MOLECULAR EVALUATION OF *EHRLICHIA CHAFFEENSIS*

by

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B.V.Sc & A.H., A. N. G. R. A. UNIVERSITY, HYDERABAD, INDIA, 2001
M.S., KANSAS STATE UNIVERSITY, USA, 2003

AN ABSTRACT OF A DISSERTATION

submitted in partial fulfillment of the requirements for the degree

DOCTOR OF PHILOSOPHY

Department of Diagnostic Medicine/Pathobiology
College of Veterinary Medicine

KANSAS STATE UNIVERSITY
Manhattan, Kansas

2008

Abstract

Ehrlichia chaffeensis, an emerging tick-borne pathogen, causes human monocytic ehrlichiosis (HME). The relationship between *E. chaffeensis* and its target cells in ticks and vertebrates is critical as the organism must persist in them. We hypothesize that *E. chaffeensis* alters gene expression in support of adapting to dual hosts. In support of testing this hypothesis, we developed an ORF-based microarray and performed global transcriptional analysis on the pathogen grown in macrophage and tick cells. The analysis revealed the expression of about 30% of all the predicted *E. chaffeensis* genes, in macrophages or tick cell. Two-thirds of the transcribed genes are common for both host cell backgrounds. About 20% of the commonly expressed genes also varied in expression levels which ranged from two to five fold. Microarray data was verified by RT-PCR for a subset of randomly selected genes. Together, this is the first report describing the global host cell-specific gene expression patterns in *E. chaffeensis*.

Differential gene expression may be an important adaptive mechanism used by *E. chaffeensis* for its continued survival in dual hosts. To test this hypothesis, we established many basic protocols and tools needed for performing mutational analysis in *E. chaffeensis.* Four antibiotic selection markers; gentamicin, chloramphenicol, spectinomycin and rifampin, and two promoters constitutively expressed in *E. chaffeensis*, genes *rpsL* and *tr*, were identified. Two regions of the genome were also identified for performing initial mutational analysis. Several plasmid constructs were also made. The optimal conditions for introducing these plasmids into host cell-free viable *E. chaffeensis* organisms were also established. The molecular evaluation of several *E. chaffeensis* transformants using these plasmids suggested that the plasmids gained entry, but failed to get integrated into the genome or remain in the bacteria for longer periods of time.

In summary, we demonstrated global host cell-specific differential gene expression in *E. chaffeensis* by employing microarray analysis. Numerous host-specific expressed genes will be important for studies leading to effective methods of control. We also established several basic protocols and tools needed for performing mutational analysis useful in evaluating the impact of the loss of expression of uniquely expressed genes.

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In summary, we demonstrated global host cell-specific differential gene expression in *E. chaffeensis* by employing microarray analysis. Numerous host-specific expressed genes will be important for studies leading to effective methods of control. We also established several basic protocols and tools needed for performing mutational analysis useful in evaluating the impact of the loss of expression of uniquely expressed genes.

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CHAPTER 1 - Literature and Significance

Vector-Borne Infections

Vectors in General

Living organisms that carry disease causing agents from one host to another are called vectors. There are two types of vectors; mechanical and biological. Mechanical vectors are defined as organisms that physically carry disease causing agents from one place to another, such as to carry a pathogen from a site where a pathogen may be present or from an infected host to another place. Mechanical vectors do not support the development or replication of a pathogen. The transfer of pathogens by mechanical vectors may lead to a disease in naïve animals and people. For example, a housefly can serve as a mechanical vector for carrying bacteria from one place to another and contact of such bacteria by a host can lead to an infection or disease (41). Inanimate objects such as gloves, clothing, equipment and shoes may also serve as vehicles to transfer infectious agents to animals or people (62). However, inanimate objects are not considered as mechanical vectors, but are referred to as fomites (12). Biological vectors differ from mechanical vectors as they also serve as hosts for the pathogens they carry and aid in their development within them. Many blood-sucking invertebrates are known to transmit infections to people and animals. Biological vectors may or may not be affected by the presence of pathogens they harbor. For example, *Anopheles* species mosquitoes, biological vectors for the malaria parasite, are not affected by the presence of *Plasmodium* parasites (27). In contrary, the human body louse, a biological vector for a rickettisial pathogen, *Rickettsia prowazeki*, dies within two weeks after acquiring infection (81).

Arthropods, a large group of invertebrates that belong to the phylum Arthropoda in the animal kingdom, are characterized by a rigid external skeleton, the presence of paired and jointed legs and a haemocoel. The phylum arthropoda includes four subphyla; Trilobita, Crustacea, Chelicerata and Uniramia. The subphylum Trilobita includes the mostly extinct

arthropods; Crustacea includes mostly aquatic arthropods; Chelicerata includes arthropods with six pairs of appendages, four pairs of which are legs; Uniramia, the largest subphyla includes arthropods with one pair of antennae and one or two pairs of maxillae. Several of the arthropods have been identified as important vectors for transmitting disease causing agents. For example, the sand fly, *Phlebotomus argentipes*, is a transmitting vector for *Leishmania donovani*, the causative agent for visceral leishmaniasis in people (113). Lice can transmit a variety of pathogens responsible for causing diseases in people. Louse-borne infections in people include epidemic typhus, relapsing fever and trench fever (57). Fleas are parasitic insects and transmit agents causing deadly diseases like plague (130). The major vector-borne diseases in humans, and associated etiological agents and arthropod vectors are listed in Table 1.1.

Arthropod-borne infections in people are of major public health concern and are responsible for heavy mortalities and morbidity. For example, arthropod-transmitted infectious agents causing malaria, dengue, yellow fever, plague, trypanosomiasis and leishmaniasis resulted in more human diseases and deaths in the 17th through early 20th centuries than all other causes combined (1). Currently, *Plasmodium* species parasites transmitted by mosquitoes infect 350-500 million people each year resulting in more than one million deaths world wide (WHO). Likewise, mosquitoes transmit several viruses which are responsible for serious infections in people and animals (55). *Aedes* species mosquitoes transmit chikungunya virus to people and severe outbreaks of this viral infections have been reported in several parts of the world (48). Eastern equine encephalitis (EEE) is a viral disease transmitted by *Aedes, Coquillettidia*, and *Culex* species mosquitoes (110). EEE virus infects humans, horses, and several bird species and results in encephalitis (110). *Culex* species mosquitoes can also transmit Japanese encephalitis virus and St. Louis encephalitis virus

Table 1.1: Important vector-borne diseases of humans, and associated etiological agents and arthropod vectors (except tick vectors)

Disease	Pathogen/Parasite	Arthropod Disease Vector
Viral diseases		
Dengue haemorrhagic Fever	DEN-1, DEN-2, DEN-3, DEN-4 flaviviruses	*Aedes aegypti* mosquito
Yellow fever	Yellow fever flavivirus	*Aedes aegypti* mosquito
Encephalitis*	Flavi-, alpha- and bunyaviruses	Various mosquito and Ixodid tick species
Bacterial diseases		
Epidemic Typhus	*Rickettsia prowazeki*	Mites
Protozoal diseases		
Malaria	*Plasmodium falciparum* *Plasmodium vivax* *Plasmodium ovale* *Plasmodium malariae*	*Anopheles* species mosquitoes
Leishmaniasis	*Leishmania* spp	*Lutzomyia* and *Phlebotomus* species Sandflies
Trypanosomiasis	*Trypanosoma brucei gambiense* *Trypanosoma brucei rhodesiense*	*Glossina* spp. (tsetse fly)
Chagas diseases	*Trypanosoma cruzi*	*Triatomine* spp.
Filarial nematodes		
Lymphatic filariasis	*Brugia malayi, Brugia timori, Wuchereria bancrofti*	*Anopheles, Culex, Aedes* and *Ochlerotatus* mosquitoes
Onchocerciasis	*Onchocerca volvulus*	*Simulium* species Blackflies

*Including Japanese encephalitis, West Nile encephalitis, St. Louis encephalitis, La Crosse encephalitis and tick-borne encephalitis.

causing infections resulting in neurological problems in people (118). West Nile Virus (WNV) is primarily transmitted by the bite of infected *Culex* species mosquitoes (16). WNV infection can also result in a life threatening disease in people as well as in many other vertebrates (75,82,107). Mosquitoes remain as the major arthropod vector and much of the mosquito-borne illnesses are centered in the tropical regions of the world, such as Africa, Asia and South America (108). Although mosquito-born illnesses have been reported in animals and people in recent years in the USA, they are better controlled due to living conditions. For example, WNV was first reported in New York in 1999 and rapidly spread to nearly all parts of the US and is responsible for several mortalities and morbidity (117). The disease, however, is on the decline now which may be attributed to better measures of mosquito control (*www.cdc.gov*).

The arthropod vectors can also transmit pathogens to plants (51). Arthropod-borne infections in plants can have significant impact on the economy. For example, citrus triesteza virus, an aphid transmitted infection to plants, is reported to have killed several millions of citrus trees worldwide and is now considered to threaten an estimated $912 million in orange crops of central California (61). Likewise, arthropod transmitted infections in animals also have significant economic impact. Bluetongue is a viral infection transmitted by *Culicoides* species insects to cattle and sheep. Bluetongue infections cause an estimated annual loss of $125 million to the livestock industry in the US alone and $3 billion world-wide (47). Several other arthropod transmitted diseases such as heart water fever, caused by *Ehrlichia ruminantium*; theileriosis, caused by *Theileria parva;* and babesiosis, caused by *Babesia* species cause heavy losses to the cattle industry world wide (10,11,63,73,93,98,150). These pathogens are transmitted by ticks. Ticks are the second most important arthropod vectors in transmitting infectious agents to people and animals (92,124,135).

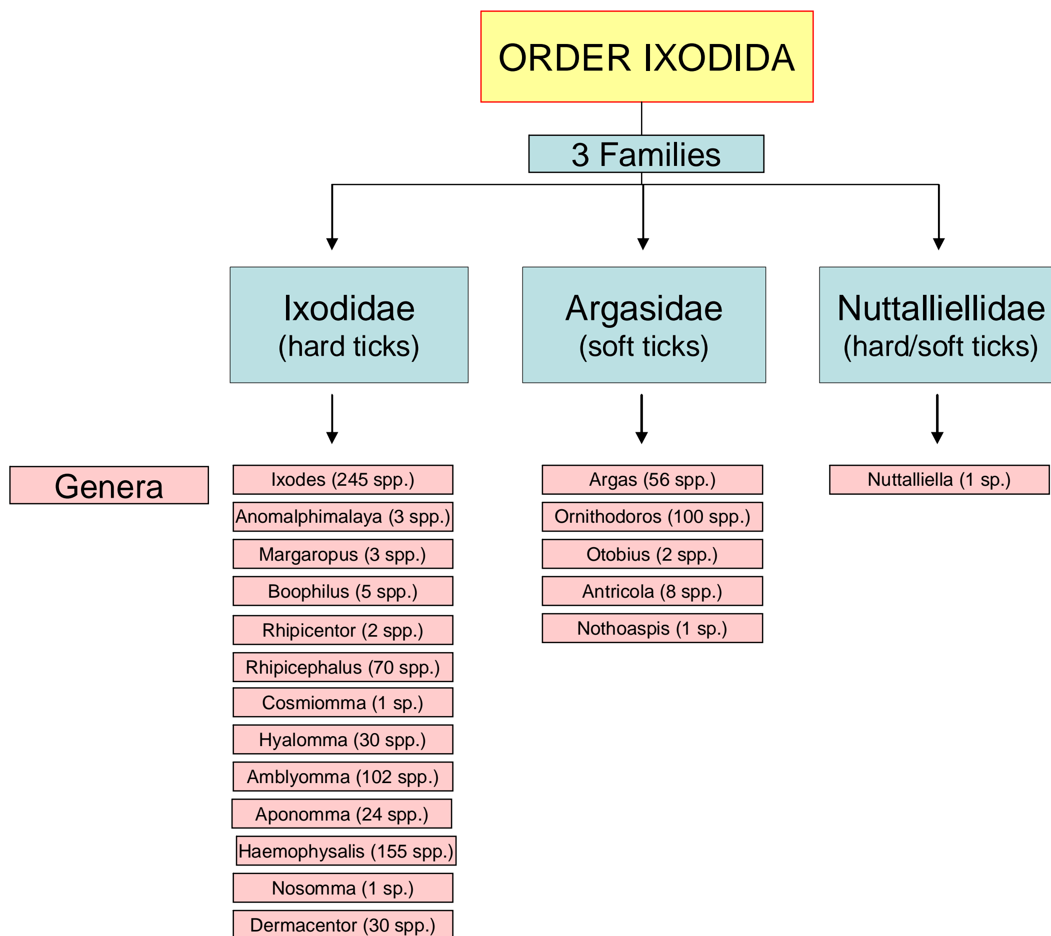
Biology of Ticks

Tick is the common name of small arthropods that belong to the subphylum *Chelicerata*, class *Arachnida* and order *Ixodida* (119). Ticks are the second most important group of arthropods that are reported to be acting as a vector for disease causing agents, mosquitoes being the first (119). There are about 850 known species of ticks that have been identified (119). The ticks are grouped into three families; *Ixodidae*, *Argasidae* and *Nuttalliellidae* based on morphological differences in body parts (Figure 1.1). The family *Ixodidae* consists of 13 genera and 650 species (Figure 1.1). Ticks belonging to this family are called hard ticks because of the tough sclerotized plate on the dorsal body surface, termed scutum. The *Argasidae* family includes 5 genera and approximately 170 species (Figure 1.1). The ticks of this family lack the scutum and instead have a leathery cuticle and so are termed as soft ticks. The family *Nuttalliellidae* is represented by one species, *Nuttalliela manaqua* and has morphological features of both hard and soft ticks. *N. manaqua* has a pseudoscutum which resembles a scutum but does not have the smooth appearance of the real scutum. Ticks belonging to the families *Ixodidae* and *Argasidae* act as vectors to transmit infectious agents to animals and people (119).

The life cycle of the tick has four different life stages; egg, larva, nymph and adult (Figure 1.2) (119). The life stages of the tick have significant morphological differences among them. For example, larval ticks have six legs where are nymphal and adult ticks possesses eight legs. Adult female ticks lay eggs following a blood meal and mating with a male. The eggs hatch to become larvae. The larvae obtain a blood meal from a smaller vertebrate host and molt to the nymphal stage. Nymphs also acquire a blood meal and molt to become adults. Adult female ticks require a blood meal and mating with a male tick before it can lay eggs. The total time needed to complete the life cycle may vary from less than a year to over three years depending on the climatic conditions, host availability and tick species. The life cycle of

Figure 1.1. Classification of ticks.

The ticks are grouped into three families; *Ixodidae*, *Argasidae* and *Nuttalliellidae* based on morphological differences in body parts. The family *Ixodidae* consists of 13 genera and 650 species. Ticks belonging to this family are called hard ticks because of the tough sclerotized plate on the dorsal body surface, termed scutum. The *Argasidae* family includes 5 genera and approximately 170 species. The ticks of this family lack the scutum and instead have a leathery cuticle and so are termed soft ticks. The family *Nuttalliellidae* is represented by one species, *Nuttalliela manaqua* and has morphological features of both hard and soft ticks. Ticks belonging to the families; *Ixodidae* and *Argasidae* act as vectors to transmit infectious agents to animals and people.



soft ticks differ by having multiple nymphal stages and molts before they finally molt to adult stage. So, the soft ticks may need to acquire several blood meals during the nymphal stage of its life cycle to finally molt to adults. The total time to completion of the entire life cycle for soft ticks is much longer than that of hard ticks and also depends on the climate, host availability and tick species (119).

Ticks are ectoparasites and depend on a blood meal from various vertebrate animals including humans, wild and domestic mammals, birds and reptiles. The ticks have evolved to have mouth parts that facilitate them to acquire a blood meal from vertebrate hosts (119). The mouth parts of the ticks include a pair of palps, a pair of chelicerae and a hypostome. All these mouth parts are mounted on a large cylindrical structure called the basis capituli (Figure 1.3). Chelicerae are the cutting organs used to penetrate the host's skin and gain access to fluids. The palps have gustatory, mechanosensory and thermosensory functions and act as sensory organs to facilitate feeding (119). The hypostome is a rod shaped structure and is a primary attachment organ of the host. It enters into the host's skin during feeding and has backward directed projections that prevent easy removal of the attached tick. Though hard ticks and soft ticks have similar mouth parts, they differ in their feeding habits. The hard ticks attach to the hosts for several days to few weeks and acquire the blood meal slowly. The salivary glands of the hard ticks produce a cement-like substance which glues the feeding tick in place. Soft ticks are fast feeders and they attach onto the host for few minutes to hours and acquire complete blood meals quickly. The cuticle of the female hard ticks continues to grow to accommodate the large volume of the ingested blood. This result in an increase in body size of several hundred times more than their unfed body weight. In contrast, the cuticle of an adult soft tick can only expand but can not grow and so the fed tick weight can increase only up to 5-10 times more than their unfed body weight. The meal size for nymphs and

Figure 1.2 Life cycle of hard ticks.

The life cycle of the tick has four different life stages; egg, larva, nymph and adult. Adult female ticks lay eggs upon blood meal and mating with a male. The eggs hatch to become larvae. The larvae obtain a blood meal from a smaller vertebrate host and molt to the nymphal stage. Nymphs also acquire a blood meal and molt to become adults.

www.iassistdata.orgimagesticklifecycle.jpg

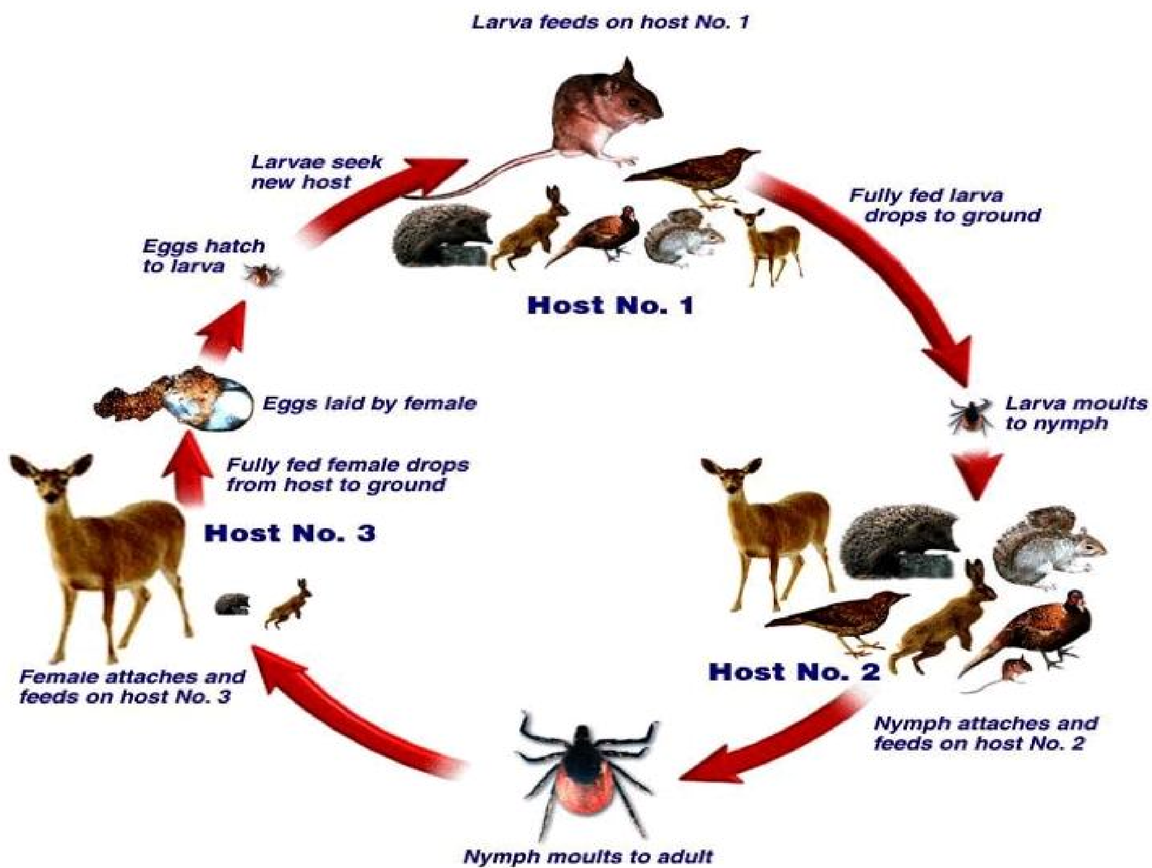
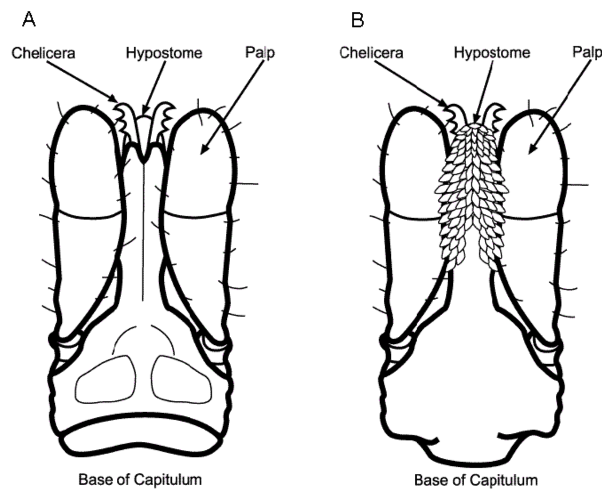


Figure 1.3 Generalized mouthparts of a hard tick, based on a species of *Ixodes*. The mouth parts of the ticks include a pair of palps, a pair of chelicerae and a hypostome mounted on a large cylindrical structure called capitulum. Chelicerae are the cutting organs used to penetrate the host's skin and gain access to fluids. The palps have gustatory, mechanosenseory and thermosensory functions and act as sensory organs to facilitate feeding. The hypostome is a rod shaped structure and is a primary attachment organ of the host. It enters into the host's skin during feeding and has backward directed projections that prevent easy removal of the attached tick. A, dorsal view; B, ventral view.

http://www.entm.purdue.edu/publichealth/images/tick/tick02.gif



adults of argasid ticks, and Ixodid adult males is typically much smaller than that acquired by adult female Ixodid ticks (119).

Tick as an ectoparasite can impact the health of a vertebrate host. For example they can suck large quantities of blood from a vertebrate host which may result in anemia. Ticks may also provoke hypersensitivity reactions including anaphylaxis (9). Some tick species secrete a neurotoxin from their salivary glands as they feed on the hosts; this may lead to paralysis followed by death (50). This condition is commonly referred to as tick paralysis (50). Damage caused by infested ticks to the host is reported to facilitate infection of the host with other infectious agents like *Dermatophilus congeolensis* (66,133).

Ticks as transmitting vectors for pathogens:

The life span of the ticks is very long ranging from several months to years; they can survive for several months, without a blood meal, waiting for a suitable vertebrate host (119). Ticks have evolved strategies to survive while waiting for hosts; for example they can absorb moisture directly from the atmosphere by using hygroscopic substances secreted by the salivary glands. This longevity and exceptional resistance to adverse conditions help ticks not only to perpetuate for several years, but also to carry the infectious agent in nature for long times. Ticks can lay large numbers of eggs. For example, *Amblyomma nuttalli* is reported to lay more than 22,000 eggs in its life time. The ability of laying large numbers of eggs is another survival feature for ticks to maintain their populations and spread effectively in nature. Another important feature is the digestion process in ticks. The blood acquired from the host is stored in the midgut of ticks and is digested slowly over several months to years. The digestion process is primarily intracellular within midgut cells, i.e., digestive enzymes are not secreted into the

midgut thus providing the pathogens that may be present in the blood to escape into the tissues of the tick (119). All these unique features allow the persistence of tick in nature, and also the pathogens they carry.

Ticks, during their life stages, can acquire pathogens during feeding on an infected vertebrate host. The pathogens acquired during one stage of the life cycle, for example larva or nymph can be maintained in the subsequent life stages like nymph or adult; this phenomenon is called transstadial transmission. *Ehrlichia chaffeensis*, a rickettsial pathogen is transstadially transmitted by *Amblyomma americanum* ticks (5,87). If the pathogen acquired by the tick is transmitted to the future generations through the eggs, it is termed as transovarial transmission. *Rickettsia rickettsii* transmitted by ticks, is passed on from parent to offspring tick by transovarial transmission (74). As ticks acquire blood meals from a variety of vertebrate hosts during their life cycle, they can transmit the infectious agents to different vertebrate hosts such as wild and domestic animals and people (77,92,119).

Ticks are known to transmit a variety of pathogens to vertebrate animals such as protozoa, bacteria including rickettsiae and viruses (40,92,119,124,127). For example, protozoan parasites belonging to the genera *Babesia* and *Theileria* are transmitted by ticks (11,98). The *Babesia* species parasites cause infections in several animals (mice, dogs, horses and cattle). *Theileria parva* and *Theileria annulata* are responsible for the disease theileriosis in cattle (98). Several bacterial pathogens transmitted by the ticks can significantly impact the health of animals. *Ixodes* species ticks can transmit the Lyme disease agent, *Borrelia burgdorferi*, to dogs, horses and cats (128,132). Similarly, *Ehrlichia ruminantium,* causative agent of Heart water fever in cattle, is transmitted by *Amblyomma* species ticks. *Anaplasma marginale*, the causative agent of bovine anaplasmosis, is transmitted by ticks (151). Tick borne infections are also responsible for economic losses to the animal industry (135). A recent

estimate by International Livestock Research Institute suggests that ticks alone are responsible for 14-17 billion dollars of economic loss annually to the livestock industry worldwide (59). In the cattle industry, tick transmitted infectious agents causing diseases, such as theileriosis, babesiosis, anaplasmosis, and Heart water fever results in the loss of several millions of dollars annually in sub-Saharan African countries (60). The economically important infections transmitted by ticks to animals are listed in Table 1.2.

Ticks also harbor and transmit protozoan, bacterial and viral pathogens to people (135). For example, *Ixodes dammini* ticks transmit a protozoan parasite *Babesia microti*, the causative agent of human babesiosis to people in the USA (106). *Ixodes* spp. ticks transmit lyme disease agent, *Borrelia burgdorferi*, to people (83). *Dermacenter* and *Haemophysalis* species ticks can transmit the bacterial pathogen *Francisella tularensis* responsible for tularemia in people (49). *Dermacentor* species ticks are primarily responsible for transmitting *Rickettsia rickettsii*, the causative agent of Rocky mountain spotted fever in the USA (137). Ticks belonging to genera *Amblyomma* and *Ixodes* transmit *Ehrlichia* and *Anaplasma* species pathogens responsible for Ehrlichiosis in people (2,5,7,15,30,31). Ticks can also act as a vector for transmitting several viral agents (such as *Tick-born encephalitis virus* (TBEV) and other TBEV complex viruses belonging to *Flavivridae*) causing encephalitis and hemorrhagic fever in people in many parts of the world (76). The important human pathogens and the tick vectors responsible for the infections are listed in Table 1.3.

In summary, ticks can transmit several disease causing agents to people. Lyme disease agent is one of the few pathogens that is considered an important tick transmitted agent in USA. Lyme disease has been reported from several parts of USA

with predominant number of cases from northeastern states. Another important human disease, Rocky mountain spotted fever

Table 1.2 Economically important infections transmitted by ticks to animals

Disease	Etiological agent	Tick species	Distribution
Bovine Babesiosis	*Babesia bovis* *Babesia bigemina*	*Boophilus* species	World-wide
Equine Babesiosis	*Babesia equi*	*Dermacenter* species	World-wide
Bovine Anaplasmosis	*Anaplasma marginale* *Anaplasma centrale*	*Dermacenter* species *Rhipicephalus* species *Boophilus* species	World-wide
Ovine and Caprine Anaplasmosis	*Anaplasma ovis*	*Dermacenter* species	World-wide
Theileriosis	*Theileria* species	*Amblyomma* species *Hyalomma* species *Dermacentor* species *Rhiphicephalus* species *Haemophysalis* species	World-wide
Heart water disease in ruminants	*Ehrlichia ruminantium*	*Ixodes* species	Sub-Saharan Africa, Asia and Caribbean

Table 1.3 Important tick transmitted etiological agents and the human disease

Disease	**Etiological agent**	**Tick species**
Rocky Mountain Spotted Fever	*Rickettsia rickettsii*	*Dermacentor variabilis* *Dermacetor andersoni*
Tularemia	*Francisella tularensis*	*Dermacentor variabilis* *Dermacetor andersoni*
Lyme disease	*Borrelia burgdorferi*	*Ixodes scapularis* *Ixodes pacificus*
Human monocytic ehrlichiosis	*Ehrlichia chaffeensis*	*Amblyomma americanum*
Human granulocytic ehrlichiosis	*Anaplasma phagocytophilum* *Ehrlichia ewingii*	*Ixodes scapularis* *Ixodes pacificus*
Babesiosis	*Babesia microti*	*Ixodes scapularis*
Relapsing fever	*Borrelia hermsi* *Borrelia turicatae*	*Ornithodoros hermsi* *Ornithodoros turicatae*
Colorado tick fever	Colorado tick fever virus	*Dermacenter andersoni*
Powassan virus encephalitis	Powassan virus	*Ixodes* species
Tick-borne encephalitis	*Tick-borne encephalitis virus*	*Ixodes* species
Haemorrhagic fever	Several hemorrhagic fever viruses belonging to *Flaviviridae,*	*Ixodes* species *Haemophysalis* species *Dermacentor* species *Argas* species *Hyalomma* species *Rhipicephalus* species

caused by a rickettsial pathogen was first reported in late 1800s and is still a concern in the USA. Until 1980s, *Rickettsia rickettsii,* the RMSF agent, was the only known important rickettsial pathogen transmitted by ticks to people. The discovery of human monocytic ehrlichiosis, human granulocytic anaplasmosis, and human ewingii ehrlichiosis has expanded the role of the tick as a vector.

