids pACD4K-C-gene 14/19/126 and a *tuf* promoter plasmid of pBlue-TOPO were digested by *Hin*d III and digested products were isolated and analyzed in 0.9 % agarose gel to verify the recovery of DNA fragments. Fragments of 7,677 bp of

pACD4K-C- gene 14/19/126 linear DNA and 374 bp of *tuf* promoter were isolated and observed under UV light (Figure 3.6).
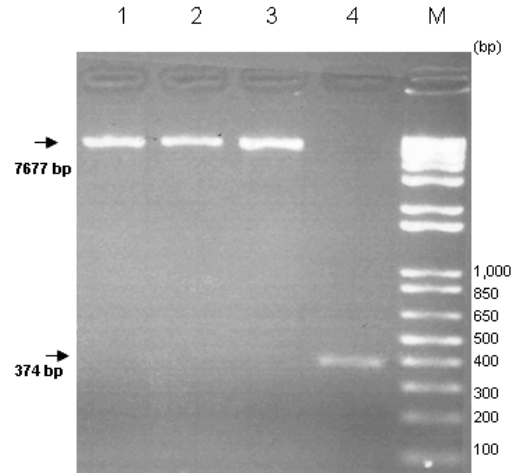

Figure 3.6 *Hin*d III digestion of plasmids pACD4K-gene 14/19/126 and *tuf* promoter. Lanes 1, 2, 3, and 4 are plasmid pACD4K- Ech_1136, plasmid pACD4K- Ech_1143, plasmid pACD4K- Ech_0126 and *tuf* promoter, respectively.

1.4.2 Cloning of *tuf* promtor in front of group II introns in pACD4K-C plasmids

Purified *Hin*d III digested pACD4K-C plasmid segments with retargeted group II introns for *E. chaffeensis* genes for Ech_1136, Ech_1143, and Ech_0126, and *tuf* promoter DNA were ligated, cloned into *E. coli* (TOP 10 strain), and plasmids with correct orientation were selected by following standard molecular cloning methods. The constructed plasmids pACD4K-C-*tuf* were picked from bacteria culture plates containing chlorephenicol (25 µg/ml) and cultured overnight in 3 ml of LB plus chlorephenicol (25 µg/ml). The plasmid DNAs from liquid cultures were extracted by boiling preparation method and digested with *Afl* II and *EcoR*V to confirm the orientation of *tuf* promoter inserts. For gene 19 and gene 126, fragments of 7,219 bp plus 814 bp should be

observable in the plasmids with forward insertion (and a 18 bp fragment should also be released); and fragments of 6849 bp plus 814 bp and 388 bp should be observable in the plasmids with reverse insertion. For gene 14, fragments of 7219 bp plus 832 bp should be observable in the plasmids with forward insertion; and fragments of 6849 bp plus 1202 bp should be observable in the plasmids with reverse insertion. The result indicate that both gene 14 plasmids are in forward orientation; one each of the gene 19 plasmids are in forward and reverse orientation (lanes 3 and 4); and one of the gene 126 plasmids has forward orientation (lane 6) and the other does not have any inserts (lane 5).
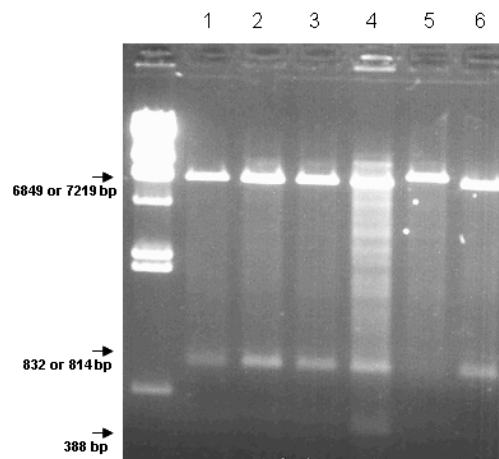

Figure 3.7 Confirmation of pACD4K-C-gene 14/19/126-*tuf*. Lanes 1 and 2, Ech_1136; lanes 3 and 4, Ech_1143; and lanes 5 and 6, Ech_0126.

The final constructs of pACD4K-C-gene 14/19/126-*tuf* were verified by sequencing analysis in CEQ-8000 gene analytic system. The verified constructs containing *E. coli* cultures in TOP 10 strain were stored as glycerol stocks at -80℃ for long term use.

1.5 Insertion of *rpsl*-CAT

1.5.1 PCR for *rpsl*-CAT

Rpsl-CAT fragment was amplified by PCR, using previously prepared plasmid construct containing the *E .chaffeensis rpsl* promoter and CAT gene coding sequence (available in our laboratory). After PCR reaction, 3 µl of PCR products were analyzed in an agarose gel to identify predicted fragment of 948 bp.
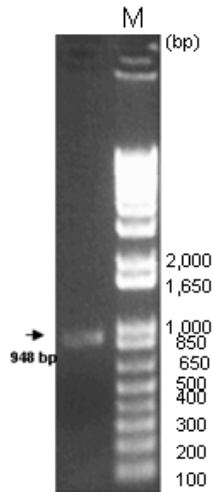

Figure 3.8   PCR for rpsl-CAT.

1.5.2 Restriction enzyme digestion of the PCR products of *rpsl*-CAT and the plasmids of pACD4K-C-gene 14/19/126-*tuf*. PACD4K-C constructs contain a kanamycin gene cassette downstream to group II intron and within the intron encoding protein coding region. This cassette can be replaced with an alternate antibiotic selection marker, such as chloramphenicol acetyltransferase (CAT), as it is present between two *Mlu*I restriction enzyme sites as a 1.37 kb insert. The PCR products of *rpsl*-CAT (0.95 kb) were also engineered to contain *Mlu*I sites at both ends of the segment. The PCR products of *rpsl*-CAT and the plasmids of pACD4K-C-gene 14/19/126-*tuf* were digested by *Mlu*I. Digested products were resolved in an agarose gel to identify predicted fragments (6.67 kb and 1.37 kb from plasmids and 0.95 kb for *rpsl*-CAT (Figure 3.9)).
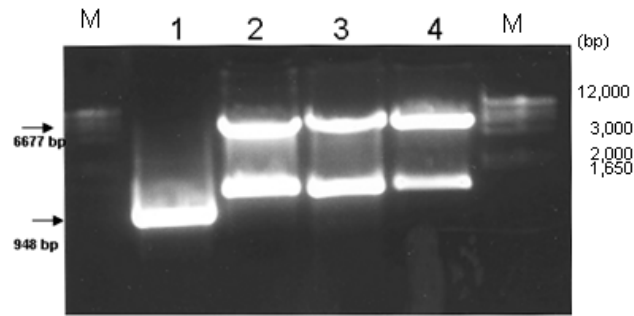
Figure 3.9   Double digestion with *Mlu*I. Lanes 1, 2, 3 and 4 are digested PCR products of *rpsl*-CAT,

the digested plasmid of pACD4K-C-gene 14, the digested plasmid of pACD4K-C-gene 19, and the

digested plasmid of pACD4K-C-gene 126, respectively.