General introduction about Rickettsiales

Rickettsiales belong to the class *Alphaproteobacteria* in the phylum *Proteobacteria* under the Kingdom *Bacteria* (32). The order *Rickettsiales* includes several intracellular bacteria that cause fatal and chronic infections in people and many vertebrate animals (32). Initial classification of *Rickettsiales* included several intracellular bacteria under this order. With the recent studies based on the analysis of 16S rRNA, GroESL and several other surface protein gene sequences, this order has been reclassified to include two families *Rickettsiaceae* and *Anaplasmataceae* (32). The family *Rickettsiaceae* includes obligate intracellular bacteria that grow in the cytoplasm of the eukaryotic host cells, whereas the *Anaplasmataceae* family has obligate intracellular bacteria that replicate in vacuoles derived from the host cell membrane (32). There are two genera included in the *Rickettsiaceae* family; *Rickettsia* and *Orientia*. The family *Anaplasma*taceae contains four genera; *Anaplasma, Ehrlichia, Wolbachia* and *Neorickettsia* (32).

Twenty five *Rickettsia* species and one *Orientia* species have been documented in the *Rickettsiaceae* family (32). Morphologically the *Rickettsia* and *Orientia* species vary in shape from coccobacillary to short rods and measure 0.8-2.0 µm long and 0.3-0.5 µm wide (32). The *Rickettsia* species are further subdivided into the spotted fever group and the typhus group.

The spotted fever group organisms have the ability to polymerize actin in the host cytoplasm and enter into the host's nucleus whereas the typhus group bacteria do not (54). The spotted fever group organisms are mainly associated with arthropods such as ticks, mites and fleas (134). These arthropods serve as biological vectors of transmission for pathogenic *Rickettsia* (91). The typhus group pathogens are mainly transmitted to vertebrates from the infected feces of lice and fleas and by gaining entry into a host through mucous membranes or abraded skin (91). Upon internalization into host cells, the Ricketssial pathogens escape from phagosomes by lysing the phagosomal membrane with phospholipase D and hemolysin C (101,139). Rickettsial pathogens are highly adapted to live in the host cell cytoplasm by acquiring ATP, nucleotides and amino acids from the host. *Orientia tsutsugamushi* causes a life-threatening illness called scrub typhus in people (52). *O. tsutsugamushi* differs from *Rickettsia* species both phenotypically, by not having peptidoglycans or lipopolysaccharides in the cell wall, and genotypically, by having only 90 % similarity in the 16S ribosomal gene sequences (125). So, this pathogen was classified under a new genus (125). This pathogen is maintained in nature in mites and is transmitted to humans by the larval mite during feeding (52). These pathogens enter into host macrophages by phagocytosis, escape into the cytoplasm, and replicate by binary fission.

The family *Anaplasmataceae* includes several important human and veterinary pathogens belonging to three genera; *Anaplasma, Ehrlichia, Neorickettsia* and non-pathogenic, symbiotic organisms belonging to the genus *Wolbachia* (32). Morphologically the bacteria in this family vary from small rod, coccoid to pleomorphic forms ranging from 0.3-1.3 μm in size (32,102). The primary target vertebrate host cells for *Anaplamataceae* organisms include erythrocytes, monocytes, macrophages, neutrophils, platelets and endothelical cells (32,102). The genera *Anaplasma* and *Ehrlichia* include several pathogenic organisms that cause infections in people and animals and are primarily transmitted by Ixodid ticks (32). Several wild

and domestic animals like cervids, mice and rodents serve as reservoir hosts for these pathogens that serve to maintain them in nature (32). The genera *Ehrlichia* and *Anaplasma* have similar cell tropisms and morphological appearance. However, the sequence analysis of 16S ribosomal protein genes revealed significant differences and so they were separated as two separate genera (32). Three *Neorickettsia* species have been reported as pathogens causing diseases in animals and people. They are *Neorickettsia helminthoeca*, *Neorickettsia risticii* and *Neorickettsia sennetsu*. Vertebrate hosts like dogs, horses and humans acquire *Neorickettsia* by ingestion of insects or fish infested with infected trematodes or metacercaria (14,70,102). The Wolbachia species, non-pathogenic and symbiotic bacteria, reside in many invertebrates like insects, spiders, mites, ticks and filarial nematodes (such as *Dirofilaria immitis*, and *Onchocerca volvulus)* (122).

Several rickettsial pathogens have been reported to cause infections in people and animals. The oldest known tick transmitted rickettsial pathogen having significant impact in the USA is *Rickettsia rickettsii,* the causative agent of RMSF (137). Several rickettsial pathogens belonging to the genera *Anaplasma* and *Ehrlichia* have also been identified as disease causing agents in animals and people (32). *Anaplasma marginale* and *Anaplasma centrale* are the tick transmitted pathogens responsible for bovine anaplasmosis, an economically important disease. *Ehrlichia ruminantium*, another economically important tick-transmitted pathogen causes heart water disease in both domestic and wild ruminants. This sub-Saharan African pathogen is also established in the some Caribbean islands posing a continuous threat to ruminants on the US mainland. A pathogen that is highly homologous to *E. ruminanitum* has been identified recently from the ticks and goats of Georgia (68). *Ehrlichia canis*, canine monocytic ehrlichiosis agent, is the oldest known rickettsia species having a wide spread distribution through out the world (except Austarlia). *Ehrlichia ewingii* is another pathogen causing ehrlichiosis in dogs. *Ehrlichia equi* and *Ehrlichia phagocytophilum* were reported as causative agents of diseases in animals in

Europe and USA, respectively. Human granulocytic agent commonly referred to as HGE agent is causing infections in people. These three pathogens were found to be identical, and more closely related to *Anaplasma* genus upon molecular analysis, and named *Anaplasma phagocytophilum*. *Ehrlichia* and *Anaplasma* pathogens have been known for quite some time as animal pathogens only and their potential as human pathogens was not established until the first report in 1987 for *Ehrlichia chaffeensis*. *Anaplasma phagocytophilum* was reported in 1990 to cause infections in people and the disease was termed human granulocytic anaplasmosis (8,19). *Ehrlichia ewingii* also was reported to be causing infections in people in 1998 and the disease is referred to as human ewingii ehrlichiosis (15). In 1986, peculiar intracytoplasmic inclusion bodies were seen in the monocytes of a patient with an unknown illness and a history of tick bites (71). They were initially identified as *Ehrlichia canis* organisms based on morphology and serological assays (3,24). Later on, molecular characterization of these pathogens from human patients confirmed that this bacterium is significantly different and was formally named *Ehrlichia chaffeensis* in 1991 (3). Subsequently, the transmitting vector (*Amblyomma americanum* tick) and the natural reservoir hosts (white tailed deer) were identified for this pathogen. *E. chaffeensis* causes human monocytic ehrlichiosis (HME) in people. HME infections are well documented in people and HME is considered as an emerging disease in the USA (136). The newly identified diseases caused by *Ehrlichia* and *Anaplasma* pathogens can be severe and fatal in people and have significant impact on the health of people in the USA (32,87,102,105). So, this led to the expansion of research on these pathogens.

Ehrlichia chaffeensis as an emerging human pathogen

Increased numbers of HME cases have been diagnosed in the USA since its first identification in 1986 (71). The initial cases of HME were reported from the southeastern United States, where the *A. americanum* tick is most abundant (30,138). However, in later years, the

infections were reported from all over the USA. This is not due to the presence of tick vectors in all the states but due to infections acquired during travel to states with a known risk for *E. chaffeensis* exposure (58). The incidence rate of HME is increasing continuously and in the year 2005 alone, about 471 cases in the US were reported to the CDC (33). However, these numbers are likely to be an underestimate of the actual incidence as active surveillance studies revealed higher infections rates. For example, a study conducted in Missouri revealed 0.02%-0.06% infection rates and a similar study in Tennessee revealed an incidence rate of 0.3%-0.4% in the human population (33). The numbers translate to approximately 25,000 cases in these two states. *A. americanum* is present in several southern and Midwestern states (Figure 1.4). Assuming that the infection rates in these states are similar to Missouri and Tennessee, the likely incidence of HME in these areas is anticipated to be much higher than reported to CDC.

E. chaffeensis pathogen is maintained in nature by transmission between white tailed deer (reservoir host) and *A. americanum* tick (vector) (Figure 1.5). In addition to people, *E. chaffeensis* is also reported to be infective to several other vertebrate animals such as dogs, coyotes, deers, raccoons and other wild animals (29,145). The wide spread distribution of this pathogen in other vertebrate hosts may also contribute to the maintenance of this pathogen in nature. *E. chaffeensis* causes persistent infections in white tailed deer and tick vectors (transstadial transmission) (6,22,25,26,34,42,123,131,155). Ticks acquire infection upon feeding on an infected animal during larval or nymphal stages. The bacteria remain in the tick as it changes from larva to nymph or from nymph to adult. Infected nymphs and adult ticks transmit *E. chaffeensis* to vertebrate hosts. Humans are considered as accidental hosts for *E. chaffeensis* (87) (Figure 1.5).

Figure 1.4 Geographical distribution of *Amblyomma americanum* tick in the USA.

The *A. americanum* tick is found in the regions that are colored in dark green in the map.

http://www.cdc.gov/ncidod/dvrd/*Ehrlichia*/Natural_Hx/Natural_Hx.htm

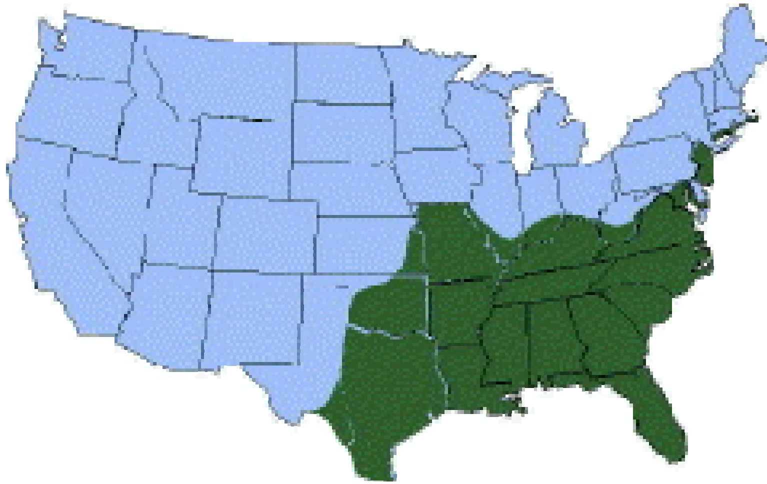
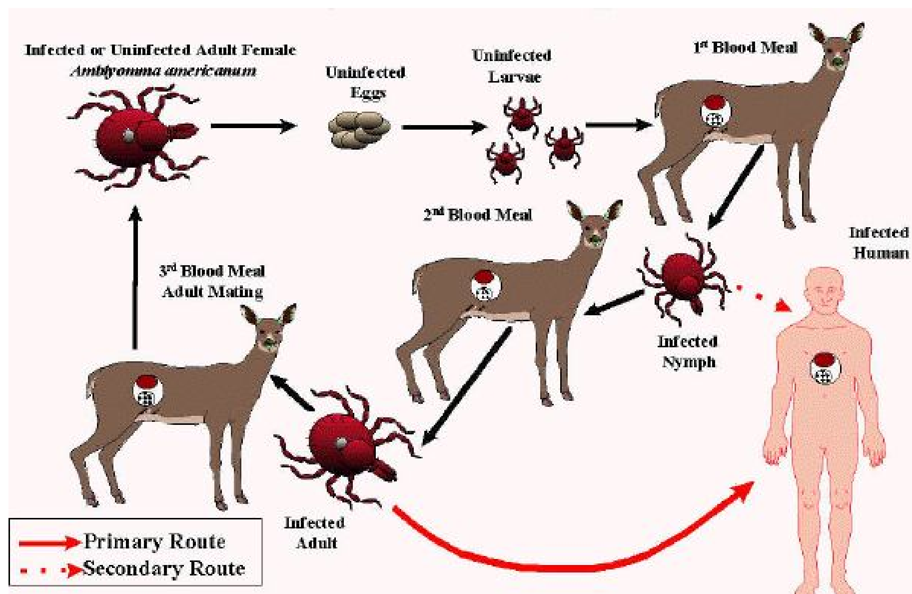


Figure 1.5 Proposed life cycle for *Ehrlichia chaffeensis.*

The life cycle of *E. chaffeensis* involves a vertebrate host and a tick vector. The pathogen is maintained in nature by transmission between the *A. americanum* tick and white tailed deer (reservoir host). Ticks acquire infection while feeding on an infected animal and maintain the infection through its life cycle stages. Human beings and other vertebrate hosts are considered as accidental hosts and will acquire infection upon bite from an infected nymph or tick.

http://www.cdc.gov/ncidod/dvrd/Ehrlichia/Natural_Hx/nathx1.htm#E.%20chaffeensis



People of all ages are likely to acquire *E. chaffeensis* infection from the bite of an infected tick (87). However, severe illness is more common in people with compromised immunity resulting from various causes, such as HIV infection or immunosuppressive medical therapy (88). Published reports also suggest that elderly people are at higher risk of developing a severe form of HME illness (88). Clinical signs of the disease range from a mild viral-like illness to severe life threatening disease and usually begin within 7-10 days following the bite of a tick (33). However as the disease progresses, multiple organs get involved and complicate the clinical course and result in the possibility of a life-threatening condition (87). Analysis of the clinical signs in patients revealed many commonly reported clinical signs which include fever (97%), malaise (82%), headache (80%), nausea (64%), myalgia (57%) and arthralgias (41%). Studies also revealed that about 42% of the patients need hospitalization with 17% developing a life-threatening condition (33). About 3% of the infections will result in mortality. Neurological signs are also observed in 20% of the patients. When disease progresses to a multisystemic condition, it results in acute renal failure, metabolic acidosis, respiratory failure, disseminated intravascular coagulopathy, hepatic failure, myocardial dysfunction and finally death (33).

Diagnosis of HME is complicated because of the absence of a specific diagnostic clinical feature (33). The tentative diagnosis is primarily based on the clinical findings combined with a history of tick bite. Serum chemistry and blood panels may be helpful in arriving at a presumptive diagnosis. Several other diagnostic approaches are available for the confirmatory diagnosis of the infection. They include peripheral blood smear examination, clinical laboratory findings, serology based tests, PCR tests and isolation of the bacteria (4). The examination of a stained peripheral blood smear of a patient is a routinely followed method. Intracytoplasmic inclusion bodies are likely to be found in the monocytes of positive samples (33). However, blood smear examination is a very insensitive method and inclusion bodies can be detected in less than 10% of the infected patients (33). The most commonly found laboratory findings

include thrombocytopenia (71%), leucopenia (62%) and elevated liver enzymes alanine aminotranferease and aspartate aminotransferase (83%) (33). The blood panel may show multilineage cytopenias in the course of the disease. Hemorrhagic abnormalities are seen in some patients resulting in coagulopathies and even epistaxis (33). The serological assays test for the presence of antibodies against *E. chaffeensis*. The most commonly available serological test is indirect immunofluorescence assay (IFA). The IFA test is performed on paired serum samples, collected within two to four week intervals, and the increase in the serum titers in the convalescent phase serum can be confirmatory to a clinical infection. However, small percentages of patients do not develop antibodies to *E. chaffeensis* and so IFA negative does not necessarily mean that a patient is negative for infection. The sensitivity of the immunological assay, ranges from 20 % to >90%, depending on the immune response from the host and the number of days since the acquisition of infection (33). PCR assays are reported which tend to have higher sensitivity (up to 85%) than IFA. However, these tests may only be available to clinicians practicing at or near a University hospital. *In vitro* isolation of the pathogen from the clinical samples is another way of confirming the diagnosis of *E. chaffeensis* infection (18,46). However, this technique requires substantial resources and time (6-8 weeks) and the sensitivity is highly variable (23). This method is not routinely used in the clinical setting.

If a tentative diagnosis is made for infection with *E. chaffeensis*, antibiotic therapy is initiated by the clinician without the need of a confirmatory diagnosis. Empirical studies provided evidence that HME agent is highly susceptible to tetracyclines. Doxycycline is preferred to other tetracyclines because of the relatively lower risk for adverse drug reactions and better patient tolerance in people. Several *in vitro* studies confirmed the susceptibility of *E. chaffeensis* to doxycycline. It is currently the drug of choice for *E. chaffeensis* treatment in all age groups of people including children. Rifampin is another antibiotic that is effective against

E. chaffeensis infection. Rifampin is prescribed to patients having contraindications to doxycycline, i.e., allergy or pregnancy. Generally a course of 1-2 weeks of doxycycline or rifampin antibiotics is recommended for complete clearance of the pathogen. Chloramphenicol is also shown to be inhibitory to the growth of *E. chaffeensis*. However, it is not used in clinical setting because of the higher dose requirement of chloramphenicol.

E. chaffeensis is an obligate intracellular bacterium that predominantly infects monocytes and macrophages in vertebrate hosts. However, infections in other cell types such as lymphocytes, atypical lymphocytes, premyelocytes, metamyelocytes and band and segmented neutrophils are also reported (87). The bacterium lacks pili or capsule and so may bind to the host cells by its outer membrane and internalized by phagocytosis (65). This internalization may be receptor mediated or by an independent mechanism which remains to be established (104). The internalized bacteria replicate in a vacuole rather than cleared by phagolysosomal degradation (104). Because of its intracellular nature, *E. chaffeensis* may depend for nutrients and metabolites on the host cell cytoplasm (46). This may be accomplished by proteins expressed on the cell surface (46). The initial interactions between *E. chaffeensis* and its host are likely through the surface exposed proteins of the pathogen (46). Electron microscopic studies suggest that similar to *Chlamydia* species, *E. chaffeensis* has two morphologically distinct forms that are observed in the phagosomes (153). Recent studies by Zhang et. al, suggest that the dense core bodies are the infectious forms that are found within the phagosomes and also are released outside, while the reticulate bodies are metabolically active, replicating cells that are only seen within the phagosomes of infected cells (153). Several studies have been performed to identify the outer membrane proteins of *E. chaffeensis* (28,46,148,149,152). A glycoprotein outer membrane protein of about 120 kDa size termed gp120 is reported in *E. chaffeensis* (144,148,149). This protein is mainly expressed in the dense core stages of *E. chaffeensis*. The function of this protein is unknown, but recent studies

suggest that it is immunogenic and may be involved in the attachment and invasion of host cells. Similarly, an immunodominant 28 kDa outer membrane protein has been reported to be expressed on the *E. chaffeensis* cell surface (21,85,99,100,147). Multiple forms of this proteins are encoded from the p28-Omp multigene locus. Recent studies also suggest that the p28-Omp proteins are differentially expressed in a host cell-specific manner (21,85,99,100,147). Moreover, multiple post-translationally modified forms of the p28-Omp 19 and 14 have been reported in *E. chaffeensis* originating from vertebrate and tick cell backgrounds, respectively (114). The biological significance of the protein products of the p28-Omp locus genes remains to be determined. Several new proteins such as DO/DeqQ family serine proteases, GroEL, type IV secretion subunit (VirB9-1), a hypothetical protein and conserved proteins have also been reported recently as the surface expressed proteins in *E. chaffeensis* (46). Though, studies to date did identify some surface expressed proteins of *E. chaffeensis*, much more remains to be understood about their function and role in pathogenicity. Similarly, understanding the pathogenicity of *E. chaffeensis* requires the details of *E. chaffeensis* protein expression and molecular characterization of differentially expressed proteins relative to its growth in vertebrate and tick cells. In addition, understanding the host response against the pathogen and the pathogen's ability to survive by evading host clearance requires detailed investigations of the pathogen *in vitro* as well as *in vivo* using infection studies performed in vertebrate and tick hosts. *In vivo* experiments using an animal model will be valuable in assessing host response to a pathogen and will provide insights about how *Ehrlichia* may evade the vertebrate immune system. The pathogenicity of *E. chaffeensis* can be better studied with the availability of a suitable animal model that mimics infection observed in people. Though white tailed deer (WTD) are considered a natural reservoir host, it is not suitable as an animal model because the clinical presentation in this host is not same as observed in people (22,26,36). For example, people develop several clinical signs that are not observed in WTD when experimentally infected with *E. chaffeensis* in a laboratory. Secondly, humans are considered accidental hosts

where infection is mostly short lived, with the exception of people with a compromised immune system. Thus it is ideal to select an animal model that acquires infections with *E. chaffeensis* and the infection is cleared or persistent similar to that in immunocompetent and immunocompromised people. Several laboratories have been using the mouse as a suitable model for understanding *E. chaffeensis* infections and host response (39,44,64,140). *E. chaffeensis* in mice induces variable immune responses, depending on the genetic background and immunological status of the murine host (39,45,64,86,112,141).

It is unclear what components of the immune system are responsible for either the clearing or persistence of *E. chaffeensis* infection in people. It is also unclear what pathogen factors, if any, contribute to the pathogen's persistence in a vertebrate host. The role of innate (macrophage and cytokines), humoral and cell mediated responses have been evaluated using the mouse model. The studies reported that macrophage activation and cell-mediated immunity, orchestrated by $CD4^+$ T cells are critical for conferring rapid clearance of *E. chaffeensis* (44). However, $CD4^+$ T-cell help can be complemented by other types of helper T cells, because CD4 knockout mice also can cure an infection two weeks late compared to wild type. The studies also showed that $CD4^+$ T cells are not absolutely necessary for *E. chaffeensis* resistance, but the most effective host response is accomplished only with the $CD4^+$ T cells (44).

E. chaffeensis causes persistent infections in MHC-II-knockout mice compared to cleared infection in wild type mice suggesting the importance of MHC-II antigen expression for activating the helper T cells (45). The presence of $CD4^+$ T-cells was also demonstrated to be helpful in clearing the infection faster but not absolutely necessary to clear the infection, as CD4-knockout mice also cleared the infection, albeit slower. When mice were treated with anti-CD4 monoclonal antibody, the clearance of infection was delayed by about one week (121).

Therefore, it has been concluded that there is some redundancy in the immune responses that allows for host resistance in the absence of $CD4^+$ T-cells. An increase in the $CD8^+$ T-cells is demonstrated in fatal HME infections and also in canine ehrlichiosis cases (53). So, studies were investigated to understand the role of $CD8^+$ T-cells in host responses. Experiments revealed that $CD8^+$ T-cells are also not required but help in clearing the infection faster (39). Toll-like receptor-4 (*Tlr4*) molecules contribute to the clearance of *E. chaffeensis* by a yet undefined mechanism (45,126). Natural killer cells have been shown to not have a role in clearing *E. chaffeensis* infection in mice (44,45). The role of $CD4^-$ and $CD8^-$ and γδ T-cells has not been studied and is not yet defined.

Antibodies against *E. chaffeensis* are shown to confer transient protection against an experimental challenge infection (64,85,146). Antibodies may act on *E. chaffeensis* when released from the infected cells and before entering another host cell. Several reports have been published defining the role of antibody-mediated protection (85,146). SCID mice show fatal infections with *E. chaffeensis*, but adoptive transfer of pathogen-specific immunoglobulins induces a transient protection and survival in these mice (17). Other studies reported that the adaptive transfer of antibodies against an outer membrane protein resulted in significantly faster clearance of *E. chaffeensis* upon challenge compared to control groups in *Balb/c* mice (64,85). The mechanism of protection by antibodies is believed to be due to formation of immune complexes triggering microbicidal activities in infected macrophages leading to the elimination of bacteria. Alternatively, it is also proposed that antibodies may opsonize bacteria upon release from infected cells resulting in clearing the pathogens. However, antibodies alone not are sufficient to completely clear an *E. chaffeensis* infection as demonstrated in a SCID mice model where mice succumbed to infection when adaptive transfer of antibodies was withdrawn.

Together the comprehensive immunological studies using mouse models demonstrated that host clearance against *E. chaffeensis* is complex and involves macrophage activation, cytokines, antibodies, helper T cells responses and possibly other members of the host immune system that are yet to be defined. However, infection in the mouse model appear to be very similar to those observed in people who clear *E. chaffeensis* infection fairly rapidly as in the case of immunocompromised individuals. It is entirely unknown how in a subset of people, *E. chaffeensis* infection results in serious disease which can be fatal. One possible explanation for this could be the *E. chaffeensis* originating from tick cells may alter host response particularly in elderly people with compromised immune systems. So, the important aspect for understanding persistent infections requires detailed knowledge about how the pathogen adapts itself to a tick host. Secondly, infection resulting from Ehrlichia transferred from a tick host to a vertebrate such as a human may provide an opportunity for the bacteria to persist longer as a result of altered immunity caused by tick feeding. Recent studies from our laboratory provided preliminary evidence that *E. chaffeensis* protein expression differs considerably when it is growing in a tick cell background compared to a macrophage environment (20,43,84,114,115). Tick-specific gene expression may be one of the possible means by which *E. chaffeensis* may adapt to vertebrate host and persistently evade host response.

Differential clearance patterns of *E. chaffeensis* depending on the origin of bacteria have also been reported in murine hosts (43). For example, mice infected with tick cell-derived *E. chaffeensis* exhibited higher rickettsemia and slower clearance compared to macrophage-derived bacteria (43). Mice inoculated with tick cell-derived *E. chaffeensis* produced significantly higher concentrations of IgG antibodies compared to mice challenged with macrophage-derived bacteria (43). The differences observed may be because of the differential host-cell specific protein expression in *E. chaffeensis*. Differential host cell-specific gene expression is reported to be playing a role in causing persistent infections in several other

closely related tick transmitted pathogens such as, *Borrelia burgdorferi* (115). Differential host cell-specific protein expression has been reported from the p28-Omp gene locus of *E. chaffeensis*. However, the transcriptional studies performed in *E. chaffeensis* so far were focused on a subset of genes such as, p28-Omp locus genes. The mechanisms employed by *E. chaffeensis* in evading host cell responses can be better understood if we know all the host-cell specifically expressed genes. The role of the differentially expressed genes can be better studied if we can identify such genes. In an effort to fill the gap of knowledge in this area, studies aimed at identifying the global transcriptional profiles of *E. chaffeensis* in vertebrate and tick host backgrounds will be valuable.

To precisely map the role of such differentially expressed genes in the pathogenesis of *E. chaffeensis*, blocking the expression of such genes and then study the differences in the pathogenicity of the bacteria will also be important. Silencing of the genes can be accomplished by several methods. RNA interference (RNAi) approach, a novel technique that has gained considerable attention recently, is reported to silence the expression of a specific gene in eukaryotes (120,129). However, this approach has not been described for use in prokaryotic organisms due to the lack of dicer system. Another way is to block the function of a protein of interest by using antibodies raised against that particular protein. However, this method may be feasible to block the surface expressed proteins, but may not be feasible for cytoplasmic proteins. Moreover, such studies may also be difficult for intracellular bacteria such as *E. chaffeensis*. This is further complicated in that *E. chaffeensis* has a complex life cycle in tick and vertebrate hosts. The third method is to knockout or disrupt a specific gene of interest and study its impact on the growth and pathogenicity of the bacteria. However, genetic manipulation systems are yet to be established for *E. chaffeensis*. Therefore, studies aimed at developing a genetic manipulation system useful in creating targeted mutations in the genome of *E. chaffeensis* will be most valuable to understand the function of *E. chaffeensis* proteins.

In this chapter, I provided a detailed description summarizing the impact of arthropod vectors on animal and human health. Ticks are the second largest group of arthropods responsible for vector-borne diseases. Several tick species have been identified in recent times as the vectors for transmitting various rickettsial pathogens to animals and people. HME, caused by *E. chaffeensis*, is an emerging tick-transmitted infection in USA. An increased incidence of HME infections is reported from across the USA since its first report in 1986. *E. chaffeensis* can persist and result in serious illness in immunocompromised and elderly people. The pathogen primarily infects monocytes/macrophages and evades the host clearance mechanisms to achieve persistent infections. The pathogenic mechanisms employed by *E. chaffeensis* and the host responses in clearing the infection or causing the persistent infections is not well understood. Recent studies also suggest that *E. chaffeensis* protein expression differs in a host cell-specific manner. Furthermore, studies conducted using a mouse animal model suggested differential clearance patterns for *E. chaffeensis* originating from macrophage and tick cells. Mice infected with tick cell-derived *E. chaffeensis* exhibited higher rickettsemia and slower clearance compared to macrophage-derived bacteria. However, more remains to be understood with regard to the differentially expressed proteins and their role in the pathogen's survival in vertebrate and tick host; also how will the differential expression of protein aid in the pathogen's persistence in the vertebrate host.

CHAPTER 2 - Scope of the Thesis

E. chaffeensis is a tick-transmitted rickettsial pathogen and is the causative agent of human monocytic ehrlichiosis (HME). *E. chaffeensis* infections in people can persist and result in serious illness with fatal outcome, particularly in immune compromised and elderly people. An increased incidence of HME pathogen infections is reported in the USA since its first identification in 1986. An active surveillance study conducted in Missouri revealed 0.02%-0.06% infection rates and a similar study in Tennessee revealed even a higher incidence rate of 0.3%-0.4% in the human population. On average, these numbers translate to approximately 25,000 cases each year for these two states, suggesting that the true prevalence of the disease is much higher in the US for the regions where the *E. chaffeensis* transmitting tick (*A. americanum*) is more prevalent. With a steady increase in reported cases as well as the much higher estimated HME case load, research focused on understanding the pathogenesis and molecular basis for persistent infections with *E. chaffeensis* is necessary. It is also important to understand how the pathogen is able to adapt to dual host environments. However, very little has been understood about the pathogenesis of *E. chaffeensis*. For example, to date there are only a few outer surface proteins that have been identified and partially characterized. The function of these limited number of identified membrane proteins and their role in the pathogen's adaptability and survival is not known. Immunological studies, primarily coming from three independent laboratories, revealed considerable understanding of the components of the host immune system responsible in clearing *E. chaffeensis* infection. However, the mechanism by which the pathogen causes persistent infection and adapts to two different hosts, vertebrate and tick, remains largely unknown. Recent studies suggest the role of differential protein expression by *E. chaffeensis* as one of the contributing factors in adapting the pathogen to different hosts and causing persistent infections in them. However, the studies that described the host cell-specific differential expression are limited primarily to characterizing a few outer membrane protein genes. It is, therefore, unclear if the differential expression is limited to a subset of a few gene products or a common theme for many gene products.