The *Mlu*I digested fragment of 6.67 kb for each of the three plasmid constructs for Ech_1136, Ech_1143, and Ech_0126 genes and 0.95 kb of *rpsl*-CAT were isolated and used in ligation and cloning experiments in *E. coli* (TOPO 10 strain) to recover plasmids containing the *rpsl*-CAT inserts (Figure 3.10). The constructed plasmids pACD4K-C-gene 14/19/126-*tuf*-*rpsl*-CAT were prepared by boiling preparation method. The presence of *rpsl*-CAT inserts of 0.95 kb fragments were confirmed by digesting with *Mlu*I enzyme. The presence of correct sequence in the *rpsl*-CAT inserts was further confirmed by sequence analysis. The final constructs were stored in -80℃ for later use.
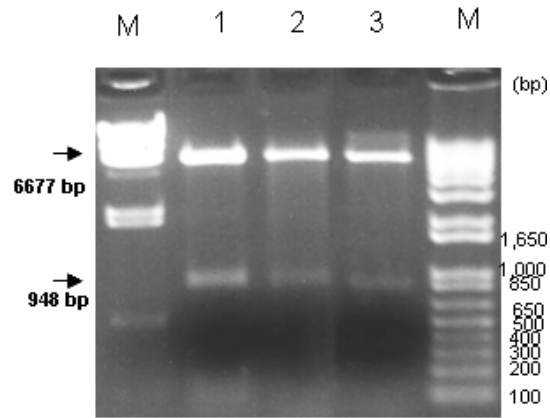
Figure 3.10   Confirmation of pACD4K-C-gene 14/19/126-*tuf-rpsl*-CAT. Lanes 1, 2, 3 are the plasmid contructs for Ech_1136, Ech_1143, and Ech_0126 genes, respectively.

2. Evaluating the transcription from Group II intron in *Escherichia coli*

PACD4K-C-gene 14/19/126-*tuf* -*rpsl*-CAT plasmids containing *E. coli* cultures were grown in 5 ml of LB media containing chlorephenicol (25 µg/ ml) for almost 14 h at 37℃. Total RNA from the cultures was isolated using Tri-reagent method (Sigma Chemical Corporation, St. Louis, MO) (Figure 3.11). The total RNA was treated with RQ1 Rnase-free Dnase enzyme. RNAs were analyzed for the presence of transcripts of group II intron driven by *E. chaffeensis tuf* promoter. Specifically, a primer set targeted to retargeted segments (Forward primers are IBS and reverse primers are EBS1d) was used to assess the expression (Figure 3.12). No products were detected when no template was used or when RNA template was used in a PCR for the RNA from retargeted plasmid containing *E. coli* of gene 14 and gene 19. Weak positive product was observed in PCR product of gene 126. When RNA was used in RT-PCR, all three RNAs yielded the predicted amplicons. For gene 126 construct RNA, the amplified product for RNA is significantly more than that observed in PCR. This suggests that the presence of positive PCR product in gene 126 construct RNA in the absence of reverse transcriptase may be due to residual plasmid DNA contamination. Together, these results demonstrate that the

group II intron constructs prepared for genes 14, 19, and 126 are functional in making transcripts.
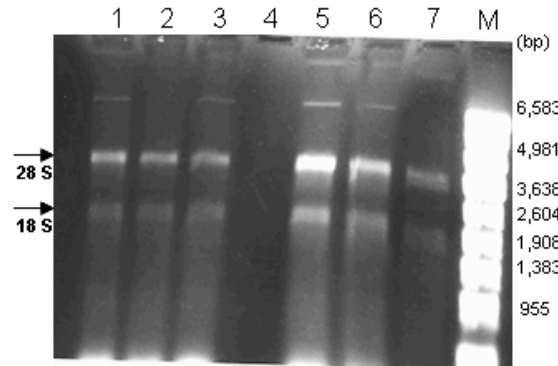

Figure 3.11   RNA gel of plasmids pACD4K-C-gene 14/19/126-*tuf* -*rpsl*-CAT. Lanes 1, 2, 3, 4, 5, 6

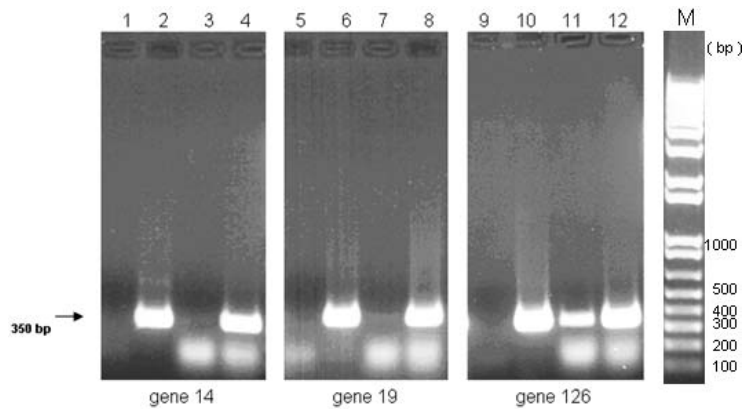and 7 are the total RNA isolated using Tri-reagent methods.

Figure 3.12 RT-PCR, PCR for extracted RNA and PCR for plasmid DNA. Lanes 1, 5, and 9 are

negative controls using Rnase free water as the template; lanes 2, 6, and 10 are PCR for plasmid DNA;

lanes 3, 7 and 11 are PCR for extracted RNA; lanes 4, 8 and 12 are RT-PCR for extracted RNA.

3 Transformation and mutation analysis

The modified Targetron plasmids for use in creating mutations in *E. chaffeensis* genes, Ech_1136, Ech_1143, and Ech_0126 were used in transformation experiments. Typically, 20 μg of purified plasmid DNA was electroporated into purified *E. chaffeensis* organism recovered from about 10 ml of 80-90% infected macrophage cultures representing ~80% confluency. The experiments of transformation were performed three times. *E. chaffeensis* was subjected to electroporation with the plasmids pACD4K-C-gene 14/19/126-*tuf-rpsl*-CAT by double shock at 1800 V. The experiment also included a control electroporation reaction that contained only *E. chaffeensis* organism and a second control that contains *E. chaffeensis* organism that were not subjected to electroporation. Electroporated and non electroporated *E. chaffeensis* organism were cultured in DH82 macrophage cells and harvested at different times. In the first experiment, the cells were harvested at 6 h, 12 h and 24 h; in the second and third experiment, the harvested cultures included at 6 h, 24 h, 48 h and 6 days. The harvested cultures were used to recover total genomic DNA for assessing the integration of group II introns into *E. chaffeensis* genome at the predicted locations of Ech_1136, Ech_1143, and Ech_0126 genes (described below). After harvesting the last batch of culture for DNA isolation, chloramphenicol was added to the culture media at a concentration of 2 μg/ml. Infection in DH82 cultures were monitored for up to two months by microscopy analysis. To date, no positive infection was found from all three transformation experiments. For further testing the insertion, PCR and Southern blot were performed.

3.1 Detection of insertions assayed by PCR methods

Two types of PCRs were performed to assess the integration of group II introns into *E. chaffeensis* genome. In the first set of PCRs, primers were designed to bind to the region upstream and downstream to integration site (these primers were expected to yield positive PCR products independent of the insertions). In the second set of PCRs, one primer is targeted *E. chaffeensis* genome either upstream or downstream to the insertion sites. The second primer of the second set was targeted to group II intron segment. The strategy and expected amplified fragments were described in Figure 3.13. To assess the mutations, the PCR products were analyzed in a 0.9 % agarose gel as well as Southern blot analysis after transferring to a nylon membrane and hybridization with group III intron specific ^{32}P-labeled DNA probes.

Ech_1136 (p28-Omp gene 14)

	Expected sizes
PCR I	Native: 860 bp / Mutated: 2.92 kb
PCR II a	Native: 0 bp / Mutated: 819 bp
PCR II b	Native: 0 bp / Mutated: 2.319 kb

Ech_1143 (p28-Omp gene 19)

	Expected sizes
PCR I	Native: 240 bp / Mutated: 2.299 kb
PCR II a	Native: 0 bp / Mutated: 399 bp
PCR II b	Native: 0 bp / Mutated: 2.219 kb

Ech_0126 (p28-Omp gene 126)

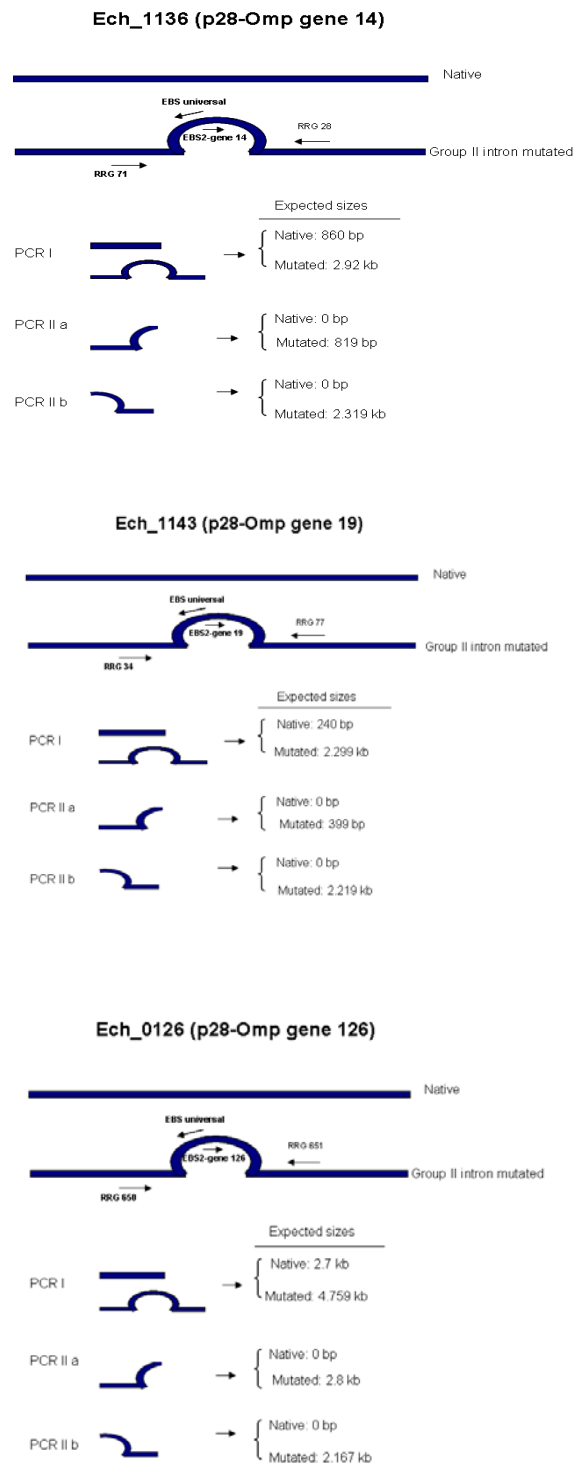|	Expected sizes
PCR I	Native: 2.7 kb / Mutated: 4.759 kb
PCR II a	Native: 0 bp / Mutated: 2.8 kb
PCR II b	Native: 0 bp / Mutated: 2.167 kb

Figure 3.13   PCR strategies for detecting mutants.