Recent advancement in *E. chaffeensis* research includes the availability of the whole genome sequence of the pathogen and the establishment of suitable animal models of infection studies. These tools can be further exploited to understand the pathogenesis of the bacteria. For example, the availability of whole genome sequence aids in functional genomics studies to map global gene expression pattern differences in the pathogen relative to its growth in vertebrate and tick cell environments, as well as to map differences in the pathogen during its replication (reticulate bodies) or in its infectious form (dense cored bodies). Studies aimed at identifying the genes that are differentially expressed in different stages of the pathogen's life cycle and in different host environments may provide important information helpful in devising better strategies for controlling pathogen infections. Differentially expressed proteins serve as a good starting point for investigations focused on identifying genes that are important for the pathogen's survival in vertebrate and tick host environments. So, the significant gap of knowledge in this field can be filled by utilizing functional genome approaches, such as to assess the global gene expression differences by transcriptome analysis. A microarray chip representing the open reading frames of the *E. chaffeensis* genome, if designed, will be valuable to evaluate the gene expression patterns of the pathogen in different life cycle stages. Microarray-based transcriptome analysis will also be valuable to validate numerous predicted genes identified by the whole genome sequence analysis. One of primary goals of this research is to fill in this important gap of knowledge by developing an ORF-based whole genome microarray chip and then utilize it to map the gene expression patterns of *E. chaffeensis* in different host backgrounds.

The importance of a gene expression to the pathogenicity of *E. chaffeensis* can be better understood if one is able to block the function of the protein made or by blocking the expression of such protein from the gene encoding it and then examine the impact of the loss of its function to the pathogen's survival and adaptability to dual host life cycle. Several approaches can be

used to achieve this goal; 1) prevent the protein expression by blocking the translation from an mRNA by using methods such as the RNA interference (RNAi) technology as demonstrated in eukaryotes, 2) inactivate the protein function by using drugs that specifically target a specific protein of interest and 3) by introducing mutations in a gene of interest so as to alter or block the expression from a gene. Knockout or knockdown of an mRNA is somewhat challenging for bacterial pathogens, as RNAi technology is not described for any bacteria due to the lack of RNA dicer enzyme pathway in them. Alternatively, a transcript may be blocked from being translated using an anti-sense oligonucleotide approach (35). However, this is not practical for an intra-phagosomal pathogen, such as *E. chaffeensis*, as it is technically impossible to transfer sufficient quantities of antisense oligonucleotides to block gene expression of the pathogen from within a phagosome. Similarly, inactivating a protein function using drugs targeted to a protein also requires the transfer of a specific drug to be accumulated in sufficient quantities within the pathogen that may be replicating in a phagosome. This is also complicated by the additional research involved in designing and evaluating specific anti-protein analogues. The third and more reasonable approach will be to create mutations within the *E. chaffeensis* genome to selectively inactivate a gene of interest. Although this is the most desirable approach, the lack of established methods of genetic manipulations for *E. chaffeensis* or other closely related tick-transmitted pathogens is a significant gap of knowledge in this field. If established, a mutational analysis system will be most desirable to study the importance of many uniquely expressed genes of *E. chaffeensis*. Although targeted mutational approaches have not been reported, recent studies reported transposon-based mutagenesis for another closely related tick-transmitted pathogen, *Anaplasma phagocytophilum*. The data described recently at two scientific meetings suggest that it is possible to transform *Ehrlichia* and *Anaplasma* species and targeted mutagenesis is likely to be achieved. If established, the availability of a genetic manipulation system in creating mutations in a specific gene will be very valuable to study the role of proteins made from various genes to the pathogenicity of *E. chaffeensis*. Therefore, the

second major goal of my research is to fill in this important gap of knowledge, i.e., evaluate the methods for establishing mutations in *E. chaffeensis*.

CHAPTER 3 - Open Reading Frame- Based Microarray Used in Evaluating Gene Expression Profiles of *Ehrlichia chaffeensis* Originating from Vertebrate and Tick cell Environments

Abstract

Ehrlichia chaffeensis is an emerging tick-borne rickettsial pathogen that causes infections in people and several vertebrate animals. This is the causative agent of human monocytic ehrlichiosis. The relationship between *E. chaffeensis* and its target cells, macrophages/monocytes in vertebrate hosts and tick cells, is critical as the organism must persist in them to complete its life cycle. We hypothesize that *E. chaffeensis* employs host cell-specific gene expression as a strategy for adapting to and surviving in dual hosts. In To test this hypothesis, we developed a novel open reading frame (ORF)-based whole genome microarray and performed global transcriptional analysis using RNA from *E. chaffeensis* grown in macrophage and tick cells. The analysis aided in the identification of about 30% of all the predicted *E. chaffeensis* genes (341-381) as expressed independent of its growth in macrophages and tick cells. About two-thirds of the transcribed genes identified by microarray analysis are common for both host cell backgrounds. Nearly 20% of the commonly expressed genes also varied in their expression levels which ranged from two to four fold. Proteins encoding commonly expressed genes include those involved in DNA metabolism, energy metabolism, protein synthesis, transcription, transport and cellular processes. In addition, a significant portion of the commonly expressed genes (60 out of 263 ORFs) included those coding for proteins with unknown function (hypothetical proteins). Host cell-specific expressed transcripts also have a similar ratio of genes coding for hypothetical proteins and those with defined function. Microarray data was verified by RT-PCR for a subset of randomly selected genes from non-expressed, commonly expressed and differentially expressed pools. Together the comprehensive transcriptional analysis represents the first report describing the global host cell-specific gene expression patterns in *E. chaffeensis*.

Introduction

E. chaffeensis causes persistent infections in both vertebrate and tick hosts (6,22,25,26,34,42,123,131,151,155). It is critical to understand how this pathogen remains in dual hosts for extended periods of time. There may be many ways by which the organism may persist. The strategies may include altering the host response, varying the expressed antigens relative to time post-infection, and differential host-specific protein expression (43,114,115,131). As ticks are heterothermic and vertebrate hosts are homeothermic, *E. chaffeensis* may also have evolved to express unique antigens in response to their host environments. Several studies reported the role of differential gene expression as an important adaptive mechanism in causing persistent infections by many tick-borne pathogens. For example, Lyme disease agent, *Borrelia burgdorferi*, is reported to express outer surface protein A in its tick host and outer surface protein C in the vertebrate host under *in vivo* conditions (109). This differential expression in *B. burgdorferi* is shown to be important for the colonization and adaptation to different hosts (89,90). Host cell-specific differential expression for a subset of *E. chaffeensis* proteins has also been reported recently (114,115). Recent studies also demonstrated that clearance by the murine host is delayed for *E. chaffeensis* grown in tick cells as compared to those grown in vertebrate host cells (43). Together, these results suggest that unique protein expression in *E. chaffeensis* grown in tick host provides an opportunity for the pathogen to evade clearance from a vertebrate host. Much of the fundamental knowledge about the host-specificity and how it contributes to the pathogen's persistence can be understood by surveying the host-specific differences in the gene expression by transcriptome and proteome analysis.

A limited number of studies have been carried out on *E. chaffeensis* in an effort to examine the contributions of expressed genes in support of persistent infection (43,154). Previous transcriptional analyses from our laboratory demonstrated the differential expression of the genes coding for p28 outer membrane proteins, termed p28-Omp genes, from *E.*

chaffeensis grown in mammalian host cells and tick cells under *in vitro* conditions (114,115). Several *in vitro* and *in vivo* studies from other laboratories also demonstrated the differential expression of outer membrane protein genes in vertebrate and tick host backgrounds (20,84,131). Our recent proteome analysis also confirmed the findings from the transcriptional studies (111). Much of the research until now is focused on few genes, mostly encoding outer membrane proteins (21,85,99,100,131,144,147-149). A significant gap of knowledge exists in understanding the pathogenic mechanisms employed by *E. chaffeensis* and the strategies it uses to evade host clearance. Comparative analysis of gene expression at the global level from *E. chaffeensis* cultivated in vertebrate and tick environments will yield significant more novel information to answer questions about the intracellular survival of this important human pathogen. Recent advances in the rickettsial field include the availability of the whole genome sequence of *E. chaffeensis* (56). However, only limited numbers of genes have been characterized at the molecular level (144,147,148). The availability of whole genome sequence greatly aids in mapping the global transcriptional profiles of *E. chaffeensis.* Such data will be valuable in defining the mechanisms employed by the pathogen for its continued survival in vertebrate and tick hosts as well as to map the molecular basis for pathogenesis.

In this study, we designed an ORF-based whole genome microarray chip of *E. chaffeensis* and utilized it to assess global transcriptional differences in the bacteria growing in vertebrate and tick cell environments. This is the first functional genome study reporting the transcriptional analysis of *E. chaffeensis.* This study aided in the identification of numerous host specifically expressed genes in *E. chaffeensis.* Findings from our study will be important to further enhance our understanding of *E. chaffeensis* pathogenesis and help in devising better control strategies.

Materials and Methods

***In vitro* cultivation of *E. chaffeensis* in macrophage and tick cell lines:** *E. chaffeensis* Arkansas isolate was cultivated at 37°C in the canine macrophage cell line, DH82, as described previously. Arkansas isolate was also cultivated in the tick cell line, ISE6, but at 34°C in the absence of CO_2 as described by Munderloh *et al.* (79).

Designing the microarray chip: We obtained the unannotated complete genome sequence of *E. chaffeensis* from The Institute of Genome Research, TIGR (www.tigr.org) and carefully analyzed it for the presence of open reading frames (ORFs) that contain 95 or longer amino acids with the aid of the Artemis genome sequence analysis program (Welcome Trust Sanger Institute, Cambridge, UK). (Note: An annotated genome sequence was not available at the time when we initiated this study). A total of 1,234 ORFs were identified and used in designing the array. Two probes, of about 45 nucleotides long, specific for each ORF were designed. Similarly, three probes targeting the variable regions in each ORF were designed for all 22 genes in the p28-Omp gene locus. Specific probes targeting several house keeping genes were also designed representing human, canine, murine, and tick hosts. The house keeping genes used as a control are: 18S rRNA genes from homosepians, mouse (*Mus musculus*), *Rhiphicephalus sanguinius* ticks and dog (*Canis familiaris*); beta actin gene from *Ixodes scapularis*, homosepians, mouse and dog; GAPDH gene from mouse, dog and homosepians; 16S mitochondrial rRNA gene from *I. scapularis* and *A. americanum*. Positive controls with perfectly matched and mismatched oligonucleotides were also included in the array. In array design, we opted to use an oligonucleotide-based *in situ* synthesized microarray. The advantage of an *in situ* synthesized oligonucleotide microarray is that it can be highly specific and minimizes non-specific hybridization signals resulting from a false priming of the targets and probes. We also reasoned that the inclusion of the probes in triplicate and distributing them randomly throughout the microarray chip will aid in accurate detection of the expressed

transcripts. This is specifically to rule out the false negatives. Several blank wells were included to serve as background controls. The microarray chips were custom manufactured from Xeotron Corporation (now affiliated with Invitrogen, Carlsbad, CA). The chip is prepared using DMT-protected phosphoramidite nucleoside monomers in the presence of photo-generated acid.

RNA isolation, labeling and hybridization: *E. chaffeensis* was grown in either DH82 cells or ISE6 cells to about 100% infectivity and allowed to undergo ~100% lysis (culture is evaluated microscopically by Hema 3 polychromatic staining of the cytospin slides). About 30 ml of *E. chaffeensis* infected culture is centrifuged at 1,200 g for 10 min at 4°C to pellet the host cell debris and the supernatant containing host cell-free *E. chaffeensis* is filtered by passing through 5 and 3 μ filters. The filtrate is centrifuged at 15,000 g for 15 min at 4°C to pellet the *E. chaffeensis* organisms. The cell pellet is resuspended in 1 ml of Tri-reagent and total RNA is extracted by following the protocols described by the manufacturer (Sigma-Aldrich, St. Louis, MO). The final purified RNA pellet is dissolved in 100 μl of nuclease free water and subjected to column purification, by passing through RNeasy mini elute columns (Qiagen Corporation, Valencia, CA), to remove residual genomic DNA that may be present in the preparations. The RNA is eluted from the column into a final volume of 10 μl of nuclease free water. An outline of the protocol for isolating host cell-free *E. chaffeensis* RNA is shown in Figure 3.1. The concentration of *E. chaffeensis* RNA was assessed by using a ND-1000 Spectrophotometer (Nano Drop Technologies, Wilmington, DE). The integrity and purity of RNA samples was assessed by using an Agilent 2100 Bioanalyzer (Agilent Technologies, Palo

Figure 3.1 Outline of protocol for isolating host cell-free *E. chaffeensis* RNA.

The host cells infected with *E. chaffeensis* were harvested when the infectivity was about 100% and the bacteria were separated from the host cell debris by filtration and differential centrifugation. RNA was isolated from host cell-free *E. chaffeensis* by Tri-reagent method and contaminating DNA was removed by using a column based purification technique.

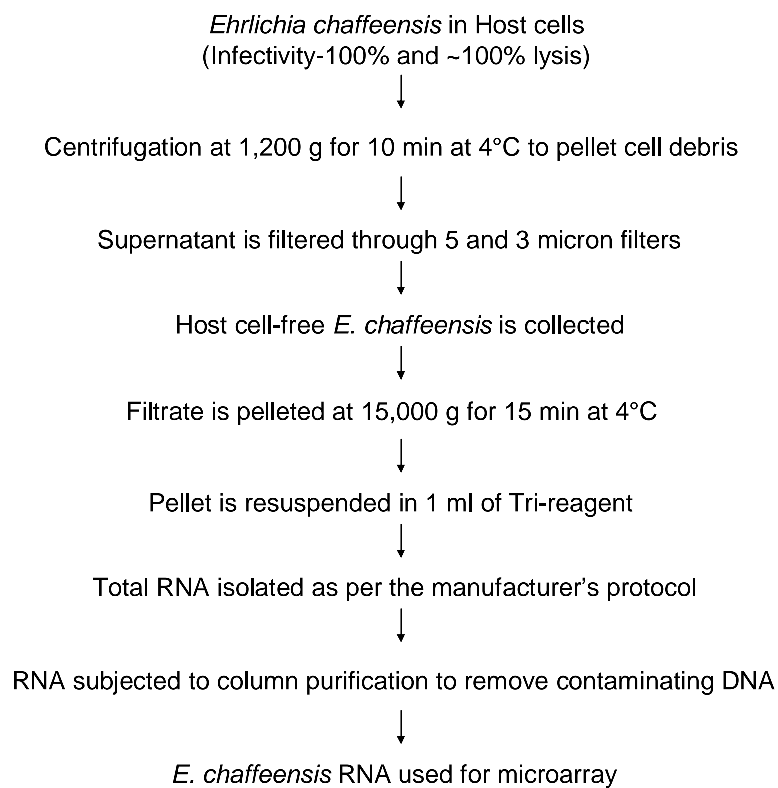
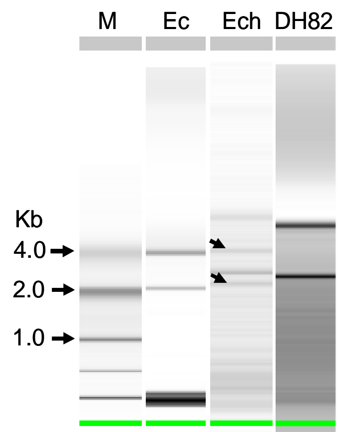


Figure 3.2 Purified *E. chaffeensis* RNA (Ech) resolved on a Bioanalyzer chip.

RNA from *E. coli* (Ec) and macrophage cultures were also isolated and analyzed to compare the resolved rRNA bands of all three species (Ech rRNA bands were identified with arrow heads). Lane M represents molecular markers.

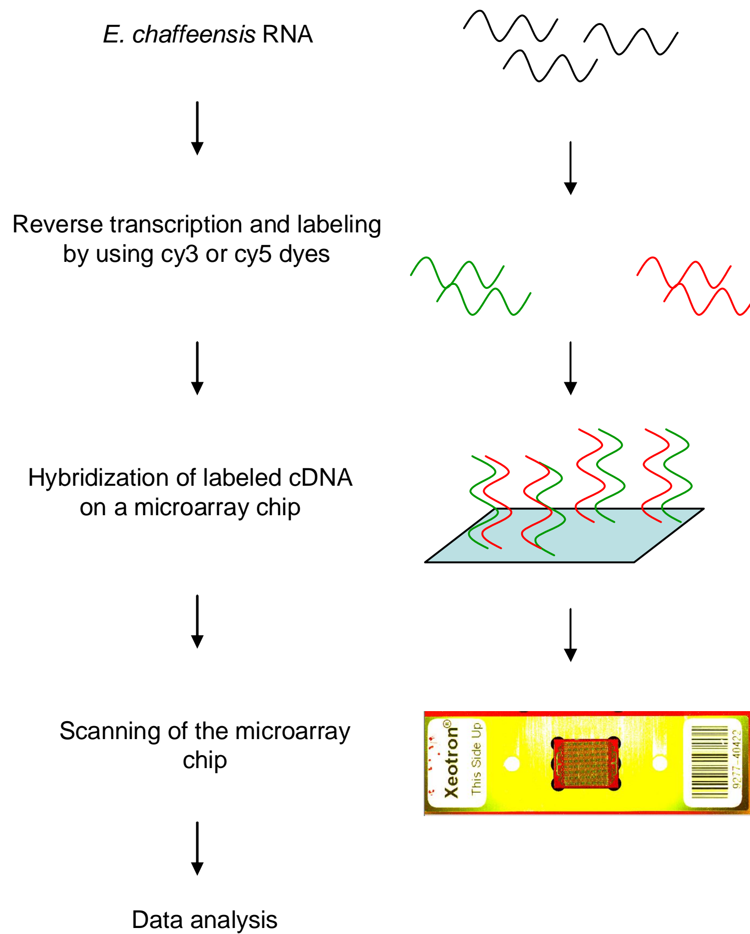


Alto, CA) (Figure 3.2). About 4 µg of RNA was reverse transcribed using superscript II (Invitrogen Corporation, Carlsbad, CA) in the presence of random hexamers, chip control spikes, and amino allyl modified dUTPs (Sigma-Aldrich, St. Louis, MO). The amino allyl-labeled targets were then coupled to aliquots of ester linked Cy3 or Cy5 dyes (GE Healthcare, Piscataway, NJ). The labeled targets were used to hybridize the microarray chips for 18 h at 35°C in a M-2 microfluidic station and washed as outlined in the Xeotron specifications (Invitrogen, Carlsbad, CA). Arrays were scanned to visualize the labeled wells using a GenePix 4000B scanner (Molecular Devices Corporation, Union City, CA). The RNA labeling, chip hybridization and initial output data analysis were performed at the Vanderbilt University Microarray Shared Resource Facility (Nashville, TN). An outline of the microarray protocol is shown in Figure 3.3.

Data analysis: The data from the GenePix scanner were analyzed using the software, Gene Traffic (Stratagene, La Jolla, CA) and MS Excel (Microsoft, Redmond, WA). We performed three independent experiments using three different batches of RNA each isolated from *E. chaffeensis* growing in either macrophage or tick cell environments. Our chip design included various internal controls, the presence of several empty wells and hybridization and labeling controls with both mismatch and perfect match oligonucleotides. Any fluorescence detected in the negative control wells is considered background fluorescence and is subtracted from the total fluorescence measured from all wells. The averages of the fluorescence intensity values of all the probes representing an ORF were calculated separately for each hybridization. The average fluorescence values were compared with the other two hybridizations performed using *E. chaffeensis* RNA derived from the same host cell background and the total average values from all three hybridizations were calculated for each ORF. This analysis resulted in the generation of the average normalized fluorescence intensity values, from the three experiments using *E. chaffeensis* RNA derived from DH82 host cell background, for each ORF.

Figure 3.3 Microarray protocol.

E. chaffeensis RNA was reverse transcribed and labeled with Cy3 or Cy5 fluorescent dyes. The labeled cDNA was then hybridized to a microarray chip, followed by washing to remove unbound molecules and the chip is scanned in a Genepix 4000 B scanner and the data were analyzed.



Similar analysis was performed using the data generated from the three hybridizations performed using ISE6 cell-derived *E. chaffeensis* RNA.

A threshold fluorescence value was determined, for each hybridization, based on the fluorescence measured in the control wells which contained mismatched and unrelated probes. The ORFs whose average fluorescence is more than the determined threshold fluorescence are identified as expressed in *E. chaffeensis*. The ORFs that were found to be expressed in both cell backgrounds were further analyzed. The ratio of the fluorescence intensity values for each ORF between macrophage and tick cell hybridizations was calculated. The ORFs with the ratio values between 0.5 and 2.0 were considered as expressed at the same level. All the ORFs with ratios greater than 2.0 and less than 0.5 were considered as expressed at higher or lower levels in the RNA obtained from *E. chaffeensis* grown in either macropahges or tick cells.

RT-PCR analysis: The gene expression data obtained from the microarrray experiments was validated by analyzing the transcriptional profiles for a subset of genes using a semi-quantitative RT-PCR assay. A subset of genes representing the commonly expressed genes, macrophage- and tick cell- specific expressed genes and genes that are found to be transcriptionally inactive or too low to be detected by microarray analysis were selected. Forward and reverse primers were designed for the selected genes for use in the RT-PCR analysis. RNA was isolated from 5 ml cultures of *E. chaffeensis* grown in macrophage or tick cells at different time points post infection (ranging from 6 h to 120 h) by using Tri-reagent method as per the manufacturer’s protocols. The final RNA pellet is dissolved in 100 µl of nuclease free water and stored at -80°C. About 10 µl of the RNAs were treated with RQ1DNase to remove any contaminating genomic DNA as per the manufacturer’s protocols. The amount of RNA used in the RT-PCR analysis was normalized by assessing the RNA concentration by real time RT-PCR targeted to 16S rRNA gene. Equal amounts of RNA were

used as templates in subsequent experiments. RT-PCR was performed by adding the forward and reverse primers under optimized conditions identified for each gene as described in Chapter 5. A semi-quantitative RT-PCR was performed by terminating the reaction at different PCR cycles. About 5 µl of PCR products are resolved on an agarose gel and the presence or absence of amplicons was evaluated by using ethidium bromide stained DNA gels as described in Chapter 5.

Results

ORF-based whole genome microarray chip for *E. chaffeensis*: We obtained the unannotated complete genome sequence of *E. chaffeensis* from TIGR. The genome sequence was carefully analyzed for the presence of open reading frames (ORFs) that contain 95 or longer amino acids in a row by using Artemis genome sequence analysis program (Welcome Trust Sanger institute, Cambridge, UK). Our analysis identified 1,234 ORFs. Two probes were designed targeting specific regions for each identified ORF. The length of each probe is about 45 nucleotides long. Similarly, three probes targeting the variable regions in the ORF of each gene were designed for all 22 genes in the p28-Omp gene locus of *E. chaffeensis.* The probes were *in situ* synthesized by randomly placing them throughout the microarray chip in triplicate. In addition, we incorporated probes for one or more housekeeping genes to represent dog, human, white tailed deer and tick host of *E. chaffeensis*. Positive controls with perfectly matched and mismatched oligonucleotides were also synthesized in the array chips. Several blank wells were left to serve as background controls.

Global gene expression analysis of *E. chaffeensis*: Six independent experiments were performed (three each) by using RNA isolated from *E. chaffeensis* grown in macrophages or tick cell environment. RNA from *E. chaffeensis* grown in tick cells or macrophages was isolated three times independently using three different culture flasks. To evaluate the reproducibility of

the data from microarray analysis, we performed self to self hybridization of the *E. chaffeensis* RNA isolated from macrophage and tick cells. The data obtained from one experiment in Cy3 and Cy5 channels is presented in the Figure 3.4 (panels A, B and C). The log fluorescence between the two dyes used in the experiment was plotted against each which shows good correlation between the signals observed in Cy3 and Cy5 channels (Figure 3.4, panel D).

To assess global gene expression differences in *E. chaffeensis*, the data from three independent experiments were analyzed carefully. The averages of fluorescence intensity values for all the probes representing an ORF were calculated and compared between the hybridizations performed using RNA from *E. chaffeensis* grown in macrophage and tick cells. with the macrophage and tick cells. The analysis using the data from three different experiments revealed that average fluorescence values for probes representing about 381 ORFs were above the background fluorescence in RNA derived from macrophage grown *E. chaffeensis*. Similar analysis revealed that average fluorescence values for probes representing about 341 ORFs were above the background fluorescence in RNA derived from tick cell grown *E. chaffeensis*. We examined the expression profiles of the transcripts from the RNA polymerase complex in macrophage and tick cell derived *E. chaffeensis* RNA (Figure 3.5). The expression levels for all transcripts are within two fold for *E. chaffeensis* grown in both cell backgrounds. These results suggest that microarray data are valid since all subunits of RNA polymerase had very similar ratios of expression levels in both host cell backgrounds.

Figure 3.4 *E. chaffeensis* microarray.

Whole genome microarray was prepared and evaluated using RNA isolated from macrophage and tick cell grown *E. chaffeensis*. Panel A contains the image of a chip scanned for Cy5 labeling and panel B contains the image of a chip scanned for Cy3 labeling. Panel C contains an image representing the ratio between the Cy5 and Cy3 labeling for the same chip. Panel D represents the correlation data for self to self hybridization.

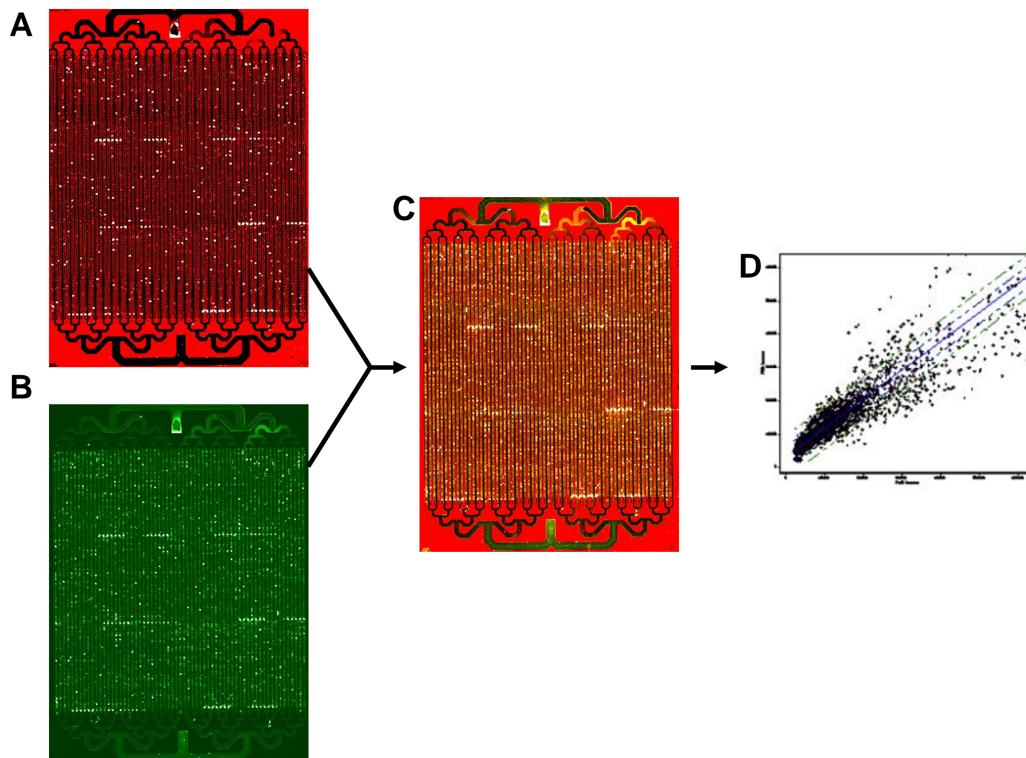


Figure 3.5 Expression of genes of the RNA polymerase complex in *E. chaffeensis* grown in macrophage and tick cells.

The expression genes coding the RNA polymerase complex was analyzed by using RNA derived from *E. chaffeensis* grown in macrophage and tick cells. The ratios of the expression (average of three independent experiments) between macrophage and tick cell derived *E. chaffeensis* along with error bars is plotted on Y-axis.