As outlined in the cartoon of Figure 3.13, predicted fragments from various PCR samples were analyzed for agarose gels or in the Southern blot results from PCR DNA hybridized with group II intron probe. Figure 3.14 has the agarose gel data for first experiment. In non-transformants and transformants for gene 14, we expected to see 0.86 kb and 2.92 kb amplicons, respectively, when PCR I is performed. In the gel, we did detect 0.86 kb, but not the 2.92 kb (Lanes 2, 4 and 6 of Figure 3.14). Predicted 0.82 kb fragment for PCR IIa was also not visible in the analyzed PCR products of gene 14 transformants (Lanes 1, 3, and 5 of Figure 3.14). Similar experiments for gene 19 yielded predicted fragments for PCR I (0.24 kb for gene 19; lanes 8, 10 and 12 of Figure 3.14) and for gene 126 as well (2.7 kb; lanes 14, 16 and 18 of Figure 3.14). As observed from results of gene 14, expected amplicons for transformants for genes 19 and 126 (2.3 kb and 4.7 kb, respectively) were also not clearly visible. The analysis was also complicated with the presence of multiple non-specific amplified DNA fragments observed in the agarose gel. PCR IIa strategy should yield positive bands only if the genomic DNA included insertion mutations (for gene 14, 0.82 kb; for gene 19, 0.39 kb and for gene 126, 2.8 kb). The predicted fragments were undetectable for gene 14 PCR IIa products (Lanes 1, 3, and 5 of Figure 3.14). However, we do detect minor predicted bands for gene 19 and 126 PCR IIa amplicons. In addition, we observed multiple non-specific amplicons in the lanes representing PCR IIa of genes 19 and 126 (Lanes 7, 9 and 11 of Figure 3.14 for gene 19 and lanes 13, 15 and 17 of Figure 3.14 for gene 126).
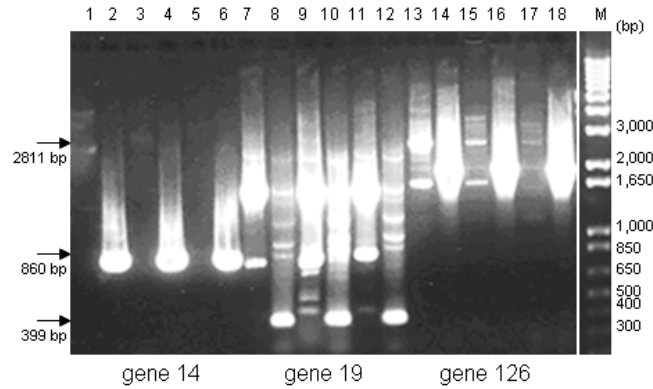
Figure 3.14   Experiment 1, PCR assay for detecting insertion. Lanes 1, 2, 3, 4, 5 and 6 are gene 14;

lanes 8, 9, 10, 11 and 12 are gene 19; and lanes 13, 14, 15, 16, 17 and 18 are gene 126; Lanes 1, 3, 5, 7,

9, 11, 13, 15, 17 are PCR IIa, and Lanes 2, 4, 6, 8, 10, 12, 14, 16, 18 are PCR I; Lanes 1, 2, 7, 8, 13, 14

are the electroporated cells harvested after 6 h; lanes 3, 4, 9, 10, 15, 16 are the electroporated cells

harvested after 12 h; lanes 5, 6, 11, 12, 17, 18 are the electroporated cells harvested after 24 h.
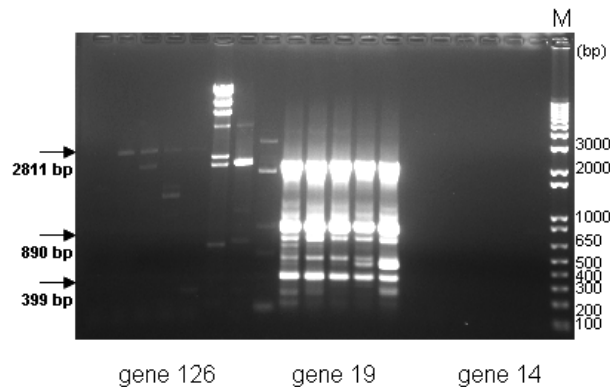

Figure 3.15   Experiment 2, PCR assay for detecting insertion. Gene 14, gene 19 and gene 126 all

have equal 6 lanes. For each of the six lanes, the templates for PCR assay are: from right to left, post 6

h harvested culture, post 24 h harvested culture, post 48 h harvested culture, post 6 days harvested

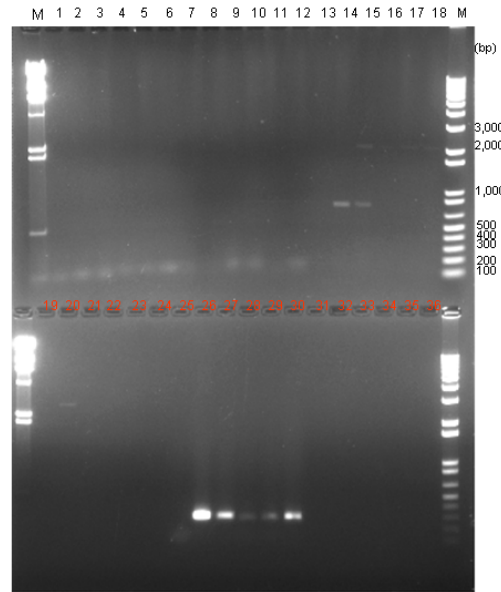culture, *E. chaffeensis* genome and nuclease free water.

Figure 3.16   Experiment 3, PCR assay for detecting insertion. Lanes 1, 2, 3, 4, 5 and 6 are PCR IIb for gene 19; lanes 7, 8, 9, 10, 11 and 12 are PCR IIa for gene 126; lanes 13, 14, 15, 16, 17 and 18 are PCR IIa for gene 19; lanes 19, 20, 21, 22, 23 and 24 are PCR I for gene 126; lanes 25, 26, 27, 28, 29 and 30 are PCR I for gene 19; and lanes 31, 32, 33, 34, 35 and 36 are PCR IIb for gene 126. For each of the six lanes, the templates for PCR assay are: from right to left, post 6 h harvested culture, post 24 h harvested culture, post 48 h harvested culture, post 6 days harvested culture, *E. chaffeensis* genome and nuclease free water.

3.2 Southern blot analysis

To further evaluate the insertion mutations, PCR DNAs resolved in the agarose gel were transferred to a nylon membrane and assessed by Southern blot using group II intron specific ^{32}P-labeled DNA probe. In the first experiment, predicted DNA amplicons in PCR IIa products were detected only for gene 19 and 126, and no detectable signals were observed for gene 14 (Figure 3.17). The signal intensity for gene 19 was the highest for 12 h post-transformation and 6 h post-transformation for gene 126. For gene 126, we also

observed smaller size non-specific amplicons. The signal intensity decreased in the samples anayzed for 24 h of gene 19 and 12 h and 24 h for gene 126. In the experiment 2 and 3, group II insertion strategy, using the modified Targetron plasmids for *E. chaffeensis* genes 14, 19 and 126, was repeated two additional times. In the second experiment (Figure 3.18), only PCR IIa strategy was followed to assess the presence of mutations in *E. chaffeensis* genome. Similar to the first experiment, agarose gel analysis had no detectable amplicons for gene 14 and multiple bands for gene 19 and 126 transformants. The resolved DNAs were transferred to nylon membrane and hybridized with group II intron probe to see if we can identify predicted amplicons. Consistent with the first experiment, Southern blot data was negative for gene 14, and positive for genes 19 and 126. Insertion-specific amplicons decreased for 6 h post-tranformation to 48 h post-transformation for gene 19 and undetectable in the DNA harvested after 6 days post-transformation. Similar analysis for gene 126 yielded positive only for 6 h time point. In the third experiment, we performed PCRs using the strategy of I, IIa, and IIb (as outlined in Figure 3.13). In addition, using the amplicons from the PCRs, we perfomed nested PCRs (two pairs of PCR primers were used. The first pair of primers amplified the locus as seen in any PCR experiments, the second pair of primers bind within the first PCR product and produce a second PCR product that will be shorter than the first one.). The aim for this experiment is to see if we can reduce the non-specific amplicons and also to enhance the signal intensity of the amplified products. The analysis for this experiment was performed for 6 h, 24 h, 48 h and 6 d similar to the second experiment.
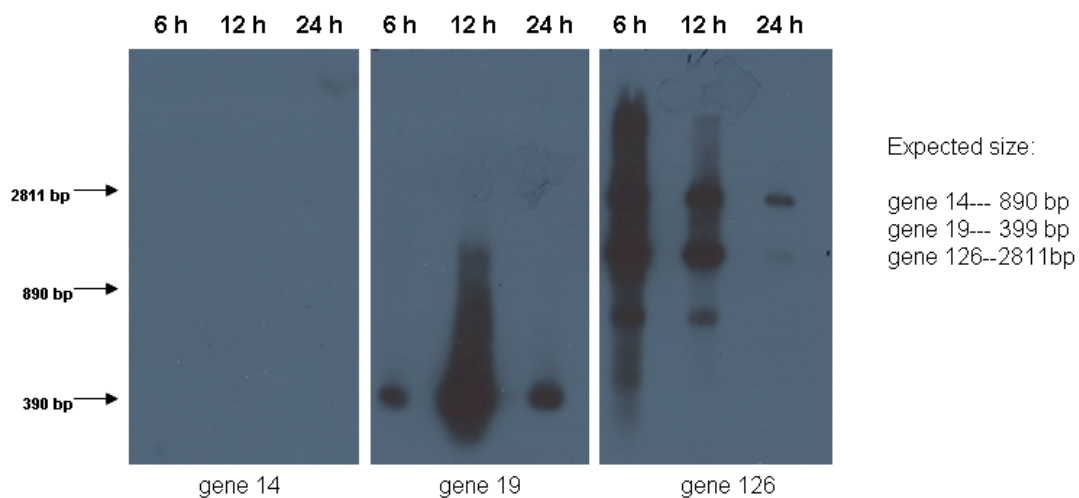
Figure 3.17   Experiment 1, Southern blot for detecting insertion. 6 h: Southern blot for post 6 h

harvested culture; 12 h: Southern blot for post 12 h harvested culture; and 24 h: Southern blot for post
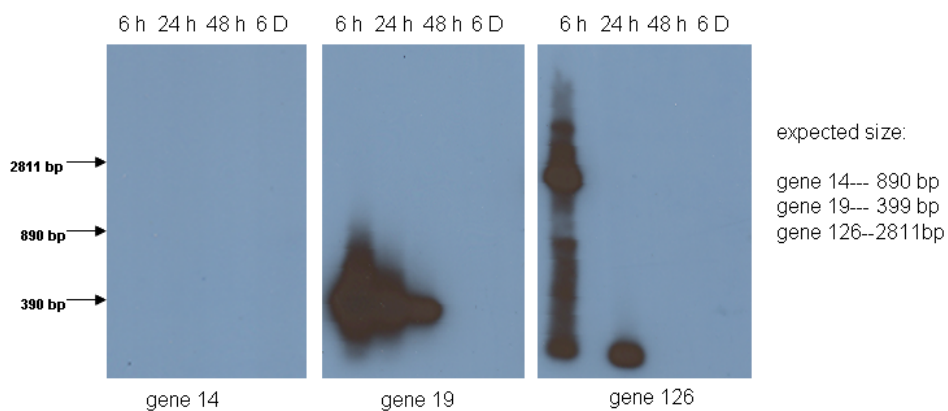
24 h harvested culture.

Figure 3.18   Southern blot for experiment 2. 6 h: Southern blot for post 6 h harvested culture; 24 h:

Southern blot for post 24 h harvested culture; 48 h: Southern blot for post 48 h harvested culture; and

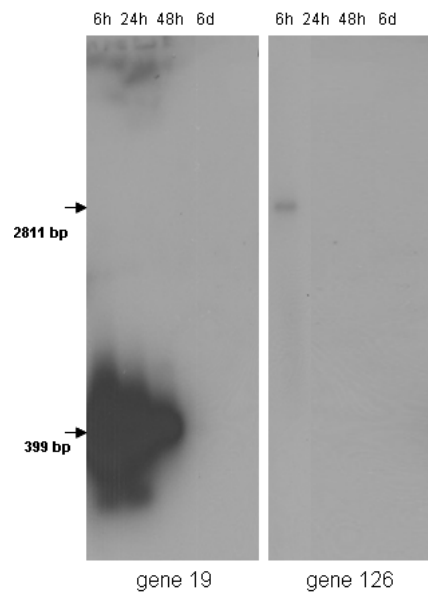6 D: Southern blot for post 6 days harvested culture.

Figure 3.19   Southern blot for experiment 3. Lanes 1, 2, 3, 4 are 6 h, 24 h, 48 h and 6 d post

transformation culture of gene 19 using PCR IIa; lanes 5 is 6 h post transformation culture of gene 126

using PCR IIa.

Discussion

E. chaffeensis is an emerging tick-borne pathogen responsible for an important disease in people, human monocyte ehrlichiosis (Dawson *et.,* 1991; Lockhart *et al.,* 1995). The disease was first discovered in 1987 and subsequently reported in many parts of the USA and world (Bakken *et al.,* 1996; Bakken *et al.,* 1998; Dumler *et al.,* 1998; Whitlock *et al.,* 2000; Standaert *et al.,* 2000; Olano *et al.,* 2003; Paddock *et al.,* 2003; McNabb *et al.,* 2008). *E. chaffeensis* also causes infections in several other vertebrate hosts (Dugan *et al.,* 2000; Yabsley *et al.,* 2004; Dugan *et al.,* 2005; Yu *et al.,* 2008). Despite recent progress, the pathogensis and how the organism persists in vertebrate hosts and ticks still remain to be known.