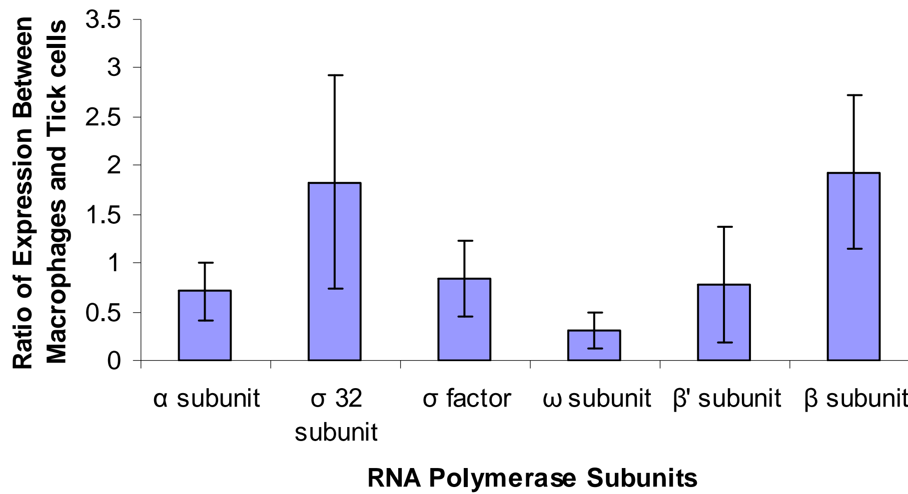
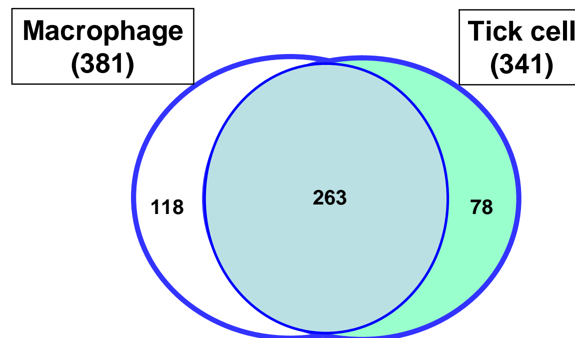


Figure 3.6. Transcriptional analysis of the *E. chaffeensis* genome.

RNA was isolated from *E. chaffeensis* grown in tick and macrophage cell environments and used in the microarray gene expression analysis. The microarray data for three independent experiments were evaluated to identify expression pattern in *E. chaffeensis* originating from macrophages and tick cells. The analysis revealed a total of 381 and 341 genes were expressed in *E. chaffeensis* growing in macrophages and tick cells, respectively. Two hundred and sixty three genes are commonly expressed in both cell backgrounds and about 118 and 78 are found to be uniquely expressed in *E. chaffeensis* growing in macrophages and tick cells, respectively.



The ORFs that were found to have signals above the background fluorescence in macrophage and tick cell-derived *E. chaffeensis* RNA were compared to identify the commonly expressed and host-specifically expressed transcripts. This analysis revealed that 263 ORFs have fluorescence above the background in both macrophage and tick cell derived *E. chaffeensis* RNA (Figure 3.6 and 3.7). About 118 ORFs were found to have fluorescence above background in macrophage derived *E. chaffeensis* RNA. Similarly, 78 ORFs were found to have fluorescence above background in tick cell derived *E. chaffeensis* RNA (Figure 3.7). We further compared the average fluorescence intensity values for the 263 genes that were found to be positive in both cell backgrounds. About 80% of the ORFs have fluorescence values within two fold difference between macrophage and tick cell derived *E. chaffeensis* RNA. About 8 ORFs have average fluorescence values more than two fold greater in macrophage derived *E. chaffeensis* RNA compared the tick cell derived *E. chaffeensis* RNA. This analysis also revealed that 47 ORFs have average fluorescence values more than two fold greater in tick cell derived *E. chaffeensis* RNA compared to the macrophage derived *E. chaffeensis* RNA (Figure 3.7)

The major portion of ORFs that have fluorescence above background in both cell environments included those coding for DNA metabolism, energy metabolism, protein synthesis, transcription, transport and cellular processes (Table 3.1 and 3.2). Several ORFs coding for hypothetical proteins are also found to have fluorescence values above background. The majority of the transcripts belonging to uniquely expressed groups include those coding for cell envelope, hypothetical and genes with unknown function. In addition, transcripts made from cofactor and vitamin biosysnthesis and protein fate genes were among the uniquely expressed group, contributing to about 20% of the group.

Figure 3.7 Gene expression analysis of *E. chaffeensis*.

Microarray data were evaluated to identify differences in the levels of transcripts in commonly expressed genes. Transcripts from which greater than 2 fold differences were observed were considered as expressed at high or low in either macrophage or tick cell backgrounds. The data from this analysis revealed that about 80% of the genes that are common to *E. chaffeensis* grown in both cell backgrounds are expressed at equal levels and included several coding for hypothetical proteins. The genes that are expressed at high or low levels in the common list of genes also include some coding for hypothetical proteins. Similarly, the host cell-specifically expressed genes also contained several coding for hypothetical, membrane proteins and proteins of unknown function.

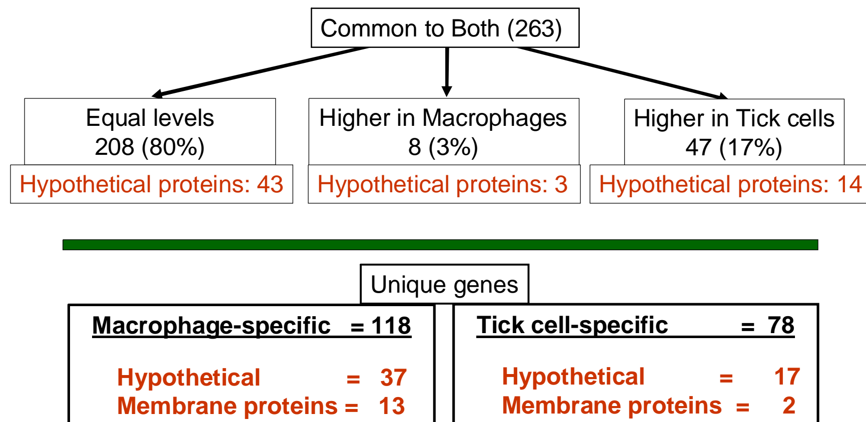


Table 3.1 *E. chaffeensis* transcripts grouped based on functional identity.

Functional Group	Genome (total number)	Macrophage (unique or higher levels	Tick cell (unique or higher levels)	Common
Disrupted reading frame	7	0	0	0
Mobile and extrachromosomal	4	0	0	0
Amino acid biosynthesis	24	4	2	4
Co-factor and vitamin biosynthesis	**65**	**10**	**7**	**7**
Cellular processes	43	3	4	7
Central intermediary metabolism	3	1	0	2
DNA metabolism	46	2	3	10
Energy metabolism	88	7	7	40
Phospholipid metabolism	22	4	2	4
Protein fate	**75**	**10**	**10**	**10**
Protein synthesis	111	9	20	36
Nucleotide biosynthesis	38	4	5	4
Regulatory functions	17	1	2	3
Signal transduction	5	0	0	1
Transcription	23	3	2	9
Transport	40	3	1	10
Cell envelope	**49**	**13**	**2**	**16**
Hypothetical	**425**	**40**	**31**	**43**
Unknown function	**85**	**12**	**15**	**13**

Table 3.2 The transcriptional analysis of *E. chaffeensis*.

The genes that were found to be expressed by microarray analysis are grouped based on their functional identity. The average fluorescence (along with standard error) detected in the microarray chips is presented in the table. Macrophage, average fluorescence measured from the chips hybridized with macrophage-derived *E. chaffeensis* RNA; tick cells, average fluorescence measured from the chips hybridized with tick cell-derived *E. chaffeensis* RNA; white background, commonly expressed; yellow background, tick cell-specifically expressed; green background, macrophage-specifically expressed. (Table continued into pages 60-70).

No.	Gene ID	Gene Name	Macrophage	Tick cell
		Amino acid biosynthesis		
1	ECH_0058	2,3,4,5-tetrahydropyridine-2-carboxylate N-succinyltransferase	14585±222	26275±256
2	ECH_0594	acetylglutamate kinase	0	4690±2418
3	ECH_0676	arginine biosynthesis bifunctional protein ArgJ	17140±9817	23742±11405
4	ECH_0680	argininosuccinate synthase	1676±2607	0
5	ECH_0016	aspartate-semialdehyde dehydrogenase	10931±9397	0
6	ECH_0443	dihydrodipicolinate reductase	6276±2541	9131±2645
7	ECH_0089	glutamine synthetase, type I	1958±1732	0
8	ECH_1017	N-acetyl-gamma-glutamyl-phosphate reductase	1388±13	0
9	ECH_0311	serine hydroxymethyltransferase	10751±3209	18718±9489
10	ECH_0144	succinyl-diaminopimelate desuccinylase	0	1722±2192
		Biosynthesis of co-factors and vitamins		
1	ECH_0156	2C-methyl-D-erythritol 2,4-cyclodiphosphate synthase	976±152	0
2	ECH_0157	2-C-methyl-D-erythritol 4-phosphate cytidylyltransferase	796±2041	0
3	ECH_0134	3,4-dihydroxy-2-butanone 4-phosphate synthase	8425±2326	13477±9449
4	ECH_0757	4-diphosphocytidyl-2C-methyl-D-erythritol kinase	8611±2546	10005±6722
5	ECH_0783	6,7-dimethyl-8-ribityllumazine synthase	0	3476±85
6	ECH_0950	8-amino-7-oxononanoate synthase	1167±427	0
7	ECH_0666	adenosylmethionine-8-amino-7-oxononanoate aminotransferase	1091±445	0
8	ECH_0873	Coq7 family protein	6278±1141	5815±3846
9	ECH_0629	cysteine desulfurase	2867±2701	0
10	ECH_0381	dihydropteroate synthase	0	2305±398
11	ECH_0702	folylpolyglutamate synthase	10400±2645	5964±2742
12	ECH_0125	glutamate--cysteine ligase	6690±1121	9056±6577
13	ECH_0822	glutamine-dependent NAD(+) synthetase	2035±190	0
14	ECH_0651	GTP cyclohydrolase I	5538±6224	0

15	ECH_0689	iron-sulfur cluster assembly accessory protein_0689	28916±10414	39554±13924
16	ECH_0806	nicotinate (nicotinamide) nucleotide adenylyltransferase	0	3973±294
17	ECH_0701	porphobilinogen deaminase	2604±1419	0
18	ECH_1004	protoheme IX farnesyltransferase	0	3041±321
19	ECH_0225	putative ATP-NAD kinase	1067±501	0
20	ECH_0455	putative geranyltranstransferase	2211±998	0
21	ECH_0931	pyridoxamine 5'-phosphate oxidase	0	4599±3499
22	ECH_0798	thiamin biosynthesis protein ThiC	0	4178±2626
23	ECH_0206	thiamin biosynthesis ThiG	0	6161±2180
24	ECH_0893	thiamine-phosphate pyrophosphorylase	12289±3797	18787±12629
		Cell envelope, Surface structures		
1	ECH_0039	120 kDa immunodominant surface protein	0	20243±17672
2	ECH_0645	immunogenic protein	6634±2164	19133±10687
3	ECH_0462	OmpA family protein	0	5504±3750
4	ECH_0482	putative lipoprotein_0482	19875±1872	18691±3571
5	ECH_0558	putative lipoprotein_0558	19235±1376	17992±8388
6	ECH_0625	putative lipoprotein_0625	6024±8070	7087±9837
7	ECH_0929	putative lipoprotein_0929	18443±7934	25927±8367
8	ECH_0188	putative surface protein	7102±1136	7000±3066
9	ECH_0170	variable length PCR target protein	27673±10111	15239±8623
		Cellular processes		
1	ECH_0734	antioxidant, AhpC/Tsa family_0734	0	2048±181
2	ECH_1090	cell division protein FtsA	18113±1365	11914±3591
3	ECH_1153	cell division protein FtsZ	7794±2390	14658±6312
4	ECH_1156	chromosome partitioning ATPase, ParA family	10805±11191	0
5	ECH_0648	dimethyladenosine transferase	0	7942±3953
6	ECH_0031	hemolysin	5751±3094	0
7	ECH_0890	putative cell division protein FtsK	6342±1402	6062±3316
8	ECH_0337	putative cell division protein FtsQ	8193±2350	8178±5211
9	ECH_0143	putative competence protein F	0	8013±3680
10	ECH_0335	putative osmotically inducible protein	6137±1347	5898±3519
11	ECH_0871	putative twitching motility protein PilT	6774±1638	8889±3696
12	ECH_0042	type IV secretion system protein VirB10	6689±1275	16441±9341
13	ECH_0041	type IV secretion system protein VirB11	1120±211	0
14	ECH_0497	type IV secretion system protein, VirB6 family	13597±2986	13064±4826
		Central intermediary metabolism, Amino sugars		
1	ECH_0541	5-formyltetrahydrofolate cyclo-ligase family protein	9845±2821	8827±3278
2	ECH_1014	inorganic pyrophosphatase	7490±1641	8523±47391
3	ECH_0018	S-adenosylmethionine synthetase	3031±1615	0
		DNA metabolism		
1	ECH_0809	chromosomal replication initiator protein DnaA	13008±6457	6543±3616
2	ECH_0028	crossover junction endodeoxyribonuclease RuvC	0	5880±5105
3	ECH_0858	DNA gyrase, A subunit	6749±6457	7111±3616
4	ECH_0620	DNA gyrase, B subunit	6405±1261	12269±6063

5	ECH_0080	DNA polymerase I	3095±474	0
6	ECH_1009	DNA polymerase III, beta subunit	12272±6823	14543±9547
7	ECH_0761	DNA primase	26580±12101	21736±11143
8	ECH_0750	DNA topoisomerase I	0	7629±3304
9	ECH_0857	endonuclease III	10311±195	7697±5276
10	ECH_1109	recA protein	7267±2472	14770±9667
11	ECH_0843	recR	6111±1355	10473±4782
12	ECH_0451	replicative DNA helicase	6249±1261	13822±6063
13	ECH_0082	ribulose-phosphate 3-epimerase	16641±5056	15040±10469
14	ECH_0173	tyrosine recombinase XerD	6172±1310	16923±8077
15	ECH_0074	uracil-DNA glycosylase, family 4	1273±444	0

		Energy metabolism		
1	ECH_0097	fructose- biphosphate aldolase, class I	1132±176	0
2	ECH_1031	aconitate hydratase 1	21169±12766	19023±9817
3	ECH_0138	aminomethyl transferase family protein	2631±545	0
4	ECH_1089	ATP synthase F0, B chain	940±711	0
5	ECH_1088	ATP synthase F0, B' subunit	6932±1262	5746±23722
6	ECH_0132	ATP synthase F1, alpha subunit	7578±3179	9833±1958
7	ECH_0573	ATP synthase F1, beta subunit	10965±4382	18508±6889
8	ECH_0131	ATP synthase F1, delta subunit	10182±3952	24845±11051
9	ECH_0574	ATP synthase F1, epsilon subunit	6871±1617	5651±2240
10	ECH_0652	ATP synthase F1, gamma subunit	8819±4161	17029±8420
11	ECH_0124	citrate synthase I	9193±2971	11655±9158
12	ECH_1068	C-type cytochrome family protein	0	3079±916
13	ECH_1055	cytochrome c oxidase assembly protein CtaG	0	4311±1953
14	ECH_1003	cytochrome c oxidase, subunit I	8859±1821	7703±2766
15	ECH_1002	cytochrome c oxidase, subunit II	11471±4471	18134±8904
16	ECH_0029	cytochrome c oxidase, subunit III	7549±2361	16452±5468
17	ECH_0327	cytochrome C, membrane-bound	6047±1622	10906±6229
18	ECH_0789	cytochrome c-type biogenesis protein CcmE	8479±2590	13161±10459
19	ECH_0137	cytochrome c-type biogenesis protein CcmF	13705±5675	39329±16690
20	ECH_0509	dihydrolipoamide dehydrogenase	6638±1222	8029±2167
21	ECH_0992	dihydrolipoamide dehydrogenase	8804±2774	5608±4228
22	ECH_0368	dioxygenase family protein	0	13871±5209
23	ECH_0544	enolase	1371±498	0
24	ECH_0038	ferredoxin A	0	5285±2893
25	ECH_0376	fumarate hydratase, class II	9302±3088	7685±3565
26	ECH_0175	malate dehydrogenase	6163±1091	10876±6991
27	ECH_0641	malate dehydrogenase, NAD-dependent	9176±3202	30728±10644
28	ECH_0786	NADH dehydrogenase I, A subunit	7373±195	6139±5276
29	ECH_0787	NADH dehydrogenase I, B subunit	27209±8291	30484±2252
30	ECH_0788	NADH dehydrogenase I, C subunit	6700±1786	9985±3487
31	ECH_0548	NADH dehydrogenase I, F subunit	9130±2135	5774±2527
32	ECH_0618	NADH dehydrogenase I, G subunit	1022±4444	16698±5546
33	ECH_0617	NADH dehydrogenase I, H subunit	1475±669	0
34	ECH_0552	NADH dehydrogenase I, J subunit	2474±774	13427±4787

35	ECH_0554	NADH dehydrogenase I, L subunit	18090±10362	14902±10414
36	ECH_0555	NADH dehydrogenase I, M subunit	12805±8341	1138±3572
37	ECH_0556	NADH dehydrogenase I, N subunit	8616±4025	1322±4624
38	ECH_0474	NADH-ubiquinone/plastoquinone oxidoreductase family protein_0474	7747±3077	11203±7588
39	ECH_0667	proline dehydrogenase/delta-1-pyrroline-5-carboxylate dehydrogenase	2146±71	0
40	ECH_0487	propionyl-CoA carboxylase, alpha subunit	6696±1976	6026±1207
41	ECH_0599	propionyl-CoA carboxylase, beta subunit	18937±8071	11061±6516
42	ECH_1079	putative cytochrome c oxidase, subunit I	13917±5405	17289±6709
43	ECH_0442	putative flavin reductase	27094±7391	24636±13633
44	ECH_0179	putative NADH dehydrogenase I, N subunit	26806±15317	32773±17222
45	ECH_0385	quinone oxidoreductase	8494±2111	16318±9090
46	ECH_0638	ribose 5-phosphate isomerase B	13091±5958	21188±9633
47	ECH_0316	succinate dehydrogenase and fumarate reductase iron-sulfur protein	0	4394±474
48	ECH_0918	succinate dehydrogenase, cytochrome b556 subunit	21495±9984	49282±11686
49	ECH_0315	succinate dehydrogenase, flavoprotein subunit	10646±3085	17691±8636
50	ECH_0979	succinyl-CoA synthetase, beta subunit	7278±1444	5904±3174
51	ECH_0646	triosephosphate isomerase	11413±4523	43251±11480
52	ECH_0521	ubiquinol-cytochrome c reductase, cytochrome b	10504±4392	15120±8207
53	ECH_0522	ubiquinol-cytochrome c reductase, cytochrome c1	1567±0555	0
54	ECH_0520	ubiquinol-cytochrome c reductase, iron-sulfur subunit	10447±3086	15872±10451
		Fatty acid and phospholipid metabolism		
1	ECH_0072	1-acyl-sn-glycerol-3-phosphate acyltransferase family protein	1254±124	0
2	ECH_1078	CDP-diacylglycerol--glycerol-3-phosphate 3-phosphatidyltransferase	0	2818±864
3	ECH_0811	enoyl-(acyl-carrier-protein) reductase	12947±5815	17174±7513
4	ECH_0447	fatty acid/phospholipid synthesis protein PlsX	10387±2705	16352±10471
5	ECH_0741	holo-(acyl-carrier-protein) synthase	904±183	0
6	ECH_0227	malonyl CoA-acyl carrier protein transacylase	37771±12533	38221±13635
7	ECH_0905	phosphatidylglycerophosphatase A	2264±66	0
8	ECH_0780	putative CDP-diacylglycerol--serine O-phosphatidyltransferase	11866±2528	20735±13714
9	ECH_0269	putative phosphatidate cytidylyltransferase	10557±4037	26537±14874
10	ECH_0875	putative phosphatidylglycerophosphatase A	2323±530	0
		Hypothetical		
1	ECH_1155	conserved hypothetical protein TIGR00043	4001±442	0
2	ECH_0009	hypothetical protein ECH_0009	40583±11987	43890±10865
3	ECH_0010	hypothetical protein ECH_0010	11740±2881	27477±17486
4	ECH_0021	hypothetical protein ECH_0021	7127±5719	0
5	ECH_0049	hypothetical protein ECH_0049	7056±2080	16520±10443
6	ECH_0079	hypothetical protein ECH_0079	7408±1312	9287±4179
7	ECH_0084	hypothetical protein ECH_0084	32835±15835	29558±17989
8	ECH_0087	hypothetical protein ECH_0087	2106±267	0
9	ECH_0106	hypothetical protein ECH_0106	349±1200	0

10	ECH_0113	hypothetical protein ECH_0113	2031±77	0
11	ECH_0114	hypothetical protein ECH_0114	1114±1730	0
12	ECH_0115	hypothetical protein ECH_0115	9178±7485	0
13	ECH_0116	hypothetical protein ECH_0116	1397±443	0
14	ECH_0119	hypothetical protein ECH_0119	7171±3417	0
15	ECH_0120	hypothetical protein ECH_0120	5590±5643	0
16	ECH_0122	hypothetical protein ECH_0122	0	11154±8844
17	ECH_0147	hypothetical protein ECH_0147	607±1173	0
18	ECH_0148	hypothetical protein ECH_0148	0	884±953
19	ECH_0158	hypothetical protein ECH_0158	0	6836±6320
20	ECH_0159	hypothetical protein ECH_0159	7059±1377	18569±12432
21	ECH_0166	hypothetical protein ECH_0166	31639±12975	15097±9975
22	ECH_0176	hypothetical protein ECH_0176	6651±1084	13798±11048
23	ECH_0199	hypothetical protein ECH_0199	0	4183±860
24	ECH_0230	hypothetical protein ECH_0230	23963±10542	17911±8518
25	ECH_0237	hypothetical protein ECH_0237	7683±2227	9200±4350
26	ECH_0240	hypothetical protein ECH_0240	7230±1856	15400±9368
27	ECH_0243	hypothetical protein ECH_0243	12420±4984	10544±7220
28	ECH_0246	hypothetical protein ECH_0246	29273±3192	19278±7579
29	ECH_0247	hypothetical protein ECH_0247	1226±564	0
30	ECH_0252	hypothetical protein ECH_0252	2541±1588	0
31	ECH_0253	hypothetical protein ECH_0253	2952±1580	0
32	ECH_0257	hypothetical protein ECH_0257	2121±789	0
33	ECH_0270	hypothetical protein ECH_0270	6378±2501	10728±9233
34	ECH_0272	hypothetical protein ECH_0272	6402±1784	18481±7960
35	ECH_0275	hypothetical protein ECH_0275	2437±4662	0
36	ECH_0278	hypothetical protein ECH_0278	0	5708±5490
37	ECH_0284	hypothetical protein ECH_0284	1036±47	0
38	ECH_0285	hypothetical protein ECH_0285	35406±8415	47265±8745
39	ECH_0288	hypothetical protein ECH_0288	1059±230	0
40	ECH_0289	hypothetical protein ECH_0289	27256±6273	33043±6540
41	ECH_0292	hypothetical protein ECH_0292	11744±3050	13042±4524
42	ECH_0329	hypothetical protein ECH_0329	486±311	0
43	ECH_0343	hypothetical protein ECH_0343	0	19260±7614
44	ECH_0348	hypothetical protein ECH_0348	17090±6425	12034±6990
45	ECH_0377	hypothetical protein ECH_0377	13267±5425	18589±8799
46	ECH_0388	hypothetical protein ECH_0388	6796±2332	15329±8944
47	ECH_0391	hypothetical protein ECH_0391	11871±7668	6040±2879
48	ECH_0439	hypothetical protein ECH_0439	6129±2369	5691±3393
49	ECH_0454	hypothetical protein ECH_0454	0	2820±340
50	ECH_0477	hypothetical protein ECH_0477	0	9085±9671
51	ECH_0519	hypothetical protein ECH_0519	1065±46	0
52	ECH_0523	hypothetical protein ECH_0523	13334±7206	12420±8758
53	ECH_0526	hypothetical protein ECH_0526	6008±1383	11345±3955
54	ECH_0531	hypothetical protein ECH_0531	0	10478±3649
55	ECH_0549	hypothetical protein ECH_0549	0	5913±2760
56	ECH_0570	hypothetical protein ECH_0570	896±193	0
57	ECH_0576	hypothetical protein ECH_0576	8099±2855	5748±1693

58	ECH_0578	hypothetical protein ECH_0578	0	3420±246
59	ECH_0587	hypothetical protein ECH_0587	6342±1529	7850±684
60	ECH_0588	hypothetical protein ECH_0588	1406±780	0
61	ECH_0593	hypothetical protein ECH_0593	25753±5943	9366±5683
62	ECH_0601	hypothetical protein ECH_0601	25991±7596	17903±8857
63	ECH_0609	hypothetical protein ECH_0609	1120±397	0
64	ECH_0611	hypothetical protein ECH_0611	0	1149±510
65	ECH_0612	hypothetical protein ECH_0612	10088±1013	6526±1317
66	ECH_0627	hypothetical protein ECH_0627	26088±7844	22160±8935
67	ECH_0635	hypothetical protein ECH_0635	0	9693±2366
68	ECH_0640	hypothetical protein ECH_0640	7089±1926	6913±4453
69	ECH_0663	hypothetical protein ECH_0663	0	4393±1318
70	ECH_0664	hypothetical protein ECH_0664	10258±2272	6114±3408
71	ECH_0670	hypothetical protein ECH_0670	2689±2631	0
72	ECH_0681	hypothetical protein ECH_0681	1464±1578	0
73	ECH_0695	hypothetical protein ECH_0695	15672±6329	54738±7002
74	ECH_0697	hypothetical protein ECH_0697	1254±1286	0
75	ECH_0700	hypothetical protein ECH_0700	20170±10683	0
76	ECH_0706	hypothetical protein ECH_0706	2689±2910	0
77	ECH_0707	hypothetical protein ECH_0707	20146±6161	13719±9514
78	ECH_0708	hypothetical protein ECH_0708	25131±10546	18037±12619
79	ECH_0709	hypothetical protein ECH_0709	2967±3198	0
80	ECH_0715	hypothetical protein ECH_0715	7158±1002	8093±5437
81	ECH_0720	hypothetical protein ECH_0720	9571±2991	14618±6965
82	ECH_0722	hypothetical protein ECH_0722	10284±4469	23110±12660
83	ECH_0725	hypothetical protein ECH_0725	6844±1861	7024±4290
84	ECH_0739	hypothetical protein ECH_0739	872±174	0
85	ECH_0744	hypothetical protein ECH_0744	7639±1394	7023±3104
86	ECH_0753	hypothetical protein ECH_0753	0	3289±2029
87	ECH_0778	hypothetical protein ECH_0778	6426±674	5817±802
88	ECH_0825	hypothetical protein ECH_0825	6287±1379	6578±3540
89	ECH_0836	hypothetical protein ECH_0835	1519±458	0
90	ECH_0838	hypothetical protein ECH_0838	7304±1713	21640±8392
91	ECH_0854	hypothetical protein ECH_0854	20187±11633	21651±12771
92	ECH_0865	hypothetical protein ECH_0865	18895±7729	24018±3082
93	ECH_0878	hypothetical protein ECH_0878	20321±13794	62034±2745
94	ECH_0888	hypothetical protein ECH_0888	11266±3500	15265±5015
95	ECH_0910	hypothetical protein ECH_0910	958±90	0
96	ECH_0916	hypothetical protein ECH_0916	6431±1521	13466±8962
97	ECH_0943	hypothetical protein ECH_0943	6096±1778	29838±3493
98	ECH_0947	hypothetical protein ECH_0947	1807±321	0
99	ECH_1023	hypothetical protein ECH_1023	0	3592±4832
100	ECH_1036	hypothetical protein ECH_1036	36759±11902	42291±18552
101	ECH_1037	hypothetical protein ECH_1037	1992±447	0
102	ECH_1042	hypothetical protein ECH_1042	18621±8263	15202±9949
103	ECH_1043	hypothetical protein ECH_1043	13569±4546	17071±9467
104	ECH_1044	hypothetical protein ECH_1044	16056±6897	12384±6417
105	ECH_1047	hypothetical protein ECH_1047	7307±1725	22524±12578

106	ECH_1059	hypothetical protein ECH_1059	35733±6155	11690±7864
107	ECH_1104	hypothetical protein ECH_1104	26672±7330	14927±2857
108	ECH_1105	hypothetical protein ECH_1105	2994±1977	0
109	ECH_1122	hypothetical protein ECH_1122	14385±8526	7239±4306
110	ECH_1128	hypothetical protein ECH_1128	3259±1451	0
111	ECH_1147	hypothetical protein ECH_1147	2008±124	0
112	ECH_1148	hypothetical protein ECH_1148	0	23080±7366
113	ECH_1152	hypothetical protein ECH_1152	8004±1732	9183±4028
114	ECH_1154	hypothetical protein ECH_1154	23060±6972	30716±3219
		Protein fate		
1	ECH_0178	apolipoprotein N-acyltransferase	1972±1744	0
2	ECH_0367	ATP-dependent Clp protease, ATP-binding subunit ClpB	0	5760±4376
3	ECH_0900	ATP-dependent Clp protease, ATP-binding subunit ClpX	6935±2012	9672±6565
4	ECH_0901	ATP-dependent Clp protease, proteolytic subunit ClpP	0	2764±1769
5	ECH_0899	ATP-dependent protease La	0	6330±4465
6	ECH_0471	chaperone protein DnaK	10406±3035	20485±8463
7	ECH_0853	chaperone protein HtpG	8188±2893	13495±7381
8	ECH_0364	chaperonin, 10 kDa	9390±4362	26007±15986
9	ECH_0365	chaperonin, 60 kd	9422±4248	30179±16991
10	ECH_0369	cytosol aminopeptidase	0	4660±2880
11	ECH_0997	heat shock protein HslVU, ATPase subunit HslU	623±602	0
12	ECH_1050	hflK protein	6625±2122	10222±2928
13	ECH_1064	methionine aminopeptidase, type I	2427±1007	0
14	ECH_1057	peptidase, M16 family_1057	0	2144±406
15	ECH_0073	peptide deformylase	0	10925±7688
16	ECH_0428	preprotein translocase, SecY subunit	9663±3751	15073±5955
17	ECH_1008	preprotein translocase, YajC subunit	7756±2245	13695±8125
18	ECH_1101	prolipoprotein diacylglyceryl transferase	15659±4605	8962±6984
19	ECH_1106	protein-export membrane protein SecD	6833±2444	7784±3611
20	ECH_0095	protein-export membrane protein SecF	1353±152	0
21	ECH_0233	protein-export protein SecB	7573±2368	11882±6887
22	ECH_0295	putative heme exporter protein CcmA	0	2489±609
23	ECH_0065	putative heme exporter protein CcmB	3147±1594	0
24	ECH_0939	putative polypeptide deformylase	0	6887±2033
25	ECH_0731	rotamase family protein	3260±2873	0
26	ECH_0690	signal peptidase I	9623±2156	12559±6323
27	ECH_1060	signal peptidase II	1863±831	0
28	ECH_0401	signal peptide peptidase SppA	1119±66	0
29	ECH_1111	signal recognition particle-docking protein FtsY	923±14	0
30	ECH_0970	type I secretion membrane fusion protein, HlyD family	938±525	0
		Protein synthesis		
1	ECH_0484	50S ribosomal protein L28	19097±8276	17734±11315
2	ECH_0981	alanyl-tRNA synthetase	15335±7512	5570±3514
3	ECH_0876	ankyrin repeat protein_0876	33212±2532	16034±9888