Recent studies demonstrate differential expression of *E. chaffeensis* proteins in a host cell specific manner (Ohusi *et al.,* 1998; Unver *et al.,* 2002; Long *et al.,* 2002; Singu *et al.,* 2005; Seo *et al.,* 2008). The protein expression differences are also shown to be influencing the host response and the pathogen persistence (Brelun *et al.,* 1996; Cotter *et al.,* 2000). In this study, we evaluated a method to create mutations *in E. chaffeensis* genome. Targetron mutagenesis system can be exploited to introduce mutations in virtually any bacterial organism (Yao *et al.,* 2006; Cui *et al.,* 2007; Heap *et al.,* 2007; Rodrigurez *et al.,* 2008). However, to adapt to a species, such as to *E. chaffeensis*, one needs to make several manipulations to the Targetron constructs prior to use in creating mutations in the organism. First, we needed to identify suitable regions to create mutations in the *E. chaffeensis* genome. Second, we had to adapt the group II intron expression system for *E. chaffeensis* using a constitutively expressing gene promoter of

this organism. Third, we were required to introduce an antibiotic selection marker cassette into the Targetron constructs. The antibiotic resistance protein expression also requires an *E. chaffeensis* promoter that is stably expressed. Once the constructs were prepared, appropriate conditions for introducing foreign DNA into *E. chaffeensis* and methods to recover viable cultures were also necessary. In this project, our efforts were focused to develop all the above described methods.

We selected three genes of *E. chaffeensis* for creating mutations: 1) a gene which appeared to be transcriptionally silent (Ech_0126); 2): a gene predominantly expressing *E. chaffeensis* when it replicates in tick cells (Ech_1136; p28-Omp gene 14); and 3) a gene primarily expressed in the organism when grown in macrophages (Ech_1143; p28-Omp gene 19). The justification for selecting these genes are as follows: if a mutation is detrimental for *E. chaffeensis*, we may not be able to recover viable cultures following the introduction of such mutations. Therefore, we selected a transcriptally silent gene (Ech_0126) for one of the experiment. Assuming that this gene is not important for *E. chaffeensis* survival, we may be able to recover positive mutants if introduced in this gene. The choice of tick cell specific gene expression may allow the mutant *E. chaffeensis* to grow well in macrophage cultures, but may not allow its growth in tick cell cultures. Thus, we selected Ech_1136 for this type of mutations. This gene, also referred to as p28-Omp 14 gene, is primarily expressed in the organism replicating in ticks cells or in infected ticks (Ganta *et al.,* 2002; Ganta *et al.,* 2004; Ganta *et al.,* 2007). In the third selection, Ech_1143 was selected as it is abundantly expressed in macrophage cultures or infected vertebrate hosts (Ganta *et al.,* 2002; Ganta *et al.,* 2004; Ganta *et al.,* 2007). This

gene is also expressed at low levels in tick cell-derived bacteria (Ganta *et al.,* 2002; Ganta *et al.,* 2004; Ganta *et al.,* 2007). This gene also belongs to the p28-Omp multigene locus and is referred to as p28-Omp 19. If expression from this gene is important for the bacteria growth in both host cell backgrounds or only critical in one or the other, we may see selective growth of mutants.

We selected an *E. chaffeensis* "*tuf*" promoter to drive the expression of modified group II introns targeted to Ech genes (Ech_0126, Ech_1136, and Ech_1143). The *tuf* promoter transcription was previously verified by our research team. This is a strong *E. chaffeensis* promoter which is responsible for the transcription of more than 22 genes, all of which are housekeeping genes (GeneBank cp 000236.1). In this study, we confirmed the promoter activity of *tuf* promoter in *E.coli* for driving expression of β-galactosidase gene activity (Figure 3.5). Subsequently, the promoter was transferred to Targetron constructs. The RNA expression from *tuf* promoter driving the expression of group II modified intron and intron encoded protein transcripts were also demonstrated in this study (Figure 3.11). These results were important prior to investigating the Targetron constructs in creating mutations in *E. chaffeensis*.

In support of identifying *E. chaffeensis* mutants, we inserted chloramphenicol actyltransferase (CAT) gene coding sequence of *E. coli* with another constitutively expressed *E. chaffeensis* promoter, *rpsl.* The *rpsl* promoter belongs to protein synthesis complex genes, ribosomal proteins S12 (Ech_0963). The CAT gene expression from *rpsl* promoter was also confirmed in *E. coli* by selecting clones resistant to chloramphenicol.

The transformant methods for *E. chaffeensis* required high volumes of purified, viable organisms. Our standardized protocols were effective in recovering viable *Ehrlichia* organism. In all three transformation experiments, we were able to demonstrate the growth of *E. chaffeensis* following electroporation. We also standardized conditions for transforming *E. chaffeensis* with Targetron constructs. Several electroporation conditions were tested which varied from 1200 V to 2400 V and single and double shocks. We found the most effective method of transformation is by providing two electric shocks at 1800 V.

Several pilot experiments were also performed to purify high quality DNA for use in transformation experiments. We found the Qiagen plasmid Endofree maxi kit (Qiagen, CA) to be the best in isolating high quality DNA. We also evaluated several plasmid DNA concentrations, which ranged from 2 μg to 20 μg and found the higher concentration of 20 μg to be the best for transformations. The selection 20 μg was based on the published evidence of transformations in other Rickettsial agents, *Rickettsia prowazekii* (Liu *et al.,* 2007). In our final experiment of transformation, we were able demonstrate the integration of modified group II introns into *E. chaffeensis* genome at two locations: Ech_0126 and Ech_1143. Insertions at Ech_0126 (gene 126) were following for up to 6 days in all three experiments by PCR and Southern blot analysis prior to adding chloramphenicol. In the first experiment, the analysis was performed only for two days. Predicted (0.89 kb) amplicons were clearly visible following Southern blot analysis. Minor bands of expected size were also seen in the agarose gel resolved DNA of amplicons obtained for PCR IIa, in which one primer targeted to group II intron and the

second primer targeted to the genomic regions 5' to predicted insertion site. The PCR positives in the first experiment, however, decreased with increasing time following transformation. The presence of PCR positives, however, was only seen for the first harvest, i.e. after 6 h post transformation in the cultured organisms in the second experiment. Similar transformation experiments for Ech_1143 (gene 19) also led to positive insertions in the genome and are detectable for up to 24 h in culture following the transformation. However, we found no evidence of mutations for gene 14 (Ech_1136). The detection of PCR positives for mutations in genes 126 and 19 are very encouraging and clearly demonstrate that *E. chaffeensis* genome can be mutated using Targetron-based insertion mutation methods. Introduction of mutations in *E. chaffeensis* or other species of *Ehlichia* and *Anaplasma* genome were not demonstrated earlier. Therefore, this study represents an important milestone in advancing the gap of knowledge in understanding the significance of host-specific protein expression.

It is not clear why mutated *E. chaffeensis* for gene 19 and 126 were not stable beyond 24 h. It is also not clear why the intensity of PCR positive bands decreased with time post transformation. One possible explanation for the unstable mutations in culture is that the mutants of genes 19 and126 may be slow in their growth compared to wild type *E. chaffeensis*. The absence of positive mutants for gene 14 following Targetron-based mutagenesis analysis may be an indication that the loss of this gene function may lead to lethal outcome in *E. chaffeensis*.

In our studies, we followed the Targetron mutated *E. chaffeensis* of genes 19 and 126 for up to 2 months by adding the chloramphenicol at a concentration 2 µg/ml to the culture media. Our efforts to identify mutants by culture PCR did not result in positive clones. This result may be explained due to poor or no expression of chloramphenicol actyltransferase protein in *E. chaffeensis*. This may be important, due to the difference in the codons of the CAT gene coding sequences. In particular, the CAT gene construct used in our studies was derived from *E. coli*. The codon usage of *E. chaffeensis* is significantly different from that of *E.coli*. Future experiments, therefore, should involve optimizing the codons of CAT gene to *E. chaffeensis*. The second possibility is the concentration of chloramphenicol. We may consider reducing the chloramphenicol to minimum required concentration to inhibit the growth of only the wild type *E. chaffeensis*, but not the mutants. It is also important to investigate the possibility to create Targetron based mutations in other regions of *E. chaffeensis* genome. All these experiment are now underway in our laboratory.

In summary, considerable progress has been made in introducing mutation in *E. chaffeensis*. We are the first to demonstrate the feasibility to introducing mutations at two regions of *E. chaffeensis* genome. We standardized numerous protocols needed in support of transformation and to create mutations in *E. chaffeensis*. The Targetron constructs developed in this study can be adapted easily to target other genomic regions of *E. chaffeensis* and also for use in creating mutations in other tick borne rickettisal agents of the genera *Ehrlichia* and *Anaplasma*.

Reference:

Bakken, J. S., J. Krueth, C. Wilson-Nordskog, R. L. Tilden, k. Asanovich, and J. S. Dumler. 1996. Clinical and laboratory characteristics of human granulocytic ehrlichiosis. JAMA **275**:199-205

Bakken, J. S., P. Goellner, M. V. Etten, D. Z. Boyle, O. L. Swonger, S. Mattson, J. Krueth, R. L. Tilden, k. Asanovich, J. Walls, and J. S. Dumler. 1998. Seroprevalence of human granulocytic ehrlichiosis among permanent residents of northwestern Wisconsin. Clin. Infect. Dis. **27**:1491-1496.

Brehm, K., J. Kreft, M. T. Ripio, and J. A. Vazquez-Boland. 1996. Regulation of virulence gene expression in pathogenic Listeria. Microbiologia **12**:219-236.

Chen, S. M., J. S. Dumler, J. S. Bakken, and D. H. Walker. 1994. Identification of a granulocytotropic Ehrlichia species as the etiologic agent of human disease. J. Clin. Microbiol. **32**:589-595.

Cotter, P. A. and V. J. DiRita. 2000. Bacterial virulence gene regulation: an evolutionary perspective. Annu. Rev. Microbiol. **54**:519-565

Cui X, Davis G. 2007. Mobile group II intron targeting: applications in prokaryotes and perspectives in eukaryotes. Front Biosci. **12:**4972–4985.

Dawson, J. E., B. E. Anderson, D. B. Fishbein, C. Y. Sanchez, C. Y. Goldsmith, K. H. Wilson, and C. W. Duntley. 1991. Isolation and characterization of an *Ehrlichia* sp. from a patient diagnosed with human ehrlichiosis. J. Clin. Microbiol. **29**:2741-2745.

Dugan, V. G., S. E. Little, D. E. Stallknecht, and A. D. Beall. 2000. Natural infection of domestic goats with Ehrlichia chaffeensis. J. Clin. Microbiol. **38**:448-449.

Dugan, V. G., J. K. Gaydos, D. E. Stallknecht, S. E. Little, A. D. Beall, D. G. Mead, C. C. Hurd, and W. R. Davidson. 2005. Detection of Ehrlichia spp. in raccoons (Procyon lotor) from Georgia. Vector. Borne. Zoonotic. Dis. **5**:162-171.

Dumler, J. S. and J. S. Bakken. 1998. Human ehrlichioses: newly recognized infections transmitted by ticks. Annu. Rev. Med. **49:201-13**.

Ganta, R. R., M. J. Wilkerson, C. Cheng, A. M. Rokey, and S. K. Chapes. 2002. Persistent Ehrlichia chaffeensis infection occurs in the absence of functional major histocompatibility complex class II genes. Infect. Immun. **70**:380-388.

Ganta, R. R., C. Cheng, M. J. Wilkerson, and S. K. Chapes. 2004. Delayed clearance of Ehrlichia chaffeensis infection in CD4+ T-cell knockout mice. Infect. Immun. **72**:159-167.

Ganta, R. R., C. Cheng, E. C. Miller, B. L. McGuire, L. Peddireddi, K. R. Sirigireddy, and S. K. Chapes. 2007. Differential clearance and immune responses to tick cell-derived versus macrophage culture-derived Ehrlichia chaffeensis in mice. Infect. Immun. **75**:135-145.

Guo,H., Karberg,M., Long,M., Jones,J.P.,3rd, Sullenger,B. and Lambowitz,A.M. 2000. Group II introns designed to insert into therapeutically relevant DNA target sites in human cells. *Science*. **289**: 452–457

Guo, H., Zimmerly, S., Perlman, P.S. & Lambowitz, A.M. 1997. Group II intron endonucleases use both RNA and protein subunits for recognition of specific sequences in double-stranded DNA. *EMBO J.* **16:** 6835–6848.

Huberty, T. J. Kurtti, G. G. Ahlstrand, B. Greig, M. A. Mellencamp, and J. L. Goodman. 1999. Invasion and intracellular development of the human granulocytic ehrlichiosis agent in tick cell culture. J. Clin. Microbiol. **37**:2518-2524

John T. Heap, Oliver J. Pennington, Stephen T. Cartman, Glen P. Carter and Nigel P. Minton. 2007. The ClosTron: A universal gene knock-out system for the genus Clostridium. *Journal of Microbiological Methods*. **70:** 452-464.

Jun Yao, Jin Zhong, Yuan Fang, Edward Geisinger, Richard P. Novick and Alan M. Lambowitz. 2006. Use of targetrons to disrupt essential and nonessential genes in *Staphylococcus aureus* reveals temperature sensitivity of Ll.LtrB group II intron splicing. RNA **12:**1271-1281.