4	ECH_0768	cysteinyl-tRNA synthetase	0	3366±811
5	ECH_1025	glutamyl-tRNA(Gln) amidotransferase subunit C	1340±854	0
6	ECH_0813	glutamyl-tRNA(Gln) amidotransferase, B subunit	19842±8461	12162±9513
7	ECH_0023	glycyl-tRNA synthetase, alpha subunit	10942±2505	11879±5164
8	ECH_0897	methionyl-tRNA formyltransferase	0	7647±5427
9	ECH_0705	peptide chain release factor 2, programmed frameshift	8438±2221	5660±2051
10	ECH_0434	phenylalanyl-tRNA synthetase, beta subunit	11820±3341	15898±8551
11	ECH_0309	putative ribosomal protein S18	9409±2702	9160±4692
12	ECH_0142	ribosomal 5S rRNA E-loop binding protein Ctc/L25/TL5	6428±1531	18118±4764
13	ECH_0955	ribosomal protein L1	15507±7473	20744±10069
14	ECH_0954	ribosomal protein L10	9513±1589	23207±5177
15	ECH_0956	ribosomal protein L11	12232±5326	13557±7448
16	ECH_1019	ribosomal protein L13	24258±10783	48180±8737
17	ECH_0419	ribosomal protein L14	11294±5797	32810±17650
18	ECH_0427	ribosomal protein L15	0	4200±3213
19	ECH_0416	ribosomal protein L16	19829±9657	32357±14963
20	ECH_0433	ribosomal protein L17	0	3309±388
21	ECH_0425	ribosomal protein L18	15384±3245	28710±4561
22	ECH_0412	ribosomal protein L2	15438±5958	38819±9633
23	ECH_0197	ribosomal protein L20	0	4167±1430
24	ECH_0545	ribosomal protein L21	0	6816±6166
25	ECH_0414	ribosomal protein L22	17444±3568	29391±5246
26	ECH_0411	ribosomal protein L23	16136±9969	23295±11881
27	ECH_0420	ribosomal protein L24	9229±4645	32032±14932
28	ECH_0409	ribosomal protein L3	12490±5013	31580±10182
29	ECH_0226	ribosomal protein L31	1037±603	0
30	ECH_0410	ribosomal protein L4	6187±2164	12076±5513
31	ECH_0421	ribosomal protein L5	20320±9871	38531±12592
32	ECH_0424	ribosomal protein L6	7705±2821	22597±13242
33	ECH_0953	ribosomal protein L7/L12	9505±4176	14829±11237
34	ECH_0408	ribosomal protein S10	6630±1607	11505±5726
35	ECH_0431	ribosomal protein S11	10486±2694	10915±3472
36	ECH_0963	ribosomal protein S12	9178±2042	12828±5562
37	ECH_0430	ribosomal protein S13	15470±5095	20410±11539
38	ECH_0422	ribosomal protein S14p	7126±2433	13505±7791
39	ECH_0727	ribosomal protein S15	14839±7032	29271±10993
40	ECH_0192	ribosomal protein S16	17304±6993	30720±11758
41	ECH_0418	ribosomal protein S17	688±224	0
42	ECH_0071	ribosomal protein S20	10540±1402	16500±3868
43	ECH_0978	ribosomal protein S21	16993±7116	27793±13025
44	ECH_0415	ribosomal protein S3	0	4475±3555
45	ECH_0912	ribosomal protein S4	6910±2779	13483±7518
46	ECH_0426	ribosomal protein S5	14525±8711	34310±12854
47	ECH_0308	ribosomal protein S6	14497±6138	31409±13525
48	ECH_0962	ribosomal protein S7	13823±6621	33109±14454
49	ECH_0423	ribosomal protein S8	21262±8817	19555±9263
50	ECH_0267	ribosome recycling factor	0	3591±127

51	ECH_222	rRNA-23S ribosomal RNA	28995±5452	7405±3567
52	ECH_0135	SsrA-binding protein	12820±5268	23030±7573
53	ECH_0961	translation elongation factor G	18364±10271	23102±13480
54	ECH_0777	translation elongation factor P	20773±7396	17389±7019
55	ECH_0515	translation elongation factor Ts	10283±2973	12930±7618
56	ECH_0960	translation elongation factor Tu-1	14381±5266	26824±11598
57	ECH_0407	translation elongation factor Tu-2	15483±3404	29885±11883
58	ECH_0563	translation initiation factor IF-2	6933±2130	10815±4977
59	ECH_0007	translation initiation factor IF-3	6101±1539	7432±3924
60	ECH_0872	tRNA (5-methylaminomethyl-2-thiouridylate)-methyltransferase	0	8742±5397
61	ECH_0003	tRNA (guanine-N1)-methyltransferase	10284±3809	21381±6433
62	ECH_0060	tRNA modification GTPase TrmE	2260±1758	0
63	ECH_0622	tRNA pseudouridine synthase A	15330±5331	17561±5119
64	ECH_0091	tyrosyl-tRNA synthetase	5597±2580	0
65	ECH_0136	valyl-tRNA synthetase	1893±78	0
		Purines, pyrimidines, nucleosides, and nucleotides		
1	ECH_0429	adenylate kinase	8029±3071	8113±5209
2	ECH_0461	adenylosuccinate synthetase	12942±3995	6778±1157
3	ECH_0378	carbamoyl-phosphate synthase, large subunit	999±400	0
4	ECH_0171	CTP synthase	0	4146±473
5	ECH_0940	dihydroorotate dehydrogenase	6685±1815	7785±5075
6	ECH_0123	GMP synthase	1239±1334	0
7	ECH_0224	inosine-5'-monophosphate dehydrogenase	1360±895	0
8	ECH_1117	nucleoside diphosphate kinase	12291±4526	35611±7546
9	ECH_1108	orotate phosphoribosyltransferase	0	3589±310
10	ECH_0792	orotidine 5'-phosphate decarboxylase	0	3596±1290
11	ECH_0160	phosphoribosylaminoimidazole carboxylase, catalytic subunit	1388±559	0
12	ECH_0766	ribonucleoside-diphosphate reductase, beta subunit	0	3564±1017
13	ECH_0266	uridylate kinase	7344±1614	11205±7634
		Regulatory functions		
1	ECH_1012	DNA-binding response regulator	14676±5456	14502±6524
2	ECH_0162	integration host factor, alpha subunit	0	3018±314
3	ECH_1118	putative transcriptional regulator	6379±2911	6327±1372
4	ECH_0885	sensor histidine kinase	1975±282	0
5	ECH_0152	tldD protein	11680±4221	7494±5307
6	ECH_0163	transcriptional regulator, MerR family	0	5585±4178
		Signal transduction		
1	ECH_0339	putative nitrogen regulation protein NtrX	6052±1079	6981±4508
		Transcription		
1	ECH_0002	16S rRNA processing protein RimM	14502±9045	6231±341
2	ECH_0432	DNA-directed RNA polymerase, alpha subunit	8952±2297	12644±4321
3	ECH_0952	DNA-directed RNA polymerase, beta subunit	15522±2024	8043±6063
4	ECH_0951	DNA-directed RNA polymerase, beta' subunit	7572±869	9733±10139
5	ECH_0796	DNA-directed RNA polymerase, omega subunit	10626±3201	33946±5290

6	ECH_0562	N utilization substance protein A	13500±2875	14080±7363
7	ECH_1116	polyA polymerase/tRNA nucleotidyltransferase family protein	6640±1917	6645±5069
8	ECH_0726	polyribonucleotide nucleotidyltransferase	16520±6182	20490±10075
9	ECH_0263	ribonuclease HI	10647±3313	22073±9193
10	ECH_0564	ribosome-binding factor A	0	4680±2093
11	ECH_0760	RNA polymerase sigma factor RpoD	8536±1243	10249±1382
12	ECH_0957	transcription antitermination protein NusG	6636±2452	9616±5072
13	ECH_0133	transcription elongation factor GreA	25325±12916	9729±5252
14	ECH_0200	transcription termination factor Rho	11521±301	12451±9987
		Transport and binding proteins		
1	ECH_0085	ABC transporter, ATP-binding protein_0085	16937±9826	17852±12839
2	ECH_0845	ABC transporter, ATP-binding protein_0845	6401±1690	5951±2966
3	ECH_0972	ABC transporter, permease protein	17687±9424	17565±9325
4	ECH_0313	ABC transporter, permease/ATP-binding protein	0	4223±5608
5	ECH_0067	cation diffusion facilitator transporter family protein	29322±5605	35117±12550
6	ECH_0510	efflux transporter, RND family, MFP subunit	3182±3037	0
7	ECH_0193	major facilitator family transporter_0193	13598±1390	17413±1593
8	ECH_0816	major facilitator family transporter_0816	8332±1375	6396±4316
9	ECH_0818	major facilitator family transporter_0818	6160±2451	5605±1954
10	ECH_0466	monovalent cation/proton antiporter, MrpF/PhaF subunit family	7724±2150	9531±5292
11	ECH_0469	Na(+)/H(+) antiporter subunit C	2122±212	0
12	ECH_0189	putative iron-binding protein	6523±1375	10904±4676
13	ECH_0438	sodium:alanine symporter family protein_0438	2811±3802	0
14	ECH_0383	type I secretion system ATPase	12416±3440	17916±10981
		Unknown function		
1	ECH_1011	3'-5' exonuclease family protein	26552±13641	30331±17334
2	ECH_0653	ankyrin repeat protein_0653	28725±15864	41517±6870
3	ECH_0684	ankyrin repeat protein_0684	7719±15890	13230±13193
4	ECH_1033	apaG protein	0	3667±668
5	ECH_1084	AraM protein	0	7121±5372
6	ECH_0473	aromatic-rich protein family	10289±4111	5839±3782
7	ECH_0392	ATPase, AFG1 family	2183±3179	0
8	ECH_0307	CvpA family protein	6799±1369	19710±10078
9	ECH_0312	cyaY protein	9925±4254	20683±8847
10	ECH_1114	dehydrogenase, isocitrate/isopropylmalate family	0	3586±88
11	ECH_0374	DNA / pantothenate metabolism flavoprotein family protein	1438±533	0
12	ECH_0201	dnaJ domain protein	252±72	0
13	ECH_0012	Es1 family protein	0	6577±10291
14	ECH_0724	GTP-binding protein LepA	7192±4831	0
15	ECH_0693	GTP-binding protein TypA	0	2844±692
16	ECH_0332	HAD-superfamily hydrolase, subfamily IA, variant 1	8864±4638	11690±3858
17	ECH_1066	hexapeptide transferase family protein	12811±6131	23108±13039
18	ECH_0793	hydrolase, TatD family	0	8760±3980

19	ECH_0355	M23/M37 peptidase domain protein	0	3068±719
20	ECH_0985	metallo-beta-lactamase family protein	0	2211±546
21	ECH_0184	NADH:ubiquinone oxidoreductase family protein	1108±3349	0
22	ECH_0202	NifU domain protein	7078±2290	9107±5800
23	ECH_0366	oxidoreductase, short-chain dehydrogenase/reductase family_0366	0	13095±6401
24	ECH_1083	pentapeptide repeat protein	2822±965	0
25	ECH_0008	P-loop hydrolase family protein	20242±6760	41903±8397
26	ECH_0061	putative flavoprotein	2505±4794	0
27	ECH_0644	putative metalloendopeptidase, glycoprotease family	19672±8239	17295±11414
28	ECH_0211	putative methyltransferase	10908±4457	9812±7036
29	ECH_0063	putative NADH-ubiquinone oxidoreductase, homolog	6610±1617	7610±5224
30	ECH_0896	rhodanese domain protein	0	1160±3375
31	ECH_0577	RmuC family protein	148±799	0
32	ECH_0628	rrf2/aminotransferase, class V family protein	0	497±1384
33	ECH_0390	SPFH domain /band 7 family protein	714±352	0
34	ECH_0802	Sua5/YciO/YrdC/YwlC family protein	2176±3051	0
35	ECH_0210	surA domain protein	0	3157±358
36	ECH_1081	SURF1 family protein	13118±5810	15728±8851
37	ECH_0746	topoisomerase DNA-binding C4 zinc finger domain protein	1749±1563	0
38	ECH_1069	TPR repeat protein	4500±1973	0
39	ECH_0891	YGGT family protein	11157±4128	8623±2477
40	ECH_0751	YjeF family protein	7360±1398	7678±4485

Analysis of the p28 outer membrane protein genes (p28-Omp) expression in different host backgrounds under *in vitro* conditions: The p28-Omp is a multigene locus that codes for 22 tandemly arranged outer membrane protein genes. The expression of these 22 genes was assessed by analyzing the microarray data independent from the rest of the analysis. This group of genes contained different sets of probes that were designed targeting to three variable regions in each ORF. The threshold fluorescence values for these genes were carefully selected to examine the expression (Figure 3.8). Nine genes of the p28-Omp locus were found to have fluorescence values above background in tick cell-derived *E. chaffeensis* with the highest signals from the probes representing gene 14. In macrophage cell-derived *E. chaffeensis,* the fluorescence values for all the probes representing the 22 p28-Omp genes

were above the background with highest fluorescence values from the probes of gene 19 (Figure 3.8). A cartoon depicting the *E. chaffeensis* p28-Omp locus and the microarray data for one set of variable region 1 probes from a chip hybridized with the labeled cDNA of macrophage and tick cell-derived *E. chaffeensis* RNA is represented in the Figure 3.9.

Figure 3.8 Transcription from the p28-Omp locus of *E. chaffeensis* grown in macrophage (A) and tick cells (B).

The average fluorescence measured (from three independent experiments) in all the spots is plotted on the Y-axis and the corresponding gene is represented on the X-axis. (Error bars represent the standard deviation)

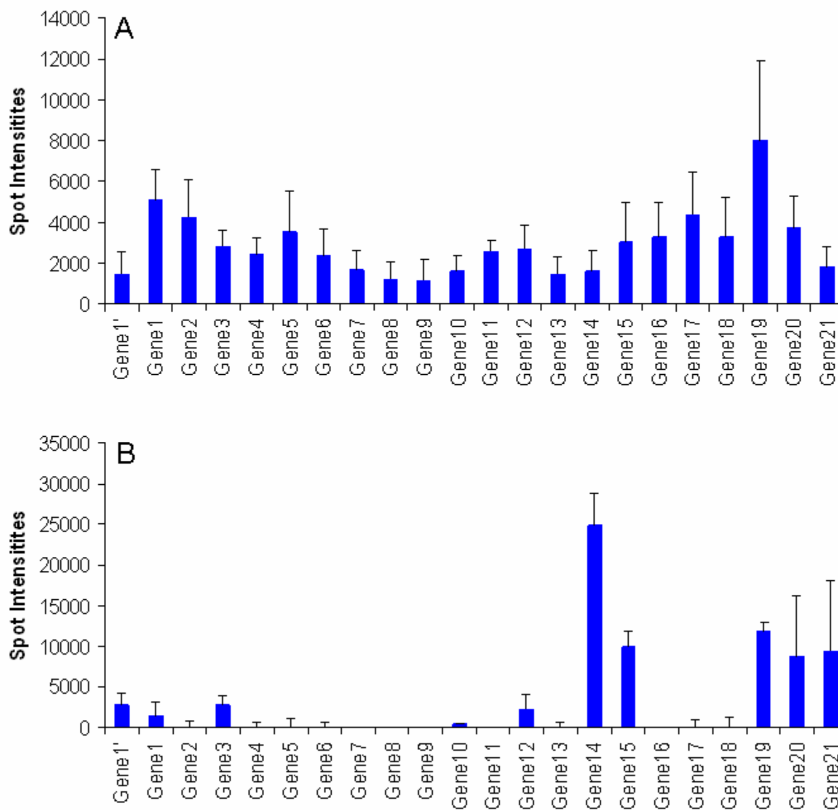
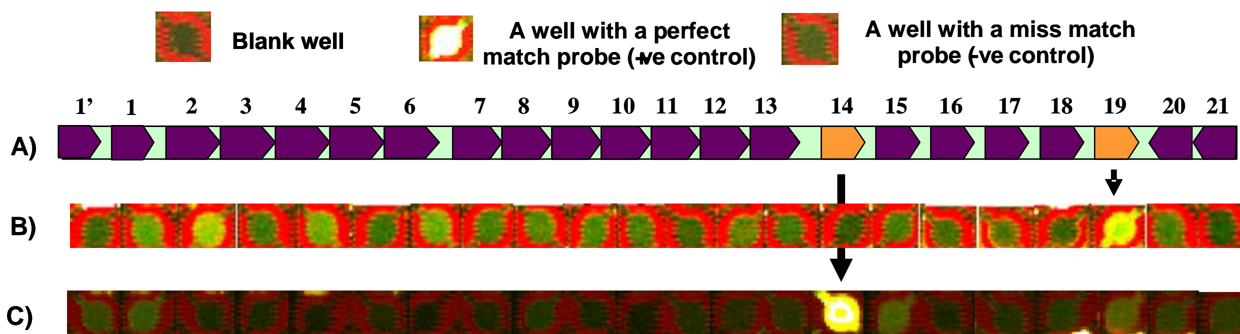


Figure 3.9 Transcription from p28-Omp locus of *E. chaffeensis* grown in macrophage and tick cells.

A, a cartoon depicting the *E. chaffeensis* p28-Omp locus; B, microarray wells for one set of variable region 1 probes from a chip hybridized with the macrophage-derived *E. chaffeensis* RNA; C, similar to B except that RNA was derived from tick cell grown *E. chaffeensis*



RT-PCR analysis to confirm the expression results from the microarray experiments: To validate the gene expression data obtained from the microarrray experiments, a semi-quantitative RT-PCR was performed for a subset of genes representing the commonly expressed, macrophage and tick cell-specifically expressed and non-expressed groups (Table 3.3). The RT-PCR data is shown in Figure 3.10. The genes from the commonly expressed lists were positive and equal levels of RT-PCR products were detected from reactions that contained RNAs derived from *E. chaffeensis* grown in both macrophage and tick cell environments as templates. The genes that contained higher fluorescence intensity values in *E. chaffeensis* RNA derived macrophages showed more RT-PCR products when RNA of macrophage grown *E. chaffeensis* was used as template. Similarly, the genes having higher fluorescence values when tick cell derived *E. chaffeensis* RNA was used also showed more RT-PCR products when tick cell derived RNA is used as a template. The ORFs having fluorescence intensity values

below the background tested negative by RT-PCR when both macrophage and tick cell derived *E. chaffeensis* RNA is used as a template.

Figure 3.10 Semi-quantitative RT-PCR for validating the microarray data.

The gene expression data obtained from the microarray experiments were confirmed by analyzing the transcriptional profiles for a subset of genes using semi-quantitative RT-PCR approach. A subset of genes representing the commonly expressed, macrophage- and tick-specific and non-expressed groups were selected. RNA isolated from *E. chaffeensis* grown in DH82 and tick cell lines at different time points post infection was used for this analysis. A semi-quantitative RT-PCR was performed where the amplification was terminated at different PCR cycles and equal volumes of the PCR products are resolved on an agarose gel and stained by ethidium bromide. The analysis was also performed on RNA isolated at different time points after infection in vertebrate and tick cell cultures. h, hours post infection; M, macrophage-derived *E. chaffeensis* RNA; T, tick cell-derived *E. chaffeensis* RNA; +, reaction positive control; -, reaction negative control.

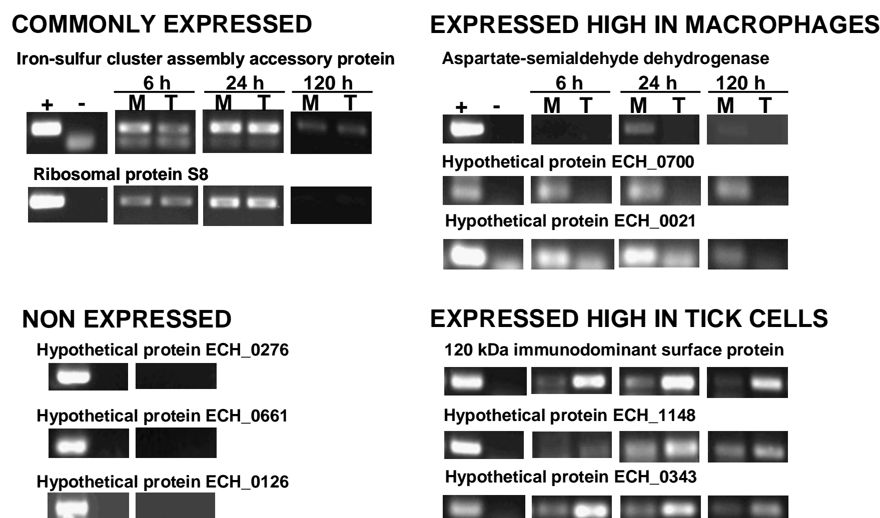


Table 3.3 Table showing the genes that were selected for RT-PCR analysis and the sequences of primers used in the assay.

A subset of genes representing the commonly expressed genes, macrophage- and tick- specific genes and non-expressed groups were selected for further analysis. The forward and reverse primers were designed targeting these selected genes for use in the RT-PCR analysis.

Gene name	Forward primer	Reverse primer	Expressed in
Aspartate-semialdehyde dehydrogenase (ECH_0016)	GATACAAATACTGGAACCCTAAC	TCAATCCAGAAGACATAGC	Macrophage
Hypothetical protein (ECH_0700)	TGTTTGCCTACTACACTTTC	GTAAGAAAAAAGTGATAGTAC	Macrophage
Hypothetical protein (ECH_0021)	GGAATTAGATTTGCTTTTG	AATACAGAATAAGTTAGCGAATC	Macrophage
*i*ron-sulfur cluster assembly accessory protein (ECH_0689)	GTACAAATACTTTACCTAACTTCC	CGAATTGTGCATGAGTTTCTAAG	common
Ribosomal protein S8 (ECH_0423)	TATGAATAAGGTAATAGTAG	CATAATTCCTTTAGATGTA	common
Hypothetical protein (ECH_0276)	CTAGAAAGTTCTATGCTGTG	CATTGTCTGTATATCCTGCTTC	not expressed
Hypothetical protein (ECH_0661)	ATTTATCCAGTTGTTCAGAAAG	GGATAAGAACACCGTAATAAC	not expressed
Hypothetical protein (ECH_0126)	GGTAGAATCAGGAGATAACT	GTACAATATAATGCTACAAC	not expressed
120 kDa immunodominant surface protein (ECH_0039)	CGTTATAAAGGAGGAAGATAAAG	CCATACATATACATTTCATAC	tick
Hypothetical protein (ECH_1148)	CACATATCTCAAAGTGACG	CTACCTTCTTCAGCAGGAG	tick
Hypothetical protein (ECH_0343)	AGGATCTGTAGGAGCAATAG	GCTTCAATAACTTAAAC	Tick

Discussion

In this study we have evaluated the possibility of designing an open reading frame based whole genome microarray chip for *E. chaffeensis*. At the time we initiated this study, the annotated genome sequence was not published and so we opted to identify the ORFs in the whole genome sequence of *E. chaffeensis*. The unannotated complete genome sequence of *E. chaffeensis* available at the TIGR website was downloaded and analyzed by using Artemis genome sequence analysis program. Our analysis revealed 1,234 ORFs which is slightly higher than the reported ORFs in the annotated sequence of *E. chaffeensis* (56). We do not anticipate this as a problem as we may have identified more ORFs than the actual reported ORFs. Upon careful evaluation we realized that some ORFs that were identified by the annotated genome were not identified by our Artemis sequence analysis. A total of 848 ORFs of the annotated *E. chaffeensis* genome were included in our analysis. Our initial analysis of examining the data of the self to self hybridization of the RNA from all six chips (three each from a specific host cell derived *E. chaffeensis* RNA) between Cy3 and Cy5 channels gave a good correlation suggesting the validity of the data for further analysis.

To further insure that the data is interpretable, we selected the RNA polymerase complex for further evaluation. We reasoned that the expression from all the subunits of RNA polymerase complex should be present as they are needed for the formation of the RNA polymerase complex. Moreover, as RNA polymerase is needed to initiate the expression of genes, it is anticipated that the expression from the subunits remain similar in both host cell backgrounds. Our analysis indeed showed that the expression levels of RNA polymerase subunits remained similar in *E. chaffeensis* grown in both macrophages and tick cell environments. These observations further authenticate the microarray data. These analyses

suggest that the microarray data represent a true reflection of the expression levels in *E. chaffeensis*.

The evaluation of fluorescence intensity signals from *E. chaffeensis* RNA identified about 381 ORFs having signals above the background in *E. chaffeensis* grown in macrophages and 341 ORFs having signals above the background in *E. chaffeensis* grown in tick cell environments. Assuming that higher fluorescence signals represent expression, these numbers represent the expressed transcripts from *E. chaffeensis* in these host cell backgrounds. The average number of expressed genes in *E.chaffeensis* in both macrophage and tick host cell backgrounds remained very similar. In fact, these numbers are very similar to our estimated number of expressed genes based on the identified protein spots on the 2D resolved gels (114). Based on evaluation of the expression levels, the expressed genes from the microarray data are grouped into three main categories; the commonly expressed, macrophage- specifically and tick cell-specifically expressed groups. The commonly expressed group may represent the genes that are required for the survival of *E. chaffeensis*. Further evaluation of these commonly expressed genes also revealed three subgroups within them; the subgroup where the expression levels remained at the same level, subgroups of the genes that contained higher signals in either macrophage or tick cell derived *E. chaffeensis* RNA. The groups that are uniquely expressed and subgroups having high or low levels of transcription in a host cell specific manner may represent important sets of proteins needed for the pathogen for its adaptation to macrophage and tick environments.

A major portion of commonly expressed transcripts belongs to categories such as DNA metabolism, energy metabolism, protein synthesis, transcription, transport and cellular processes. These categories of transcripts represent those involved in metabolic pathways needed for normal physiological homeostasis of an organism. The majority of the transcripts belonging to the uniquely expressed groups include genes coding for cell envelope, hypothetical

and proteins of unknown function. In addition, transcripts made from cofactor and vitamin biosynthesis and protein fate genes were among the uniquely expressed group contributing to about 20% of the group. It is not surprising to see uniquely expressed transcripts belonging to the above mentioned categories. For example, the cell envelope is the contact for the pathogen to its host environment. If it were to change its gene expression in support of its survival in different host background, it is likely that the proteins on the cell surface would be altered. Although, it is not clear what the function the hypothetical proteins and genes coding for proteins of unknown function may have, they may represent *Ehrlichia* specific proteins that may be involved in the pathogen's adaptation to different host environments. Possibly, it may be using its unique proteins in support of protein catabolism such as those encoded by protein fate genes. *E. chaffeensis* growth in tick cells require a more complex media which includes several minerals, co-factors and vitamins as bacteria may depend differently for its requirement in different host cells (80). Our microarray data, in fact suggest differential expression in genes encoding for various proteins involved in co-factor and vitamin biosynthesis.

We were stringent in setting the threshold levels to call a gene expressed. Thus, the genes for which the positive fluorescence signals were identified may represent true expression in *E. chaffeensis*. There may be genes that are expressed at low levels that might have been considered as non-expression because of our high stringency. For example, if we drop the threshold values by 1000 units, we saw about 200 more genes expressed from *E. chaffeensis*. This group of genes may represent truly non-expressed genes or genes that are expressed at very low levels. Careful examination of these genes is needed to further validate their expression status.

We have validated the microarray data on a subset of genes using semi-quantitative RT-PCR assay. It is important to validate the data from microarray by another independent method.

In support of this, we performed semi-quantitative RT-PCR analysis for randomly chosen genes representing the non-expressed, commonly and host cell-specifically expressed groups. The analysis was also performed using RNA isolated from cultures collected at different time points post infection (6 h to 120 h). Our RT-PCR analysis showed similar expression patterns as identified by microarray analysis for the genes studied. The genes that were identified as non-expressed were tested negative by RT-PCR. Similarly, the genes that were identified as commonly expressed had similar levels of expression as judged by RT-PCR. Likewise, RT-PCR analysis also supported the differential expression observed in microarray.