Karberg,M., Guo,H., Zhong,J., Coon,R., Perutka,J. and Lambowitz,A.M. 2001. Group II introns as controllable gene targeting vectors for genetic manipulation of bacteria. *Nature Biotechnol.* **19:** 1162–1167

Lambowitz, A.M., Caprara, M.G., Zimmerly, S. & Perlman, P.S. 1999. Group I and group II ribozymes as RNPs: clues to the past and guides to the future. In *The RNA world*, Edn. 2. (eds Gesteland, R.F., Cech, T.R. & Atkins, J.F.) 451–484(Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY).

Lockhart, J. M., W. R. Davidson, D. E. Stallknecht, J. E. Dawson, and E. W. Howerth. 1997. Isolation of Ehrlichia chaffeensis from wild white-tailed deer (Odocoileus virginianus) confirms their role as natural reservoir hosts. J. Clin. Microbiol. **35**:1681-1686.

Long, S. W., X. F. Zhang, H. Qi, S. Standaert, D. H. Walker, and X. J. Yu. 2002. Antigenic variation of Ehrlichia chaffeensis resulting from differential expression of the 28-kilodalton protein gene family. Infect. Immun. **70**:1824-1831.

McNabb, S. J., R. A. Jajosky, P. A. Hall-Baker, D. A. Adams, P. Sharp, C. Worshams, W. J. Anderson, A. J. Javier, G. J. Jones, D. A. Nitschke, A. Rey, and M. S. Wodajo. 2008. Summary of notifiable diseases--United States, 2006. MMWR Morb. Mortal. Wkly. Rep. **55**:1-92.

Munderloh, S.D. Jauron, V. Fingerle, L. Leitritz, S.F. Hayes, J.M. Hautman, C.M. Nelson, B.W. Huberty, T.J. Kurtti, G.G. Ahlstrand, B. Greig, M.A. Mellencamp and J.L. Goodman. 1999. Invasion and intracellular development of the human granulocytic ehrlichiosis agent in tick cell culture, J. Clin. Microbiol. **37**: 2518–2524.

Ohasi, N., N. Zhi, Y. Zhang, and Y. Rikihisa. 1998. Immunodominant major outer membrane proteins of *Ehrlichia chaffeensis* are encoded by a polymorphic multigene family. Infect. Immun. **66**:132-139.

Olano, J. P., W. Hogrefe, B. Seaton, and D. H. Walker. 2003. Clinical manifestations, epidemiology, and laboratory diagnosis of human monocytotropic ehrlichiosis in a commercial laboratory setting. Clin. Diagn. Lab Immunol. **10**:891-896.

Paddock CD, Childs JE, 2003. *Ehrlichia chaffeensis*: a prototypical emerging pathogen. *Clin Microbiol Rev*. **16:** 37–64

Rodriguez, S. A., J. J. Yu, G. Davis, B. P. Arulanandam, and K. E. Klose. 2008. Targeted inactivation of *Francisella tularensis* genes by group II introns. Appl. Environ. Microbiol. **74:**2619-2626.

Seo, G.-M., C. Cheng, J. Tomich, and R. R. Ganta. 2008. Total, membrane, and immunogenic proteomes of macrophage- and tick cell-derived *Ehrlichia chaffeensis* evaluated by LC-MS/MS and MALDI-TOF methods, *In.*

Singu, V., H. Liu, C. Cheng, and R. R. Ganta. 2005. Ehrlichia chaffeensis expresses macrophage- and tick cell-specific 28-kilodalton outer membrane proteins. Infect. Immun. **73**:79-87.

Standaert, S. M., T. Yu, M. A. Scott, J. E. Childs, C. D. Paddock, W. L. Nicholson, J. J. Singleton, and M. J. Blaser. 2000. Primary isolation of *Ehrlichia chaffeensis* from patients with febrile illnesses: clinical and molecular characteristics. J. Infect. Dis. **181**:1082-1088.

Unver, A., Y. Rikihisa, R. W. Stich, N. Ohashi, and S. Felek. 2002. The omp-1 major outer membrane multigene family of Ehrlichia chaffeensis is differentially expressed in canine and tick hosts. Infect. Immun. **70**:4701-4704.

Whitlock, J. E., Q. Q. Fang, L. A. Durden, and J. H. Oliver, Jr. 2000. Prevalence of Ehrlichia chaffeensis (Rickettsiales: Rickettsiaceae) in Amblyomma americanum (Acari: Ixodidae) from the Georgia coast and Barrier Islands. J. Med. Entomol. **37**:276-280.

Yabsley, M. J., T. M. Norton, M. R. Powell, and W. R. Davidson. 2004. Molecular and serologic evidence of tick-borne Ehrlichiae in three species of lemurs from St. Catherines Island, Georgia, USA. J. Zoo. Wildl. Med. **35**:503-509.

Yu, D. H., Y. H. Li, J. S. Yoon, J. H. Lee, M. J. Lee, I. J. Yu, J. S. Chae, and J. H. Park. 2008. Ehrlichia chaffeensis infection in dogs in South Korea. Vector. Borne. Zoonotic. Dis. **8**:355-358.

Zhimei Liu, Aimee M. Tucker, Lonnie O.Driskell, and David O. Wood. 2007. mariner-Based Transposon Mutagenesis of Rickettsia prowazekii. Applied and environment microbiology. **73:**6644-6649.

Chapte IV

Major conclusions

1. Targetron modified constructs for *E. chaffeensis* genes Ech_1136, Ech_1143, and Ech_0126 were prepared.

2. *E. chaffeensis* "*tuf*" promoter was cloned and confirmed its function in *E. coli* using promoterless β-galactosidase expression system.

3. The *E. chaffeensis tuf* promoter driven expression of group II intron and intron encoded protein transcripts were also confirmed in *E. coli.*

4. The *E. chaffeensis rpsl* promoter was also cloned and utilized in driving an antibiotic selection marker in *E. chaffeensis.*

5. The final modified Targetron constructs include *E. chaffeensis tuf* promoter to drive the expression of modified group II introns and *rpsl* promoter to drive the expression of chloramphenicol acetyltranferse gene.

6. The Targetron constructs for *E. chaffeensis* genes 14, 19, and 126 were examined for transformation and creating mutations in the pathogen.

7. Mutants for *E. chaffeensis* genes 19 and 126 were identified in cultures for up to 48 h in three independent times.

8. No mutants were detected for gene 14.

9. The modified Targetron constructs are useful in preparing additional constructs for making mutations in alter regions of *E. chaffeensis* genome as well as for studying mutational analysis of other tick-borne pathogens of the genera *Ehrlicha* and *Anaplasma.*