The availability of whole genome sequence data is a significant advancement in the field of E. chaffeensis. However, the data can not be used without knowing the significance and expression of the genes identified in E. chaffeensis. This is the first study that took advantage of the whole genome sequence in assessing the expression of genes in E. chaffeensis. This study also represents the first set of experiments that validated the expression from many hypothetical protein genes of E. chaffeensis. We have identified 114 genes coding for hypothetical proteins to be expressed in E. chaffeensis. Until now, the studies have only characterized a few genes (21,85,99,100,144,148). The previously reported data is very limited considering the importance of E. chaffeensis for human health. This current study represents the first and most comprehensive analysis of transcription from E. chaffeensis genome. The comprehensive transcriptional analysis also represents the first report describing the global host cell-specific gene expression patterns in E. chaffeensis. The data will be very valuable to enhance our understanding of E. chaffeensis pathogenesis and ultimately will help in devising better control strategies.

CHAPTER 4 - Evaluation of Methods for Establishing Mutations in *E. chaffeensis*

Abstract

Differential gene expression may be an important adaptation mechanism used by *E. chaffeensis* in support of its continued survival in dual hosts. One of the ways this hypothesis can be tested is by performing mutational analysis that aid in altering the expression from the differentially expressed genes and to assess their impact on the pathogen's growth in vertebrate and tick cells. However, the methods for introducing mutations in this pathogen have not yet been documented. In this study, we established many basic protocols and tools needed for performing mutational analysis in *E. chaffeensis*. These include the identification of appropriate antibiotic selection markers, choice of genes to create mutations and the selection of constitutively active *Ehrlichia* promoters useful in preparing the transformation constructs and conditions for transformation. Our analysis aided in the identification of four antibiotics; gentamicin, chloramphenicol, spectinomycin and rifampin that are inhibitory to *E. chaffeensis* growth. Resistance genes against these four antibiotics are available to serve as suitable markers for monitoring mutations in *E. chaffeensis*. The promoters of two constitutively expressing *E. chaffeensis* genes; 30S ribosomal S12 protein (*rpsL*) and putative transcriptional regulator (*tr*) were also identified to aid in driving the expression of antibiotic selection markers. We selected the p28-Omp 15 gene because protein expression from this gene is low in both in tick cell and macrophage environments. Similarly, a hypothetical protein gene, Ech_0126, was selected for creating mutations as our RNA analysis revealed no evidence for its expression in either tick cell or macrophage environments. We selected these genes as initial targets for mutational analysis; we reasoned that mutations within these genes do not impact *E. chaffeensis* growth. Several plasmid constructs were made that contained antibiotic selection markers and p28-Omp 15 or Ech_0126 gene segments for use in homologous recombination experiments. The optimal conditions for introducing these plasmids into host cell-free viable *E. chaffeensis* organisms were also established. The molecular evaluation of several transformants of *E. chaffeensis* suggested that the plasmids gained entry, but failed to get

integrated into the genome or remain in the bacteria for longer periods of time. These results suggest that the pathogen may not support the replication of plasmids or integration of foreign DNA in its genome. This hypothesis may be justified because; 1) *E. chaffeensis* and other closely related species do not harbor plasmids, and 2) although *E. chaffeensis* genome contains many genes that encode for recombinase like proteins, few appeared to be expressed. Alternatively, additional strategies or modifications to our current protocols may be needed to continue our efforts to introduce mutations in *E. chaffeensis*.

Introduction

E. chaffeensis is the causative agent of HME and it results in persistent infections in both vertebrate and tick hosts (6,22,25,26,34,42,87,123,131,151,155). It is not clear how this pathogen is able to adapt to dual hosts and continue to survive for long periods of time. One of the probable mechanisms is by inducing changes in the gene expression in a host cell-specific manner (43). Recent studies from our laboratory suggest that the pathogen indeed expresses many proteins in a host cell-specific manner (114,115). This is further confirmed by global gene expression analysis performed as a part of my Ph.D. dissertation research (discussed in Chapter 3). Whole genome microarray analysis identified host cell-specific gene expression from numerous genes of *E. chaffeensis*. Recent studies from our laboratory also suggest that *E. chaffeensis* originating from tick cells is able to survive longer in a vertebrate host compared to the pathogen originating from macrophages (43). Together, these data suggest that variation of gene expression in *E. chaffeensis* grown in tick cells may aid the pathogen in conferring resistance to host clearance. However, this hypothesis remains to be supported by providing experimental evidence.

This role of differential gene expression can be best understood if one is able to control or alter host cell-specific differential expression and then assess its impact on the pathogen's clearance by a vertebrate host. The availability of a genetic manipulation system to create targeted mutations in a gene of *E. chaffeensis* will be valuable to understand the importance of differential expression for the pathogen's dual host adaptation and pathogenicity. Alternatively, the protein expression may also be inhibited by blocking the translation from an mRNA or by inactivating the protein function using drugs that specifically target to a protein of interest. Knockout or knockdown of an mRNA is somewhat challenging for a bacterial pathogen, as the RNAi pathway described for eukaryotes is not reported for prokaryotes (120,129). Alternatively, a transcript may be blocked from being translated using an anti-sense oligonucleotide approach (35). However, this may be more challenging for an intra-phagosomal pathogen, such as *E. chaffeensis.* Similarly, inactivating a protein function using a drug is also challenging. Moreover, this is further complicated by the additional research involved in designing and evaluating specific anti-protein analogues. Thus, the more reasonable approach would be to create mutations within the *E. chaffeensis* genome to selectively inactivate a gene of interest.

E. chaffeensis is a Gram-negative organism; thus existing methods of introducing DNA into Gram-negative bacteria are likely to be effective. Although, transformation in *E. chaffeensis* is complicated, as it is an obligate intracellular pathogen, recent data on *Rickettsia* and *Anaplasma* species suggest that it is an achievable task (37,95,97,143). For example, several studies on *R. prowazekii*, *R. monacensis* and *A. phagocytophilum* documented the introduction of mutations using a transposon-based random mutational approach (37,78,94). Targeted mutation has also been reported by *Rachek et al* for *R. prowazeki* (95,97) More over, data reported from two unpublished meeting proceedings on *A. marginale* and *E. muris* suggested that it is possible to create targeted mutations in *Ehrlichia* species (38,69).

In this study we established many basic protocols and tools needed for performing mutational analysis in *E. chaffeensis*. They include the identification of appropriate antibiotic selection markers, choice of genes to create mutations and the selection of constitutively active *Ehrlichia* promoters useful in preparing the transformation constructs and conditions for transformation. In addition, numerous transformation experiments have been performed to introduce mutations in *E. chaffeensis*. The experimental outcome is described in this chapter.

Materials and Methods

***In vitro* cultivation of *E. chaffeensis* in macrophage and tick cell lines:** *E. chaffeensis* Arkansas isolate was cultivated at 37°C in the canine macrophage cell line, DH82, as described previously (114). The Arkansas isolate was also cultivated in the tick cell line, ISE6, using medium designed for its growth and maintenance at 34°C in the absence of CO_2 as described by Munderloh *et al.* (1999). The ISE6 cell line is derived from the embryos of the *Ixodes scapularis* tick (114). Another tick cell line, AAE2, derived form the embryos of *A. americanum* ticks, is also used to grow *E. chaffeensis* (115). The medium and the growth conditions for AAE2 cell line are same as those for ISE6 (115).

Purification of *E. chaffeensis*: *E. chaffeensis* was cultivated in either macrophage or tick cells. The cells are harvested when the infectivity reached 80-90 % of nearly 100% confluent flask. Infection was assessed by microscopic examination of polychromatic stained cytospin slides. About 25 ml of infected culture was collected into a tube and centrifuged at 15,000 g for 15 min. The supernatant was discarded and the pellet resuspended in 10 ml of 0.25 M ice cold sucrose solution. The cells were lysed by adding 1 mm diameter glass beads into the tube and vortexing for 30 sec two times. Alternately, cells were lysed by passing through a 27½ gauge needle five times or by sonicating at a setting of 6.5 for 30 sec each for two times. The lysed

cell suspension is transferred into a new tube free of glass beads and centrifuged at 100 g for 10 min. The supernatant is transferred into a new tube and centrifuged at 15,000 g for 15 min to spin down cell-free *Ehrlichia*. At the end of centrifugation, supernatant was discarded and the cell-free *Ehrlichia* pellet resuspended in 10 ml of 0.25 M ice cold sucrose. The centrifugation step is repeated one more time and the final *Ehrlichia* pellet is suspended in a small volume of 0.25 M sucrose solution.

Antibiotic sensitivity assays: The sensitivity of *E. chaffeensis* to different antibiotics was evaluated as described (13). Briefly, *E. chaffeensis* was cultivated in T75 flasks having 100% confluent DH82 cells. When the infectivity reached approximately 50 %, 0.5 ml culture per each well was transferred to all wells in a 24 well plate. Spectinomycin, rifampin, chloramphenicol, kanamycin, ampicillin and gentamicin were tested for their ability to inhibit *E. chaffeensis* growth. Antibiotic solutions were prepared by dissolving the powder in appropriate solvents as suggested by the manufacturer (Sigma Aldrich Corporation, St. Louis, MO). The negative control included the culture wells with no antibiotic added, or just solvent is added. Positive control wells contained tetracycline at 1 µg/ml final concentration. The concentrations of the antibiotics tested were as follows: specitnomycin at 10, 50, 100, 150 and 200 µg/ml; rifampin at 0.1, 0.2 and 0.5 µg/ml; gentamicin at 2.5, 10, 20, 40, 80 and 120 µg/ml; chloramphenicol at 1, 2, 4 and10 µg/ml; ampicillin at 1 and 20 µg/ml; kanamycin at 1 and 10 µg/ml. Different dilutions of antibiotics, including controls, were tested in triplicate wells and the experiments were repeated three independent times. Media along with freshly prepared antibiotics dilutions were replaced every 3 days. The infectivity of the DH82 cells was also monitored every 3 days for up to 12 days by microscopic evaluation of polychromatic stained cytospin culture slides. The inhibitory rates were assessed relative to control wells that received no antibiotics. Relative inhibition was also assessed by using tetracycline as a positive control. Average values of infected cell

numbers for triplicate wells were plotted against no antibiotics controls to determine the antibiotic sensitivity for each drug tested.

Identification of the antibiotic sensitivity genes: Rifampin, chloramphenicol, spectinomycin and gentamicin were identified as inhibitory to *E. chaffeensis*. To assess their use in our study, genes that conferred resistance to all four antibiotics were identified. The use of the rifampin resistant gene, *arr2*, has been described by Qin *et al* (94). The plasmid containing the rifampin resistance gene, pMW1409, was obtained from Dr. David Wood. The arr2 gene along with the *R. prowazeki rpsL* promoter was amplified by PCR using gene specific primers. The use of spectinomycin resistance gene has been described by Felsheim *et al* (37). The plasmid containing the spectinomycin resistance gene was obtained from Dr. Uli Munderloh. The open reading frame of the spectinomycin was PCR amplified by
using specific primers. The resistance genes for chloramphenicol and gentamicin were obtained from plasmid pDEST10 (Invitrogen Technologies, Carlsbad, CA) by following a similar strategy as described for rifampin. The primers used to amplify the antibiotic resistance genes are listed in Table 4.1.

Selection of genes in the *E. chaffeensis* as targets for homologous recombination: To identify an appropriate target in the *E. chaffeensis* genome for homologous recombination experiments, the expression patterns of various *E. chaffeensis* genes were analyzed. Specifically, microarray data were examined to identify genes that are low in expression or that lack expressed transcripts in both vertebrate and tick cell backgrounds. Our microarray data analysis aided in the identification of many such genes. We chose two genes in particular, i.e., p28-Omp 15 gene and ECH_0126. More detailed information about the selection is provided in the results section. The microarray data suggested that the p28-Omp 15 gene is expressed at low levels in *E. chaffeensis* growing in macrophages and tick cells. Another gene ECH_0126

was also selected as microarray analysis revealed that the transcripts for this gene are not detectable in both macrophage and tick cell backgrounds. To verify the absence of expression from Ech_0126 gene, RT-PCR analysis was performed by using specific primers targeted to the coding region of the Ech_0126 gene. The reaction was carried out in a 25 µl volume containing *E. chaffeensis* RNA as template and by following the optimal reaction conditions as described in Chapter 5. The reaction included a negative control tube where nuclease free water is added in place of RNA and a positive control tube where genomic DNA is added as the template in a PCR reaction.

Selection of constitutively active *E. chaffeensis* promoters: To drive the expression of antibiotic resistance genes, promoters at are constitutively active in both tick and vertebrate host backgrounds need to be selected. Our global transcriptional analysis (Chapter 3) identified several genes that appear to be constitutively expressed in *E. chaffeensis* derived from macrophage and tick cell backgrounds. Although our microarray analysis aided in the identification of many genes, which seemed to be constitutively expressed, we chose the promoters of the *tr* and *rpsL* genes as our choice because homologues of these promoters from closely related rickettsials, such as *Rickettsia prowazeki* and *Anaplasma phagocytophilum* have been utilized in mutational analysis experiments. The activity of these promoters was further verified by performing RT-PCR analysis. Primers specific for *tr* and *rpsL* genes coding regions were designed and used in the RT-PCR analysis to map transcription of these genes. RNAs isolated from *E. chaffeensis* originating from macrophage or tick cells were used as templates in RT-PCR analysis. The reaction also included a negative control that contained no template and a positive control having *E. chaffeensis* genomic DNA as the template, but by performing PCR reaction. The reaction was carried out in a 25 µl volume containing *E. chaffeensis* RNA as template and following the optimal conditions for the reaction as described in Chapter 5.

Table 4.1 The list of primers and sequences used in the preparation of plasmid constructs.

Primer	Sequence
Primes used in the Gene 15 construct	
RRG190-Gene 15-for 1	CCTTCTAGTTTTATTTATTTG
RRG191-Gene 15 for 2	CTTTCTGAACCAGTACAAG
RRG192-Gene 15 rev	TTCTCCAGAAGCTGTTG
RRG433-Gene 15-5'-for	GTCAAGCTTCAAAGACTACTTATGTATTATAG
RRG434-Gene 15-5'-rev	GTCGGATCCCAAACCATACAACGCGACAG
RRG435-Gene 15-3'-for	GTCACTAGTCTACTCTTAAAGCGTTTGC
RRG436-Gene 15-3'-rev	GTCTCTAGACAAATTAACAGTAGTAAACC
Primes used in the ECH_0126 gene construct	
Ech_0126-5'-for	GTCAAGCTTCATCATCAAAATTACATTCC
Ech_0126-5'-rev	GTCGGATCCGGTAGAATCAGGAGATAACTC
Ech_0126-3'-for	GTCACTAGTGATTTATGTGTATCTGTCGCAG
Ech_0126-3'-rev	GTCTCTAGAGCACTGTATTTCTATCTCTAC
Primes used to obtain antibiotic resistance genes and promoters	
Rifampin-for	ATGGTAAAAGATTGGATTCC
Rifampin-rev	GTCTAGAGGATCCTTAATCTTC
Gentamicin-for	ATGGGATCGGCCATTGAAC
Gentamicin-rev	TCAGAAGAACTCGTCAAG
CAT-for	ATGGAGAAAAAAATCACTGG
CAT-rev	TTACGCCCCGCCCTGCCA
Spectinomycin-for	ATGTTACGCAGCAGCAACG
Spectinomycin-rev	TTATTTGCCGACTACCTTGGTG
Ech-rpsl-for	TAGATTATTTTGAAAGGAAG
Ech-rpsl-rev	TAGATTATTTTGAAAGGAAG
Ech-Tr-for	AATACATCCCCCCTTACC
Ech-Tr-rev	CCAGTGATTT TTTTCTCCAT
Tr-ORF-for	ACGTTGAAGTAAGAGATATAATG
Tr-ORF-rev	CGATAGCAAAATAGATAGCAAAG
Rpsl-ORF-for	CAATTAGTCCGCAAACCGC
Rpsl-ORF-rev	TAAGCTATAACCTCACTACC

Preparation of plasmid constructs for use in homologous recombination experiments: Several constructs were prepared targeting the two genes, p28-Omp 15 and Ech_0126, of *E. chaffeensis*. The generation of constructs involved several molecular cloning techniques such as PCR, sequencing, restriction enzyme digestion and ligation. All the basic molecular biology protocols are described in Chapter 5 and the schematic outline of the plasmid constructs is described below for genes p28-Omp 15 and Ech_0126.

Construction of plasmids targeting the p28-Omp 15 gene: An approximately 1.4 kb segment of the p28-Omp 15 gene containing the entire ORF and parts of flanking regions was amplified by PCR. The PCR reaction was performed using a proof reading DNA polymerase, *E. chaffeensis* genomic DNA as template and the primers RRG 190 and RRG 192 under appropriate conditions as outlined in Chapter 5 (Figure 4.1 and Table 4.1). The PCR product is referred to as 15C. Another segment of gene 15 that lacked 5' flanking region and part of 5' ORF segment was also PCR amplified using the primers RRG 191 and RRG 192 for use in construct preparation; this PCR product is termed 15P. The plasmid BlueScript SK+ (pBS) was chosen to use as a backbone to insert the gene 15 fragments. The restriction enzyme sites for *EcoR* I and *Hind* III restriction enzymes were deleted from pBS by employing molecular methods. This was accomplished by digesting with one restriction enzyme at a time and then performing a fillin reaction using T4 DNA polymerase followed by blunt end ligation. Plasmids that lost the enzyme site were selected by transforming the ligation products into competent *E. coli* cells. Further plasmid DNA analysis was performed as described in Chapter 5. This strategy aided in generating a pBS vector lacking *EcoR* I and *Hind* III restriction sites; which is referred to as $pBS^{\#}$. The PCR products 15C and 15P were inserted into the *EcoR* V site of the $pBS^{\#}$ by blunt end ligation and transformation into *E. coli* cells. The resulting plasmids containing 15C and 15P are termed pBS-15C and pBS-15P, respectively. A schematic

representation detailing the steps involved in the generation of plasmid constructs is shown in Figure 4.2.

The plasmids pBS-15C and pBS-15P were then used to insert a gene cassette, *Rparr-2*, containing a rifampin resistance gene driven by the *R. prowazeki rpsL* promoter (conveniently referred to as RP for illustration). The *Rparr-2* cassette was obtained from the plasmid pMW1409 by PCR using the primers, forward primer *Rparr-2* and reverse primer DW416. RPwas inserted into the Sma I site of pBS-15C and pBS-15P plasmids by blunt

Figure 4.1. A cartoon representing the *E. chaffeensis* genomic region of the p28-Omp locus selected for mutational analysis.

The forward primers (RRG 190 and RRG 191) and reverse primer (RRG 192) used in the preparation of the construct and approximate size of the PCR products are represented in the Figure. Arrowhead direction is to represent the orientation of each gene.

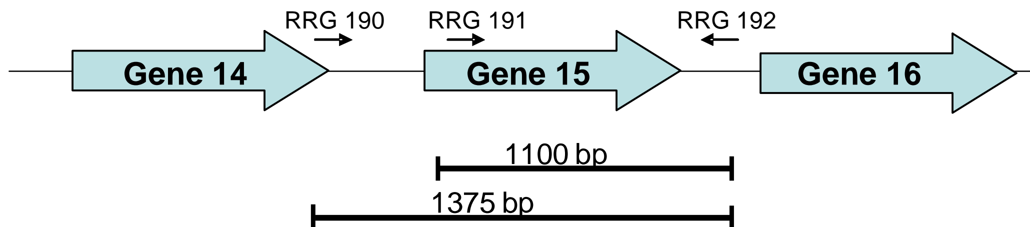
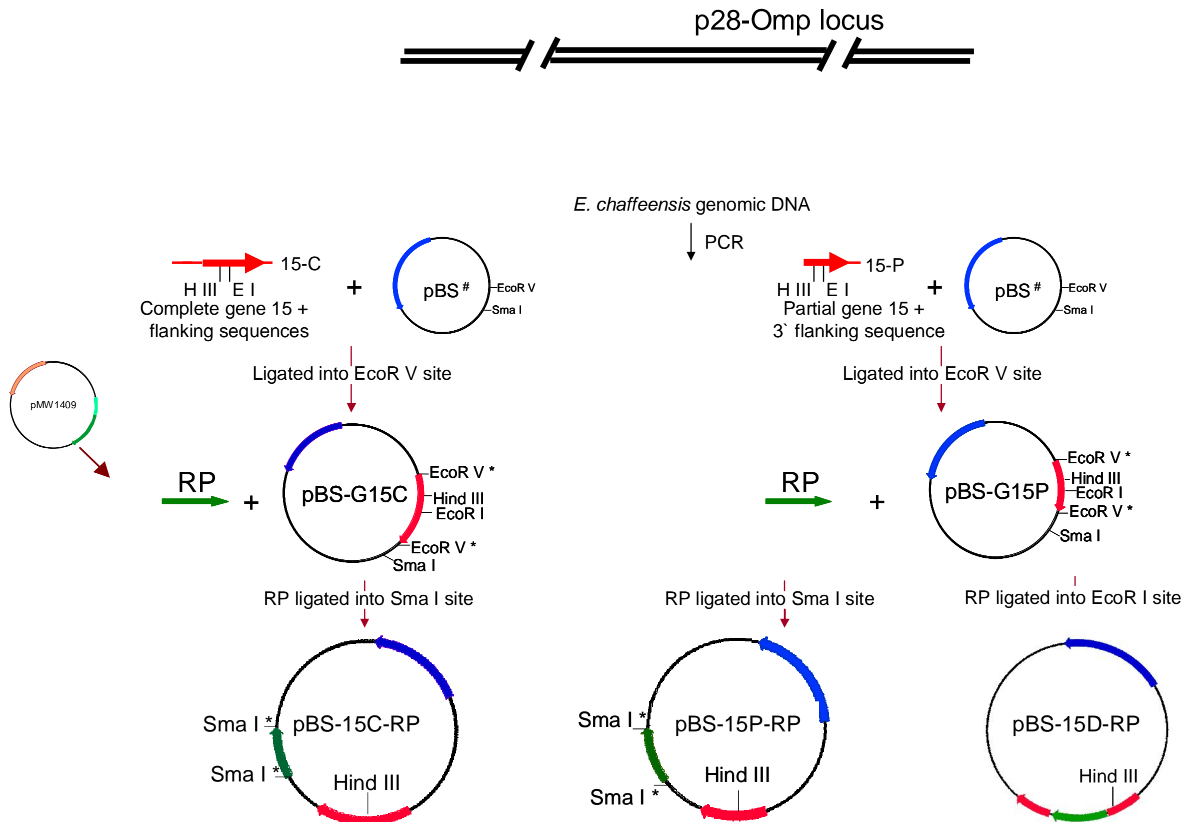


Figure 4.2 The schematic representation of the plasmid constructs prepared for use in the homologous recombination experiments targeting the p28-Omp 15 gene.

A partial or complete segment of p28-Omp 15 gene was amplified and inserted into the *EcoR V* site of the pBS plasmid. A segment (RP) containing the rifampin resistance gene along with *R. prowazeki rpsL* promoter was amplified from pMW1409 (obtained from Dr. Wood) and inserted at the *Sma* I or *EcoR* I site of pBS containing gene 15 segment. 15C, p28-Omp 15 gene complete segment plus flanking sequences; 15P, p28-Omp 15 gene partial segment plus 3' flanking sequence; RP, rifampin gene with promoter; pBS



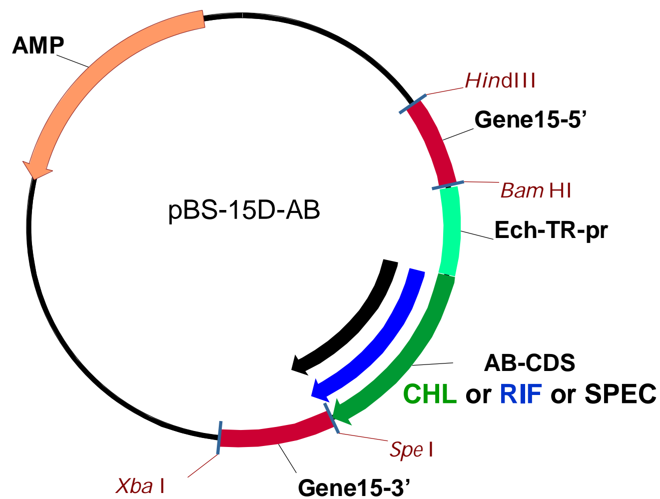
end ligation and transformation into *E. coli* cells. The newly generated constructs having the rifampin cassette in the *Sma* I site of pBS-15C and pBS-15P plasmids are termed pBS-15C-RP and pBS-15P-RP, respectively. In support of generation of another construct, RP is inserted into the *EcoR* I site of pBS-15P. To facilitate ligation of RP (blunt ended fragment) into the *EcoR* I site of pBS-15P, the plasmid was digested with *EcoR* I and a fillin reaction was performed using T4DNA polymerase to create blunt ends. Subsequently, the blunt-ended linear plasmid was used in the ligation reaction to insert the RP segment. This strategy aided in insertion of RP into the blunt-ended *EcoR* I site of pBS-15P and the resulting plasmid is termed pBS-15PD-RP. The integrity of the plasmids, particularly the presence of inserts at appropriate locations and the presence of accurate sequences was verified by plasmid DNA sequence analysis as outlined in Chapter 5.

Three additional recombination plasmids targeted to disrupt the p28-Omp 15 were also gene prepared for use in homologous recombination experiments. These constructs contained the resistance genes for chloramphenicol, spectinomycin and gentamicin. Two segments of the p28-Omp 15 gene spanning the 5' and 3' regions of the ORF were amplified by PCR technique. The 5' end segment was amplified using the primers RRG 433 and RRG 434. The primers are designed to contain *Hind* III and *BamH* I restriction sites at the 5' and 3' ends of the PCR product, respectively. The 3' end fragment was amplified using the primers RRG 435 and RRG 436; these primers are designed to contain *Xba* I and *Spe* I restriction sites at the 5' and 3' ends of the PCR product, respectively. These inserts were directionally cloned into pBS using molecular techniques such as restriction enzyme digestion, ligation and transformation as described in Chapter 5. The resulting plasmid was termed pBS-15D. The *E. chaffeensis tr* promoter was PCR amplified by using genomic DNA as the template and the primers Ech-tr-for and Ech-tr-rev. The PCR products were cloned into the plasmid Blue topo cloning vector and the resultant plasmid is termed as pBT-tr. The antibiotic resistance gene products for

gentamicin and chloramphenicol were amplified from the pDEST 10 vector by performing PCR using specific primers (Table 4.1). Similarly, the spectinomycin resistance gene is also PCR amplified by using the specific primers (Table 4.1). The antibiotic resistance gene products were inserted into the pBT-*tr* such that the antibiotic resitance genes are in frame with the *tr* promoter. The activity of the *tr* promoter was verified in *E. coli* cells by selecting the transformed organisms in media containing appropriate antibiotics. The antibiotic resistance gene cassette containing *tr* promoter driving the expression from gentamicin, chloramphenicol and spectinomycin resistance genes was released from the pBT-*tr* vector by restriction enzyme digestion and cloned into *BamH* I and *Spe* I sites of the pBS-15D by using molecular techniques as described in Chapter 5. A cartoon depicting the plasmid structure is shown in Figure 4.3.

Construction of plasmids targeting the Ech_0126 gene coding for a hypothetical protein:
A segment of the Ech_0126 gene, from the 5' end of the open reading frame, is amplified by PCR using the primers Ech_0126-5'-for and Ech_0126-5'-rev and *E. chaffeensis* genomic DNA as template and is termed 126-5'. The primers were designed to contain *Hind* III and *BamH* I restriction sites at the 5' and 3' ends of the PCR product, respectively. The 126-5' was digested with *BamH* I and *Hind* III and is ligated into similarly digested pBS vector. The resulting plasmid containing 126-5' fragment in pBS was termed pBS-126-5'. The plasmid pBS-126-5' was linearized by digestion with *Hind* III and *BamH* I. The *rpsL* gene promoter of *E. chaffeensis* is PCR amplified using genomic DNA as the template and the PCR product is cloned into pBT; the resultant plasmid is termed pBT-*rpsl*. The antibiotic resistance genes for chloramphenicol and rifampin were also amplified by PCR as described in previous section and inserted into the pBT-*rpsl* such that the resistance genes are in frame with the *rpsl* promoter. The resultant plasmids were termed as pBT-rp-CAT and pBT-rp-RIF. The plasmids pBT-rp-CAT and pBT-rp-RIF were digested with

Figure 4.3 Schematic representation of the plasmid constructed for use in homologous recombination experiments to disrupt the coding sequence of the p28-Omp gene 15. The 5’ and 3’ end segments of the p28-Omp 15 gene were generated by PCR and inserted into the pBS. A cassette containing the *E. chaffeensis tr* gene promoter driving the expression of an antibiotic resistance gene is engineered in between the inserted gene 15 segments. AMP, ampicllin resistance gene; Gene15-5, 5’ segment of the gene 15 ORF, Gene 15-3, 3’ segment of the gene 15 ORF; Ech-TR-pr, *E. chaffeensis* transcriptional regulator gene promoter; AB-CDS, antibiotic resistance gene coding sequence; CHL, chloramphenicol acetyl transferase gene; RIF, rifampin resistance gene; SPEC, spectinomycin resistance gene.

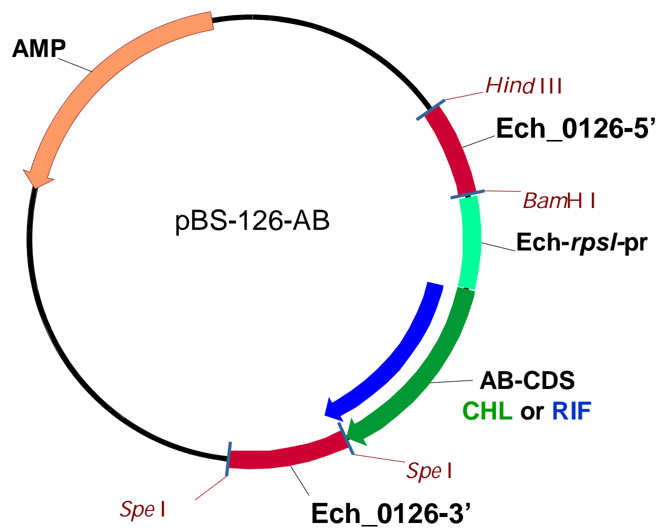


BamH I and *Spe* I restriction sites to release a cassette that contains the *E. chaffeensis rpsL* promoter driving the chloramphenicol or rifampin resistance genes, respectively. The released fragments were gel-isolated and cloned into the *BamH* I and *Spe* I site of pBS-126-5'. The resulting plasmids are termed as pBS-126-5'-rp-CAT and pBS-126-5'-rp-RIF, respectively.

Another segment of the ECH_0126 gene is amplified from the 3' end of the open reading frame using the primers Ech_0126-3'-for and Ech_0126-3'-rev RRG 560; the product was termed 126-3'. The forward primer is designed to contain a *Spe* I site at the 5' end of the PCR product and the 126-3' fragment itself has a *Spe* I site at the 3' end in the ECH_0126 gene sequence. The 126-3' and pBS-126-5'-rp-CAT and pBS-126-5'-rp-RIF were digested with *Spe* I restriction enzyme. The vector was dephosphorylated and used in ligation reactions. The *Spe* I digested 126-3' fragment is inserted into the *Spe* I site of pBS-126-5'-rp-CAT and pBS-126-5'-rp-RiF vectors and the resulting vectors were termed pBS-126-CAT and pBS-126-RIF, respectively. The proper orientation of the 126-3' segment was verified by sequencing analysis. A cartoon showing the final plasmid along with the inserted fragments is depicted in Figure 4.4.

Preparation of plasmid DNA and electroporation conditions for *E. chaffeensis*: The final plasmid constructs were transformed into a dam-/dcm- mutant strain of *E. coli*. This strain is methyltransferase deficient and suitable for growth of plasmids free of dam and dcm methylation. Plasmid DNA was isolated from 200 ml of *E. coli* culture by using endofree maxi prep Plasmid DNA isolation kit as per the manufacturer's protocols. The final plasmid DNA is eluted into 100 µl of nuclease free water. This plasmid DNA was methylated by using host-cell free extracts of *E. chaffeensis* as described by Feldschein, et al. (37). Following methylation, the plasmid DNAs were purified by ethanol precipitation and the integrity of the plasmid DNA was

Figure 4.4 Schematic representation of the plasmid constructed for use in homologous recombination experiments designed to disrupt the coding sequence of the Ech_0126 gene. The 5' and 3' end segments of the Ech_0126 gene were generated by PCR and inserted into pBS. A cassette containing the *E. chaffeensis rpsL* gene promoter driving the expression of an antibiotic resistance gene was engineered in between the inserted Ech_0126 gene segments. AMP, ampicllin resistance gene; Ech_0126-5', 5' segment of the Ech_0126 gene, Ech_0126-3', 3' segment of the Ech_0126 gene; Ech-*rpsL*-pr, *E. chaffeensis rpsl* gene promoter; AB-CDS, antibiotic resistance gene coding sequence; CHL, chloramphenicol acetyl transferase gene; RIF, rifampin resistance gene.



assessed by resolving on a DNA agarose gel. Typically, about 1 µg of plasmid DNA was added per each electroporation reaction containing 100 µl of purified cell-free *E. chaffeensis* (described above) and mixed gently by slow pipetting and carefully transferred to a prechilled 1 mm gap, 100 µl capacity electroporation cuvette (Eppendorff Corporation, Westbuty, NY). The electroporation cuvette is incubated on ice for 5 min and subjected to electric shock in an eppendorff 2510 electroporator (Eppendorff Corporation, Westbuty, NY). The settings used for electroporation are 5 µF, 5 milliseconds and voltages ranging from 1000-2500 V. The controls included in the study were *E. chaffeensis* organisms that were electroporated or not electroporated in the absence of plasmid DNA. Immediately following electroporation, the contents were transferred to a flask containing a confluent layer of host cells (macrophages or tick cells).

Southern blotting technique to assess the internalization of plasmids: To evaluate the validity of our electroporation conditions the presence of internalized plasmid DNA was assessed by using Southern blotting method (72). Briefly, *E. chaffeensis* was subjected to electroporation in the presence of 1 µg of pBS-15C-RP plasmid DNA, but at different voltage settings, ranging from 1000 V to 2000 V. The analysis also included a control electroporation reaction that contained plasmid DNA and *E. chaffeensis* but not subjected to electroporation. Immediately after electroporation, the contents of the cuvette were transferred to an eppendoff tube and centrifuged at 14,000 g for 6 min at 4°C. The supernatant was discarded and the pellet suspended in 0.5 ml of 0.25 M sucrose solution and centrifuged at 14,000 g for 6 min at 4°C. The supernatant was discarded and the pellet suspended in 85 µl of 1X DNAse buffer followed by the addition of 15 µl of DNAse (Promega Corporation) and incubated at 37°C for 1 h. The DNAse digestion was stopped by adding 4 µl of 0.5 M EDTA and incubation at 65°C for 10 min. Subsequently, 2.5 µl of Proteinase-K (10 mg/ml) was added and tube is incubated at 37°C for 4 h. *E. chaffeensis* organisms were disrupted by adding 2 µl of 10X SDS buffer and

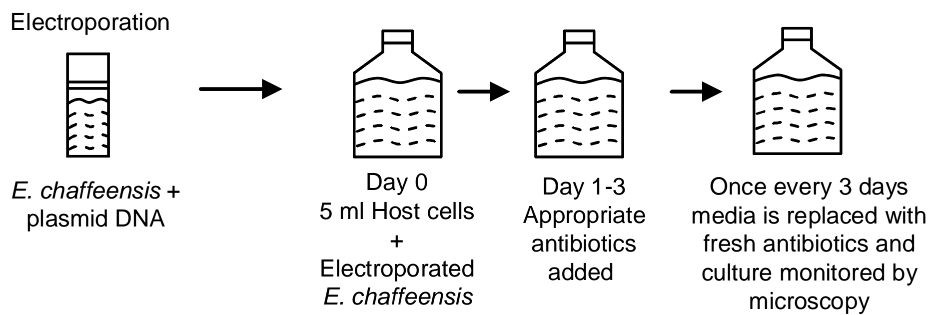
incubated at room temperature for 5 min. Total DNA from these cultures was isolated by using phenol/chloroform extraction method and the final pellet was dissolved in 9 μl of TE buffer.

To the isolated DNA, 2 μl of 10 X *EcoR* I restriction enzyme digestion buffer and 10 units of *EcoR* I enzyme were added and digested at 37°C for 2 h in a 20 μl reaction. The digested products were then resolved on a 0.9 % agarose gel run at 40 V for 6 hours and the resolved DNA was transferred to a nylon membrane (72). Digestion controls, included 0.5 μg of *E. chaffeensis* gDNA and 50 ng of pBS-15C-RP plasmid DNA, were also a part of the resolved gel. The nylon membrane was probed with ^{32}P labeled probes for the 16S rDNA and the rifampin resistance gene. The random primer labeling technique was used to synthesize radioactive probes, with PCR products of 16S rDNA gene and rifampin resistance gene segments as templates as per the manufacturer's instructions (Random Primer labeling kit, Stratagene Corporation, La Jolla, CA). After 14 h of incubation at 65°C, the membrane was washed once with 6X SSC, 0.1% SDS followed by two washes at 2X SSC, 0.1% SDS and a final wash at 0.1X SSC, 0.1% SDS. The nylon membrane was then exposed to an X-ray film at -70°C using an intensifying screen. The X-ray film was developed and presence of plasmid DNA within *E. chaffeensis* was assessed from the autoradiograph.

Methods for monitoring transformants: The culture media from flasks containing electroporated *E. chaffeensis* and from control flasks was changed once every 3-4 days. Appropriate antibiotics were added after 24-72 h post transformation at a concentration inhibitory to *E. chaffeensis* growth (Figure 4.5). The presence of genetically modified *E. chaffeensis* was assessed in cultures growing in the presence of an antibiotic by evaluating the growth or lack of growth by microscopic analysis of polychromatic stained culture slides.

Figure 4.5 Outline of transformation protocol.

The *E. chaffeensis* organisms were electroporated in the presence of plasmid DNA. The electroporated *E. chaffeensis* were inoculated into T25 flasks containing macrophages or tick cells. Depending on the plasmid used, appropriate antibiotics at inhibitory concentrations were added at days 1-3 post transformation and the media was replaced once every 3 days along with fresh antibiotics. Infectivity was monitored by microscopic evaluation of culture slides once every 3 days.



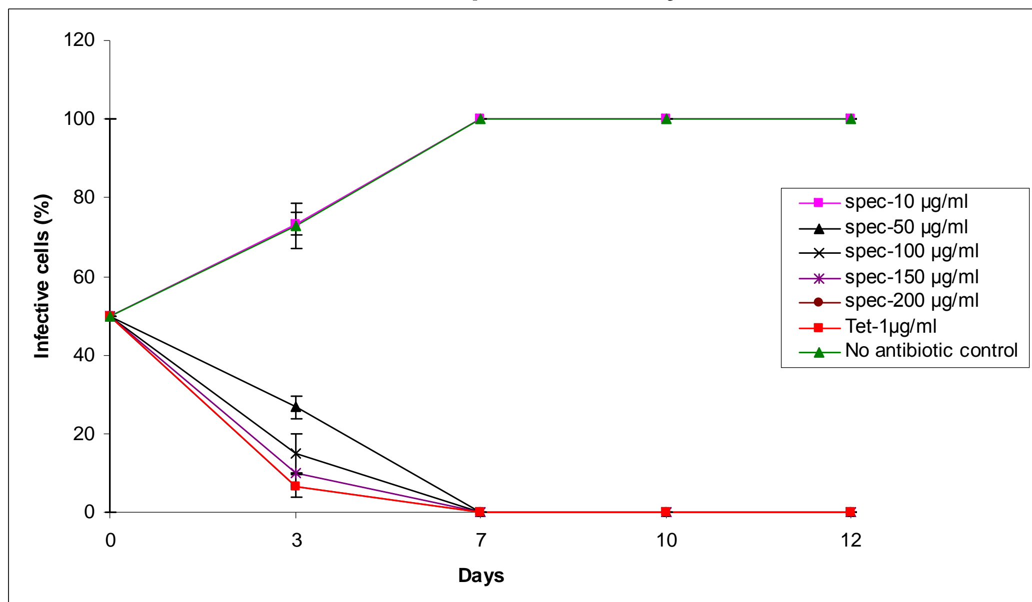
Any cultures that appeared to be positive were further evaluated by molecular techniques. Genomic DNA and total RNA were isolated from the putatively transformed *E. chaffeensis* organisms. RNA was used in RT-PCR analysis to look for the presence of the transcripts for the 16S rRNA gene. PCR analysis was performed, with genomic DNA as template, using primers spanning the expected insertion regions or with primers targeted to the antibiotic resistance gene or in combination of both.

Results

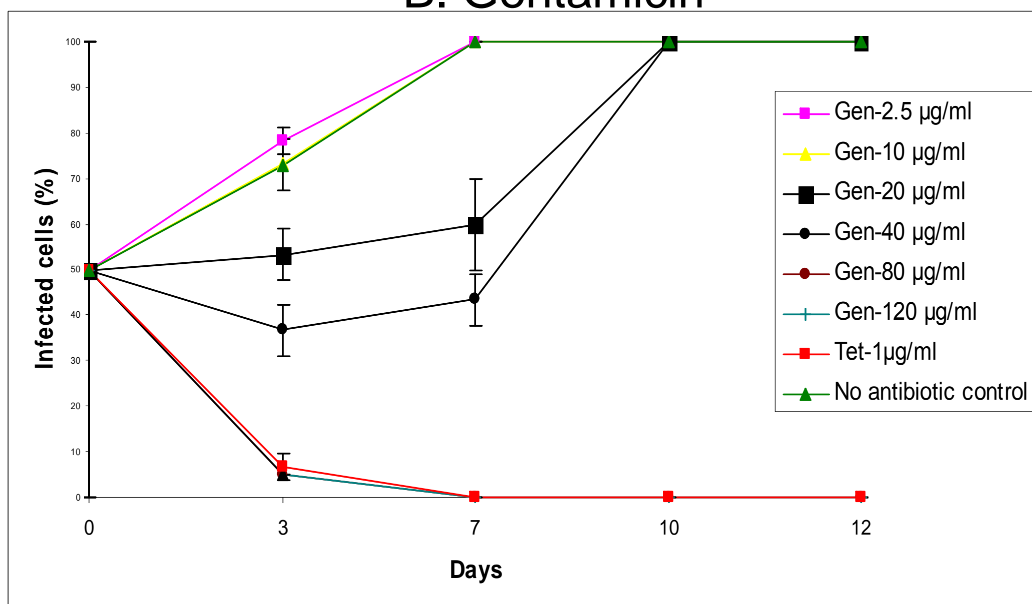
Sensitivity of *E. chaffeensis* to various antibiotics: To identify suitable selection markers for use in the transformation experiments, the sensitivity of *E. chaffeensis* to various antibiotics was evaluated. Ampicillin, chloramphenicol, gentamicin, rifampin, spectinomycin and kanamycin antibiotics were evaluated for their potential to be used as selection markers. The percentage of *E. chaffeensis* infected cells in the wells that received antibiotic treatment was compared with the control wells that did not contain the antibiotics to estimate the relative growth inhibitory effect of the antibiotics (Figure 4.6). In the control wells that did not receive the antibiotics, the *E. chaffeensis* infected host cells increased from 50% to about 75% by day 3. By day 7, nearly all the host cells were found to be infected (100%). The proportion of infected cells decreased from 50% to less than 10% in the wells that contained rifampin at concentrations of 0.1 µg/ml or higher by day 3 post antibiotic addition. *E. chaffeensis* infected host cells were undetectable by day 7 or later in the presence of rifampin at concentrations of 0.1 mg/ml or higher. The wells that contained gentamicin at concentrations of 80 µg/ml or 120 µg/ml showed a decrease in the proportion of infected cells by day 3 from 50% to about 5% and no *E. chaffeensis* organisms were detectable by day 7 until day 12. Gentamicin at lower concentrations, 20 and 40 µg/ml, was partially inhibitory to *E. chaffeensis* growth while at 2.5 and 10 µg/ml concentrations, the drug had no effect. Spectinomycin at concentrations of 50 µg/ml or higher is also completely inhibitory to *E. chaffeensis.*

Figure 4.6 Antibiotic sensitivity of *E. chaffeensis***.**

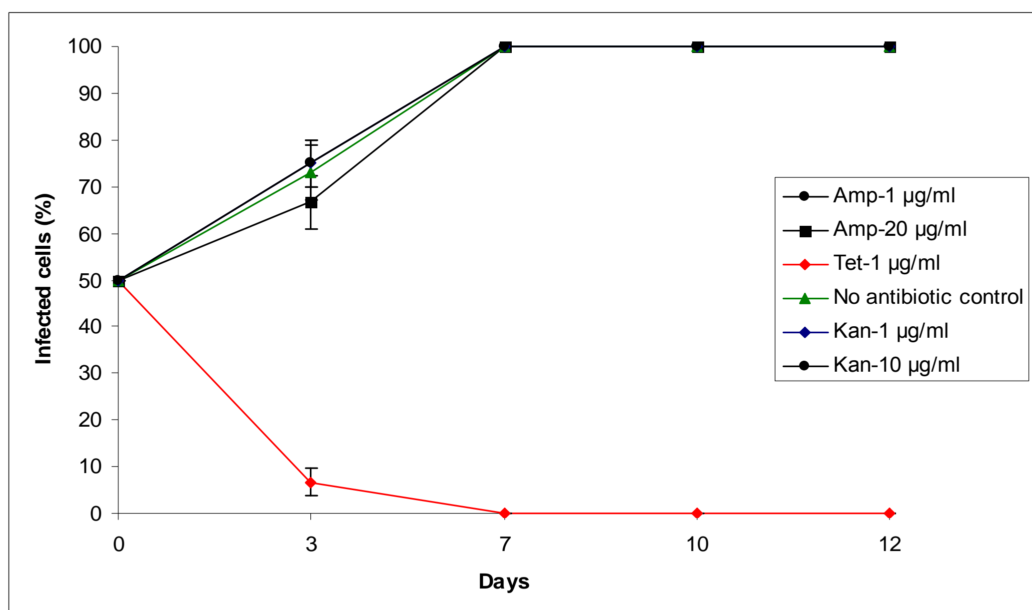
Briefly, *E. chaffeensis* was cultivated in T75 flasks having 100% confluent DH82 cells and when the infectivity reached about 50 %, approximately 0.5 ml culture per each well was transferred to a 24 well plate. Rifampin, chloramphenicol, ampicillin, kanamycin, spectinomycin and gentamicin various concentrations were added to the wells and their effect on *E. chaffeensis* growth was evaluated (Panel A-E). In positive control wells, tetracycline at 1 µg/ml is added and negative control wells did not receive any antibiotics. Media along with freshly prepared antibiotics dilutions were replaced every 3 days. The infectivity of the DH82 cells was also monitored at four time points (3, 7, 10 and 12 days) by microscopic evaluation of polychromatic stained cytospin culture slides. The inhibitory effect of rifampin was assessed relative to the *E. chaffeensis* growth in control wells. Average values of *E. chaffeensis* infected host cells were plotted on the Y-axis against the time point at which infectivity was monitored on the X-axis. The experiments were performed three independent times and the data were presented with error bars representing the standard deviation. Gen, gentamicin; Rif, rifampin; spec, spectinomycin; Kan, kanamycin; Amp, ampicillin; Chl, chloramphenicol; Tet, tetracyline. (Figure continued into Pages 103-105)



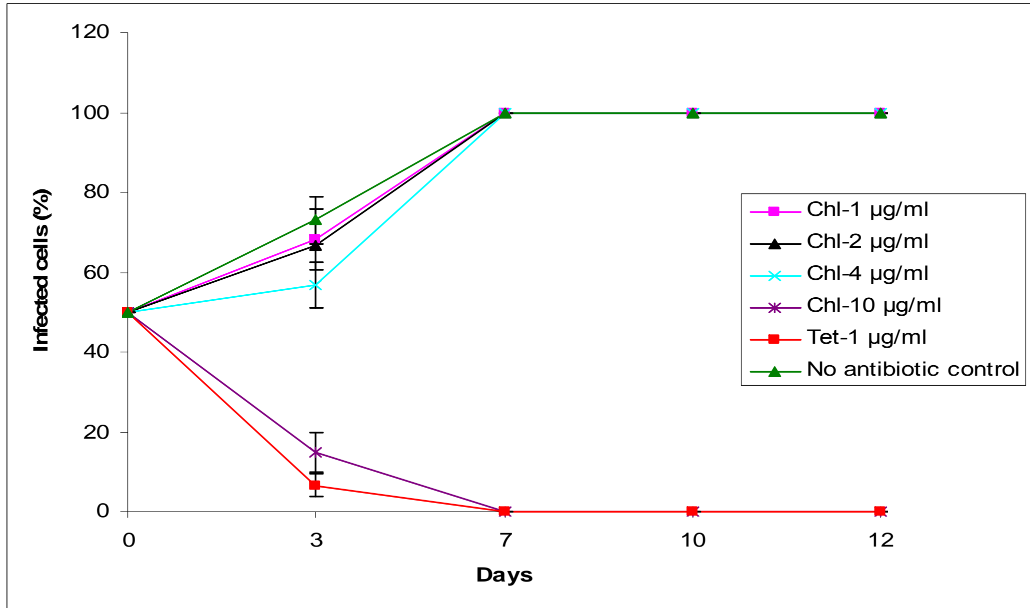
B. Gentamicin



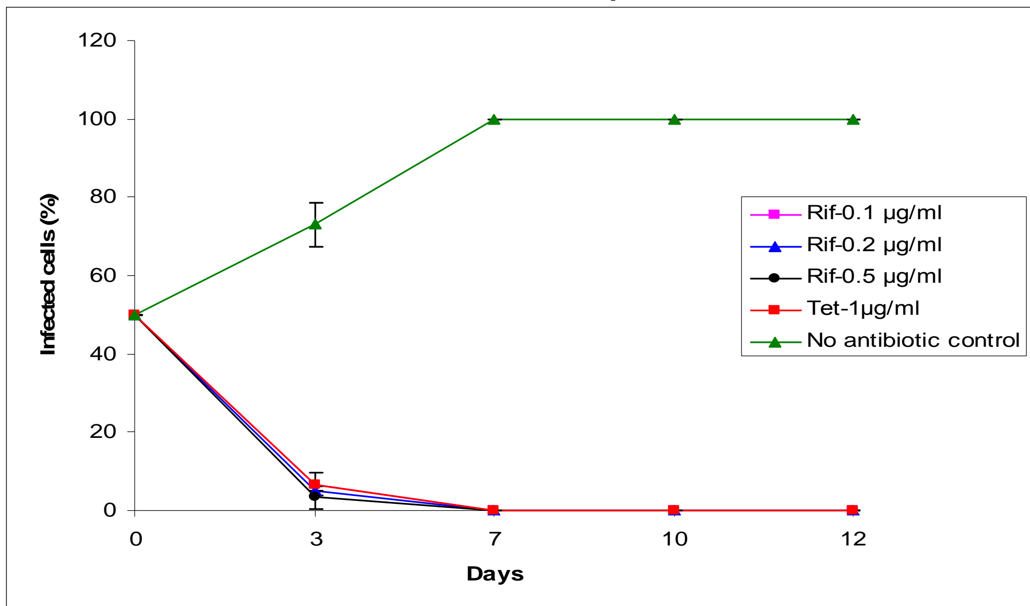
C. Ampicillin and Kanamycin



D. Chloramphenicol



E. Rifampin



chaffeensis growth by day 7 post antibiotic treatment. Similarly, chloramphenicol at a concentration of 10 µg/ml is inhibitory to *E. chaffeensis* growth, as the number of infected cells decreased from 50% to about 15% by day 3 and none were detectable by day 7. Ampicillin (at 1 and 20 µg/ml) and kanamycin (at 1 and 10 µg/ml) were not inhibitory to *E. chaffeensis* growth as the proportion of infected cells increased in the same manner as in the untreated controls. The presence of tetracycline at 1 µg/ml inhibited *E. chaffeensis* growth as no organisms were detected by day 7 post antibiotic addition. Although, we have initially identified spectinomycin to be inhibitory to *E. chaffeensis*, we have observed development of spontaneous resistance towards this antibiotic in the bacteria.

Identification of suitable targets for homologous recombination: Two genes, p28-Omp 15 and Ech_0126 were selected for use in creating mutations in *E. chaffeensis*. P28-Omp 15 was chosen for mutational analysis as our microarray data described in Chapter 3 suggested that transcripts from this gene was low in both tick cell and macrophage derived *E. chaffeensis* (Figure 3.8 of Chapter 3). Earlier proteome analysis from our laboratory suggested that p28-Omp 15 protein is not made in either tick or macrophage derived *E. chaffeensis* (115). Microarray analysis also suggested that Ech_0126 is a transcriptionally silent gene. RT-PCR analysis was performed using primers specific for the coding region of the Ech_0126 gene to verify the lack of transcription in *E. chaffeensis* cultured in macrophages and tick cells (Figure 4.7). The RT-PCR analysis included a positive control using genomic DNA as the template and no template reaction to serve as the negative control. The predicted size amplicons were detected only in the PCR products obtained from the reaction where genomic DNA is used as the template, but not in the reactions that contained *E. chaffeensis* RNA isolated from organisms cultured in tick cells and macrophages as the templates. Similarly, the no template control had no detectable amplicons. Ech_0126 is positioned in the negative strand of *E. chaffeensis* chromosome between a transcriptional

Figure 4.7 RT-PCR analysis of the ECH_0126 gene expression.

RNA isolated from *E. chaffeensis* grown in macrophage and tick cell backgrounds was used as templates in a RT-PCR reaction containing primers targeting a segment of the ECH_0126 gene. L, ladder; + reaction positive control with DNA as the template, -, reactive negative control; M, RNA from macrophage-grown *E. chaffeensis*; T, RNA from tick cell-derived *E. chaffeensis*.

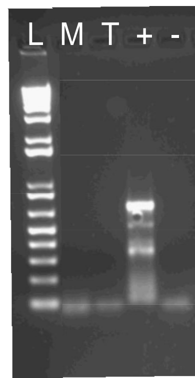
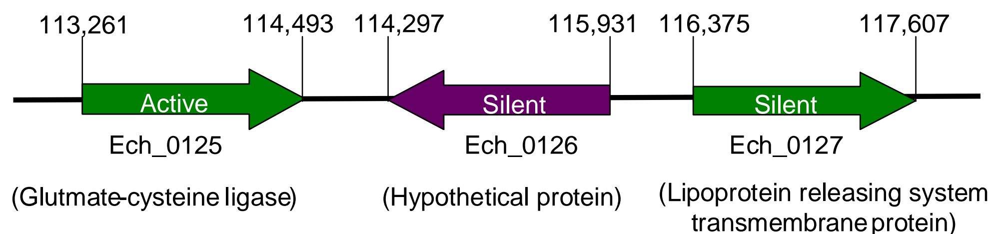


Figure 4.8. *E. chaffeensis* genomic region spanning the Ech_0126 gene selected for creating deletion mutational analysis.

The numbers above refer to the genomic coordinates of the protein coding regions of the genes. Active and silent refer to the presence or absence of mRNA transcripts in *E. chaffeensis* originating from macrophages or tick cells (as judged by the microarray analysis). For the gene in purple color (selected for mutational analysis), lack of detectable mRNA expression was also verified by RT-PCR. Arrowhead direction represents the orientation of each gene.

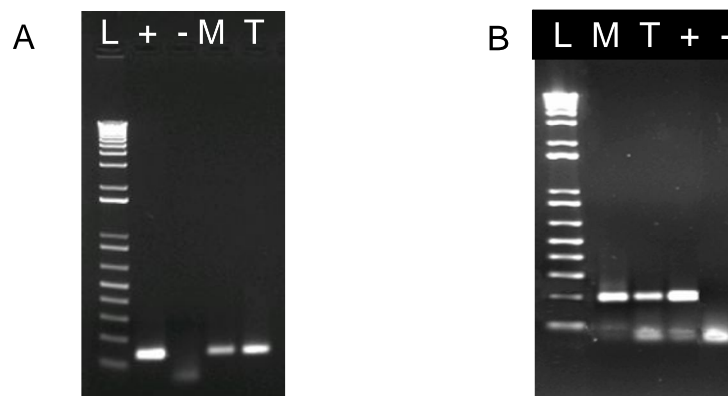


active glutamate cysteine ligase gene (located on the positive strand) and a transcriptionally silent lipoprotein releasing system transmembrane gene (located on the negative stand) (Figure 4.8).

Identification of *E. chaffeensis* promoters useful in driving the expression from antibiotic selection marker genes: To identify promoters that may be useful in driving the expression from antibiotic selection marker genes, we analyzed the transcriptionally active genes in *E. chaffeensis*. Our microarray analysis aided in the identification of 263 genes that are constitutively expressed in *E. chaffeensis* cultivated in both host cell types. While promoters from any one or more of these genes may be suitable, we selected *tr* and *rpsL* gene promoters as our choice because promoters from these gene homologues in *A. marginale* and *R. prowazeki* have already been shown to be useful in mutational analysis (37,78). To further verify the microarray results, RT-PCR analysis was performed by using RNA isolated from *E. chaffeensis*, grown in both host cell backgrounds, as templates and using primers designed to amplify segments of coding regions of the *tr* and *rpsL* genes. The RT-PCR analysis included a positive control using a genomic DNA as the template and no template reaction to serve as a negative control. The predicted size amplicons were detected in the PCR products obtained from reactions that contained the *E. chaffeensis* RNAs as templates and also in reaction positive control but not in the negative control reaction (Figure 4.9).

Figure 4.9 RT-PCR analysis of the activity of the *tr* (A) and *rpsI* (B) promoters.

RNA isolated from *E. chaffeensis* grown in macrophage and tick cell backgrounds was used as templates in a RT-PCR reaction containing primers targeting to a segment of the *tr* or *rpsI* genes. L, ladder; + reaction positive control with DNA as the template, -, reactive negative control; M, RNA from macrophage-grown *E. chaffeensis*; T, RNA from tick cell-derived *E. chaffeensis*.

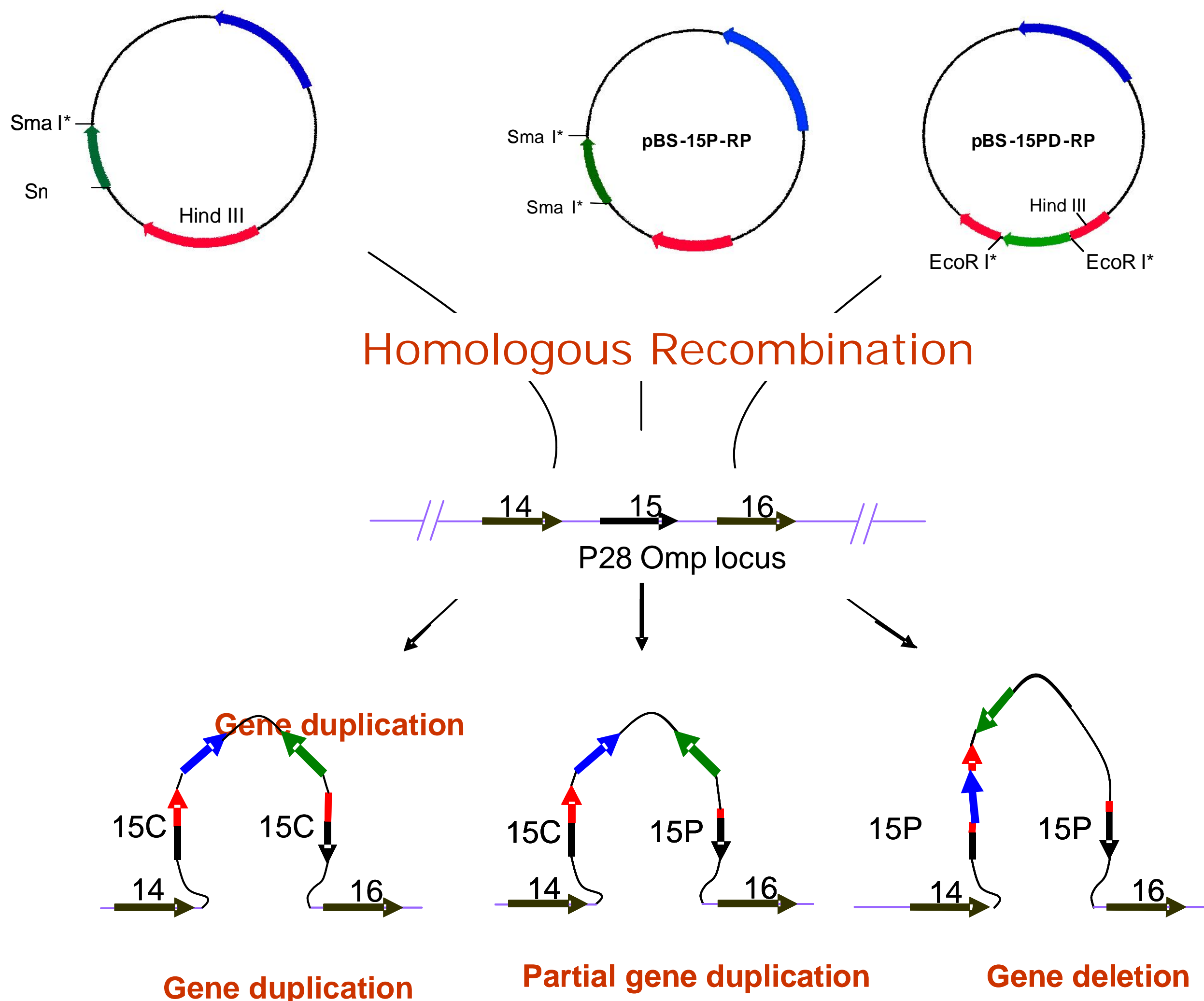


Construction of the plasmids for mutational analysis: Several plasmid DNA constructs were prepared for creating mutations in the *E. chaffeensis* genome. Initially three plasmids were constructed targeting gene 15 of the p28-Omp gene locus (Figure 4.2). The plasmid BlueScript SK+ (pBS) was used as the base vector to insert the partial or complete gene 15 coding sequences along with the 5' and 3' flanking sequences and also rifampin resistance gene driven by *R. prowazekii rpsL* promoter. Two of the plasmids, termed pBS-15C-RP and pBS-15P-RP, are designed for use in homologous recombination experiments to create duplication of either a complete gene 15 or a partial gene 15 in *E. chaffeensis* genome (Figure 4.10). The third plasmid, pBS-15D-RP which contained the rifampin resistance cassette in the middle of gene 15 segment, was constructed for use in creating disruptions in the gene 15 of *E. chaffeensis*. Similarly, three more plasmids were constructed for use in disrupting gene 15 but containing alternative selection markers (Figure 4.3). These plasmids contained *E. chaffeensis tr* promoter driving the expression from gentamicin, chloramphenicol or spectinomycin resistance genes.

We also generated plasmids targeting another gene, Ech_0126, in the *E. chaffeensis* genome. These constructs were engineered to contain the *E. chaffeensis rpsL* gene promoter driving the chloramphenicol and rifampin resistance genes. The 5' and 3' fragments of Ech_0126 gene were inserted such that they are flanking the resistance gene cassette. These plasmids are designed for use in creating disruption mutations within the coding sequence of Ech_0126 gene (Figure 4.4). The integrity of all eight plasmids, particularly the presence of inserts at appropriate locations and the presence of accurate sequences of promoters and antibiotic resistance genes was verified by employing restriction enzyme digestion and sequencing methods as described in Chapter 5.

Figure 4.10 A schematic representation of the possible homologous recombination events by insertion mutations and the resulting outcomes by using plasmids targeted to the p28-Omp 15 gene in the *E. chaffeensis* genome.

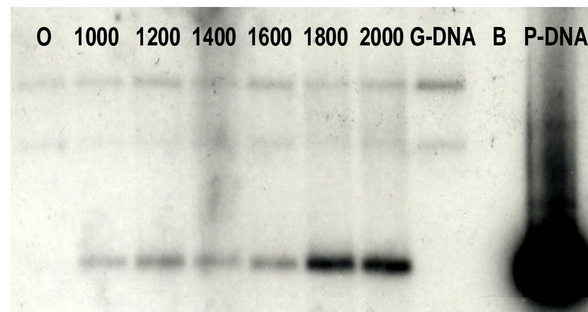
If pBS-15C-RP recombines with the *E. chaffeensis* genome, it results in integration into the genome resulting in creation of a duplicate complete gene 15. Similarly, pBS-15P-RP upon recombination results in a complete gene 15 and a partial gene 15. The plasmid pBS-15D-RP results upon recombination results in a partial gene 15 and a complete gene 15 disrupted by the rifampin resistance gene. 15C; p28-Omp 15 complete gene 15; 15P, p28-Omp 15 partial gene; 14, p28-Omp 14 gene; 15, p28-Omp 15 gene; 16, p28-Omp 16 gene.



Evaluation of electroporation conditions in internalization of recombinant plasmid DNAs into *E. chaffeensis*: Establishment of methods for *E. chaffeensis* requires the identification of conditions that would permit the entry of extracellular DNA into the organism. Different electroporation conditions were evaluated to identify the appropriate conditions to allow the internalization of the extracellular plasmid DNA. Electroporation was chosen because of its efficiency and moreover, it has been successfully used in recent studies transforming other rickettsial pathogens (37,69,78,94,95). The host cell-free *E. chaffeensis* organisms were prepared as described earlier (37,69,94). Host cell-free *E. chaffeensis* was electroporated in the presence of plasmid DNA at various voltage settings and evaluated for the internalization of the plasmid DNA upon electroporation. The viability of the host cell-free *E. chaffeensis* was initially examined for their ability to reinfect the host cells before or after subjecting to electroporation conditions. The electroporated *E. chaffeensis* were able to infect naïve host cells suggesting that electroporation did not kill the organisms (data not shown). internalized plasmid DNA in *E. chaffeensis* organisms was detected only in electroporated *E. chaffeensis* organisms at all voltage settings (Figure 4.11). Internalized plasmid DNA was not detected in the *E. chaffeensis* that did not receive electroporation treatment but contained equal amounts of plasmid DNA. Internalized plasmid DNA increased with increase in the amount of voltage used in subjecting *E. chaffeensis* to electric shock.

Figure 4.11 Southern blotting analysis to assess the internalization of plasmid DNA.

Host cell-free *E. chaffeensis* was subjected to electroporation under different voltage settings in the presence of 1 µg of pBS-15C-RP plasmid DNA. After electroporation, extracellulluar DNA residual plasmid DNA that did not internalize into *Ehrlichia* organisms was digested with DNAse. Subsequently, total DNA from the control and electroporated *E. chaffeensis* was isolated and subjected to *EcoR* I digestion. *EcoR* I digested DNA is resolved on an agarose gel and probed with ^{32}P labeled probes targeting the 16S rDNA gene of the *E. chaffeensis* and a rifampin gene segment. 0, no electroporation; 1000-2000, various voltage settings; G-DNA, 0.5 µg of *EcoR* I digested genomic DNA loaded as a control; B, blank well; P-DNA, 10 ng of *EcoR* I digested pBS-15C-RP plasmid DNA loaded as a control.



Transformation of *E. chaffeensis* using plasmids targeting gene 15 of the p28-Omp locus: To create mutations in the genome of *E. chaffeensis*, the plasmids designed for targeted mutagenesis of gene 15 in the p28-Omp locus were used in transformation experiments. About one hundred experiments were performed using pBS-15C-RP and pBS-15P-RP plasmids in *E. chaffeensis* cultivated in macrophages or tick cells (Table 4.2). About nine experiments were also performed using the plasmids pBS-15D-RP, pBS-15-CAT, pBS-15-GEN and pBS-15-SPEC (Table 4.3). Most of the transformation experiments were performed using host cell-free purified *E. chaffeensis*. A subset of experiments was also performed using *E. chaffeensis* infected macrophages or tick cells. The control for these experiments included the host cell-free purified or unpurified *E. chaffeensis* organisms that did not receive electroporation and plasmid DNA and *E. chaffeensis* organism that received electric shock but in the absence of plasmid DNA. These control flasks along with the *E. chaffeensis* electroporated in the presence of plasmid were transferred to the flasks containing naïve host cells and monitored for their growth. *E. chaffeensis* growth in host cells was detectable in the control flasks in the absence of antibiotic selection by day 3 or later. However, inclusion of antibiotics in these flasks resulted in the complete inhibition of *E. chaffeensis* within one week. The flasks that contained electroporated *E. chaffeensis* in the presence of plasmid DNA did not show any infectivity in the presence of antibiotic selection for up to two months post transformation. The infectivity was also monitored by using molecular methods PCR and RT-PCR targeting to the 16S rRNA gene of *E. chaffeensis*. Our analysis from two of the transformation experiments performed using pBS-15P-RP plasmid DNA

Table 4.2 Transformation experiments performed by using pBS-15C-RP and pBS-15P-RP plasmids designed to create mutations in the p28-Omp 15 gene locus in the *E. chaffeensis* genome. The *E. chaffeensis* grown in the host cells were purified free of host cells or used along with the host cells. The *E. chaffeensis* cultures were washed with and resuspended in 0.25 M sucrose or SPG buffer or 10% glycerol solution and subjected to electroporation in the presence of plasmid DNA. Type I restriction inhibitor was added to some of the electroporation reactions. The electroporated *E. chaffeensis* organisms were inoculated into a T25 flask containing a monolayer of naïve host cells. Rifampin antibiotic at inhibitory concentrations were added after 1-3 days post transformation and media was replaced with fresh antibiotics once every three days for about two months. The culture was monitored microscopically for the presence of infection once every 3 days.

Date	**Culture**	**No. of Expt.**	**Conditions**			
			Resuspended in	**Electroporation**	**Antibiotics**	**Type1**
2005	DH82 (whole cell)	6	SPG buffer	2500	24 h	no
2005	DH82 (purified by glass beads and centrifugation)	19	SPG buffer (2) 10% glycerol (3) 0.25 M sucrose (14)	2500 (4) 1700 (15)	24 h	No (2) Yes (17)
2005	ISE6 (purified by glass beads and centrifugation)	1	0.25 M sucrose	1700	24 h	Yes
2006	DH82 (whole cell)	1	0.25 M sucrose	1700	24 h	Yes
2006	DH82 (by glass beads and centrifugation)	10	0.25 M sucrose	1700	24 h	Yes

2006	ISE6 (purified by glass beads and centrifugation)	4	0.25 M sucrose	1700	24 h (3) 72 h (1)	Yes
2006	AAE2 (whole cell)	4	0.25 M sucrose	2500	24 h (2) 48 (1) 72 (1)	Yes
2006	AAE2 (purified by glass beads and centrifugation)	4	0.25 M sucrose	1700	24 h(3) 48 (1)	Yes
2007	DH82 (purified by needle)	1	0.25 M sucrose	1200	72 h (1)	Yes

Table 4.3 Transformation experiments performed by using pBS-15PD-RP and pBS-15D-AB plasmids designed to create gene disruptions in the p28-Omp 15 gene locus in the *E. chaffeensis* genome. The transformation experiments were performed as described in Table 4.2, except that in some of the transformations the electroporated *E. chaffeensis* organisms were inoculated into flasks containing host cells and rocked once every 15 min for about 2 h.

Date	Culture	No. of Expts	Conditions				
			Resuspended	Electroporation	Inoculation	Antibiotics	Type1
2005	DH82 (whole cell)	1	SPG	2500	Straight	24 h	no
2005	DH82 (purified by glass beads and centrifugation)	7	0.25 M sucrose (4) SPG (1) 10% glycerol (2)	2500 (4) 1700 (3)	Straight	24 h	No (2) Yes (5)
2007	DH82 (purified by needle	1	0.25 M sucrose	1200	Rocking once every 15 min for 2 hours	48 h	Yes

showed positive results for 16S rDNA gene and the rifampin resistance gene by PCR starting day 7 post-transformation until day 25 (data not shown). PCR analysis was performed to evaluate the homologous recombination in the genome. The analysis suggested no evidence of a recombination in the *E. chaffeensis* genome (data not shown).

Transformation of *E. chaffeensis* using plasmids targeting the ECH_0126 gene: The plasmids designed for targeted mutagenesis of the Ech_0126 gene were also evalauted in transformation experiments similar to the p28-Omp 15 gene targeted plasmids described in the previous section. About forty eight experiments were performed using pBS-126-CAT and pBS-126-RIF plasmid constructs in purified host cell-free or unpurified *E. chaffeensis* organism cultivated in macrophage or tick cells (Table 4.4). The experiments included controls as described in the previous section. The electroporated *E. chaffeensis* cultures that received plasmid DNA were monitored for up to two months. The control flasks showed the *E. chaffeensis* infectivity by day 3 or later post transformation. However, the flasks inoculated with *E. chaffeensis* organisms that received electric shock in the presence of plasmid DNA did not show any infectivity for up to two months in the presence of appropriate antibiotic selection.

Table 4.4 Transformation experiments performed by using the plasmids designed to create gene disruption mutations in the Ech_0126 gene locus in the *E. chaffeensis* genome.

The transformation experiments were performed as described in Table 4.2, except that all the reactions included type 1 restriction inhibitor and electroporation was performed at varying voltages in multiple experiments as shown in table. In some transformation experiments, plasmid DNAs were added and no electroporation was performed to evaluate the utility of natural transformation mechanisms by *E. chaffeensis*. In some transformation experiments, the plasmid DNA and the *E. chaffeensis* cultures were incubated for extended periods of time before electroporation.

Date	**Culture**	**No. of experiments**	**Conditions**			
			Resuspended	**Electroporation**	**Antibiotics**	**Extra incubation**
2007	DH82 (whole ell)	7	0.25 M sucrose	1000 +1000 (2) 1200 + 1200 (3) 1200 (2)	24 h	1 min (1) 5 min (2) 30 min (4)
2007	DH82 (purified by needle)	5	0.25 M sucrose	Phagocytosis (1) 1000 + 1000 (1) 1200 (3)	72 (2) 24 (3)	1 min (4) 5 min (1)
2007	AAE2 (whole ell)	5	0.25 M sucrose	1000 + 1000 (1) 1200 + 1200 (1) 1000 (2) 1200 (1)	24	5 min (2) 30min (3)
2007	AAE2 (purified by needle)	7	0.25 M sucrose	Phagocytosis (1) 1000 + 1000 (1) 1000 (1) 1200 (4)	24 (5) 72 (2)	1 min (5) 5 min (2)

Discussion

In this study, we evaluated methods for establishing conditions for creating mutations in *E. chaffeensis*. Standardization of many basic protocols is the first step towards establishing a mutational analysis system. This is particularly important as there are no reports in the literature describing protocols for creating mutations in *E. chaffeensis*. Likewise, methods are also not available for other closely related *Ehrlichia* pathogens. We focused our efforts in standardizing several basic protocols which included optimizing methods for purifying viable *E. chaffeensis* for electroporation, identification of appropriate antibiotic selection markers, and the selection of targets in the genome for initial mutational analysis where creating mutations should not have any negative effect on bacterial growth. We also identified constitutively expressed genes whose promoters can be used to drive the expression of appropriate antibiotic resistance genes. It is also important to identify appropriate antibiotics that are not used in controlling *E. chaffeensis* infections but are useful in selecting the transformed organisms. All these protocols are the prior requirement in support of creating mutations in *E. chaffeensis*.

As a first step to identify the appropriate antibiotics, we evaluated chloramphenicol, rifampin, spectinomycin, gentamicin, ampicllin and kanamycin for their inhibitory effect on *E. chaffeensis*. Gentamicin, rifampin spectinomycin and chloramphenicol were found to be inhibitory to *E. chaffeensis* growth. Our analysis confirmed the earlier reports about the sensitivity of *E. chaffeensis* to rifampin, chloramphenicol and gentamicin (13). In addition, we identified spectinomycin to be inhibiting the growth of *E. chaffeensis*. Rifampin, gentamicin, specitnomycin and chloramphenicol were chosen as they are not routinely used in clinical setting to treat the HME infections and genes that confer the resistance to these antibiotics are available (33,87).

Selecting an appropriate region(s) in the genome of *E. chaffeensis* to create initial mutations where there will be no detrimental effect caused by mutations is necessary to establish protocols for the introduction of genetic mutations in the organism. Based on the careful analysis of transcriptome data and also taking into consideration the location of the genes in the genome, two genomic regions were selected for the initial mutational analysis experiments. The region spanning the Ech_0126 is ideally suited for this kind of analysis as it met the criteria that we chose. Similarly, p28-Omp 15 was selected as it is present in the genome locus coding for multiple paralogs that encode for differentially expressed outer membrane proteins. Moreover, we were careful in designing mutational experiments that will retain the native structure of the p29-Omp 15 gene even after creating mutations by homologous recombination.

The third logical step in establishing the conditions for creating mutations is to select appropriate promoters that can be used to drive the expression of antibiotic selection marker genes. Our transcriptome analysis identified several genes that are constitutively expressed in *E. chaffeensis* in both host cell backgrounds. We focused our efforts in selecting the promoters of transcriptional regulator (*tr*) gene and ribosomal protein S12 gene (*rpsL*) as the homologues of these promoters were already used in demonstrating their utility in transformation studies in other rickettsiae and *Anaplasma* organisms (37,94).

Several constructs have been prepared by including DNA segments spanning the Ech_0126 and p28-Omp 15 genes. Six plasmid constructs were made for the p28-Omp 15 gene and two constructs were made for the Ech_0126 gene for use in mutational analysis experiments. It is possible that creating mutations in the p28-Omp 15 region may have an impact on the growth of this bacterium as this gene is shown to be expressed in *E. chaffeensis* at low levels. Therefore, a strategy was developed to create mutations that result in complete or

partial gene duplications rather than to create disruption mutations in this gene. We also designed constructs for use in disruption mutations for p28-Omp 15 gene. The constructs targeted to Ech_0126, an inactive gene, were designed to create gene disruption mutations.

The next important step in creating mutations is to have appropriate conditions for transformation where extracellular plasmid DNA can be introduced into the *Ehrlichia* cells. We essentially followed the protocols described in the literature for our experiments, but made minor changes such as altering the voltage used for transformation. The validity of the experimental protocols was tested by assessing the internalization of plasmid constructs into *E. chaffeensis* cell. This experiment was performed after eliminating extracellular (non-internalized) DNA by enzymatic digestion and then checking the internalized plasmid DNA by Southern blot analysis probes specific for *E. chaffeensis* 16S rDNA gene and a segment of plasmid DNA. The Southern blot analysis verified that we have appropriate conditions for transforming *E. chaffeensis* with recombinant plasmids. We also demonstrated in our studies that electroporation conditions used in the transforming *E. chaffeensis* did not result in the complete loss of cultivable organisms.

About 109 independent transformation experiments were performed using plasmids targeting the p28-Omp 15 gene and about 48 experiments were performed using plasmids targeting to Ech_0126 gene. The transformation experiments were either performed at one set of conditions or by introducing minor modifications to the protocols. This is to maximize the probability of creating targeted mutations in *E. chaffeensis*. In each experiment, the electroporated bacteria were cultured in the presence or absence of appropriate antibiotic and the infectivity was monitored for up to 60 days. In all the transformation experiments, *E. chaffeensis* infection of host cell cultures was detectable in control flasks. However, none of the experiments resulted in the rescue of antibiotic resistant *E. chaffeensis*. The only exception was

the two experiments where we detected rifampin resistant *E. chaffeensis* when plasmids designed for the gene duplication of the p28-Omp 15 gene were used. However, careful evaluation of these two transformation experiments revealed no evidence for homologous recombination events in the genome. Although studies led to the significant accomplishments in conducting the experiments, we were unsuccessful in identifying mutations by homologous recombination.

Genetic manipulations have already been described for *A. phagocytophilum, R. prowazekii and R. monacensis,* however, they are based on transposon based mutagenesis approach (37,78,94). Recent published reports by Rachek *et al* also described the targeting mutations in *R. prowazeki* (95,97). Two reports of targeted mutagenesis in *Anaplasma*taceae bacteria have also been reported recently at two different scientific meetings (38,69). In the absence of detailed description of these experiments from the *Anaplasma*taceae pathogens it is very difficult to conclude if targeted mutations is an achievable task or may need fine tuning of experiments. Alternatively, despite the evaluation of about 150 transformation experiments, the failure to demonstrate targeted mutations may suggest that the pathogen does not support the replication of plasmids or integration of foreign DNA in its genome or has an efficient method to restore the integrity of the genome. This hypothesis may be justified as *E. chaffeensis* and other closely related *Anaplasma*taceae organisms do not harbor plasmids (56). The *E. chaffeensis* genome contains about eight genes that encode for recombinase like proteins (56), but our microarray data provides evidence for the expression of only two genes. These data may further suggest that the *E. chaffeensis* genome does not support introduction of foreign DNA. Nonetheless, this study is a significant step forward as it aided in carefully examining various protocols and methods required for creating mutations in *E. chaffeensis*.

CHAPTER 5 - General Molecular Methods

Restriction enzyme digestions: Typically, restriction enzyme digestions were performed in a 20 µl reaction containing 0.5-1.0 µg of DNA, 1X concentration of appropriate buffer and 5-10 units of restriction enzyme (Promega Corporation, Madison, WI). The reaction was carried out by incubating at 37°C for 2 h. For double digestion with two restriction enzymes, a buffer was chosen that was optimal for both the enzymes.

Sequencing of PCR products or plasmid DNA: The sequencing of plasmid DNA or PCR products was performed using the CEQ Genetic Analysis System following the manufacturer's recommendation (Beckman & Coulter, Fullerton, CA). PCR products were subjected to presequencing treatment prior to proceeding to sequencing. Five µl of PCR product were treated with Exo-I (10 units) and shrimp alkaline phosphotase (2 units) (USB Corporation, Cleveland, Ohio) and incubated at 37°C for 15 min to remove excess primers and nucleotides. The enzymes were inactivated by incubation at 80°C for 15 min. After treatment, the DNA was purified by using a phenol purification method (72). The integrity and concentration of the DNA was estimated by resolving the purified sample on an agarose gel and using nanodrop technique. Plasmid DNA isolated by using boiling preparation method was purified free of contaminating proteins using phenol purification. Later the integrity and concentration of the plasmid DNA was evaluated by resolving on an agarose gel and spectrophotometrically by using nanodrop instrument. The purified PCR products/plasmid DNA were then used in sequencing reaction by using the DTCS sequencing kit following manufacturer's protocols (Beckman & Coulter, Fullerton, CA). The primers upstream or downstream to the insert sequences were used in the sequencing reaction of plasmid DNA. The forward or reverse primers used in the PCR reaction are used in sequencing the DNA from PCR products. The sequence generated from the above reaction was analyzed using the GCG program (142).

RNA isolation: *E. chaffeensis* Arkansas strain were grown in host cells (dog macrophage cell line or tick cells) as described by Singu et al. (115). RNA from cultures samples was isolated using Tri-reagent (Sigma Chemical Corporation, St. Louis, MO) as per manufacturer's instructions. Briefly, cells from 5 ml of culture were pelleted by centrifuging at 13,000 g for 15 min at 4C and the pellet was dissolved in 1 ml of Tri-reagent solution, incubated at room temperature for 5 min after which 200 µl of $CHCl_3$ was added, mixed and incubated at room temperature for 10 min. Subsequently, the sample was centrifuged at 13,000 g for 15 min at 4°C and the supernatant was transferred to a clean tube and an equal volume of ice cold 100% isoproponal was added. The sample was vortexed, incubated at room temperature for 10 min, and the centrifuged to pellet RNA. The pellet was washed with 70% ethanol and the RNA dissolved in 100 µl of nuclease free water and stored at -70°C in the presence of 40 units of RNase inhibitor, RNasein (Ambion Corporation, Austin, TX).

DNA isolation: *Ehrlichia chaffeensis* cultures also were used to isolate genomic DNA. Genomic DNA was isolated typically from 1-5 ml of *Ehrlichia*-infected DH82/ISE6/AAE2 cultures utilizing the sodium dodecyl sulfate-proteinase K, phenol, chloroform-isoamyl-alcohol method (72). The final DNA pellet was dissolved in 100 µl of TE buffer.

PCR: PCR was performed in a 25 µl reaction at 1X PCR reaction buffer containing 10 pico moles of forward and reverse primers, 10 nmoles of each dNTP, 1 unit of Taq DNA polymerase (Invitrogen technologies, Carlsbad, CA), 75 nmoles of $MgCl_2$, and about 50 ng of genomic DNA or 1 ng of plasmid DNA as template. The thermal cycling conditions included an initial heating of 3 min at 95°C, followed by 45 cycles of 95°C denaturaion for 15 sec, 50°C of annealing for 30 sec and 72°C extension for 60 sec (extension time is adjusted at the rate of 1 min per kb expected amplicon size). For PCR reactions where proof reading is required, slight

modifications are made to the above conditions. They are: 1) platinum Pfx DNA polymerase enzyme is added at 1 unit per 25 µl reaction containing 1X concentration of the Pfx reaction buffer (Invitrogen Technologies, Carlsbad, CA), 2) the concentration of dNTPs is changed to 5 nmoles and 3) the extension temperature is decreased to 68°C. The PCR products were resolved on an agarose gel to visualize the DNA bands.

RT-PCR: RT-PCR was performed in a 25 µl reaction at 1X reaction buffer containing 1 µl of SS-III and Taq mix (SuperScript-III, one step RT-PCR system with platinum Taq DNA polymerase, Invitrogen Technologies, Carlsbad, CA), 10 pico moles of forward and reverse primers, 10 nmoles of each dNTP (Invitrogen technologies, Carlsbad, CA), 75 nmoles $MgCl_2$ in a thermal cycler. Thermal cycling conditions included an initial reverse transcription step at 48°C for 30 min followed by an initial heating of 3 min at 95°C, followed by 45 cycles of 95°C denaturation for 15 sec, 50°C of annealing for 30 sec and 60°C extension for 60 sec. The reaction products were resolved on an agarose gel to visualize the DNA bands.

Real time RT-PCR: Taqman based Real-Time RT-PCR and PCR allows detection of amplified products in the reaction tube directly. Real time RT-PCR was performed in a 25 µl reaction at 1X reaction buffer containing 1 µl of SS-III and Taq mix (Super Script-III, one step RT-PCR system with platinum Taq DNA polymerase, Invitrogen Technologies, Carlsbad, CA), 10 pico moles of *Ehrlichia*-specific primers (116), 10 nmoles of each dNTP (*i*nvitrogen technologies, Carlsbad, CA), 40 nmoles $MgCl_2$, 7.5 pmoles of *E. chaffeensis*-specific Taqman detection probe in the Smart Cycler. Thermal conditions include an initial reverse transcription step at 48°C for 30 min and by an initial heating of 3 min at 95°C, followed by 45 cycles of 95°C denaturation for 15 sec, 50°C of annealing for 30 sec and 60°C extension for 60 sec. The fluorescence emitted is detected in the extension phase of the PCR cycles by turning on the optics built in the PCR

machine and the computer. The threshold for detecting positive signal was set at 30 fluorescent units.

Plasmid DNA isolation: Plasmid DNA was isolated from *E. coli* cultures by boiling preparation method (72). One and a half milliliters of culture was pelleted at 13,000 g for 5 min. The supernatant was carefully aspirated and the bacterial pellet suspended in 400 µl of plasmid lysis buffer (10mM Tris-HCl pH 8.0, 0.1 M NaCl, 1mM EDTA, 5% v/v Triton X-100) by agitating the pellet with a sterile toothpick. To this, 25 µl of freshly prepared lysozyme (Lysozyme was dissolved in 10 mM Tris-HCl buffer pH 8.0 to get a final concentration of 10 mg/ml) was added. The samples were vortexed for 10 sec, boiled immediately in a boiling water bath for 40 sec and centrifuged for 15 min at 13,000 g. The pellet was removed using a sterile toothpick. To the solution, 420 µl of cold (-20°C) 100% isopropanol was added, mixed well, incubated at room temperature for 5 min and centrifuged at 13,000 g for 15 min to recover plasmid DNA. The DNA pellet was washed with 70% ethanol, dried in a speed-vac system (Labconco Centrivap Concentrator, Kansas City, MO) and finally dissolved in 100 µl of TE buffer. To remove bacterial RNA, 1 µl of RNase A (1 mg/ml) was added and incubated at 37°C for 5 min.

Transformation and selection of recombinant clones/plasmids: Ligated products were transformed into chemically competent XL1 Blue MRF' strain *E. coli* cells by a chemical method. About 200 µl of competent cells are mixed with 50 µl of 100 ml $CaCl_2$ and 49 µl of sterile water. To this suspension, 1 µl of ligation mixture is added, mixed well and incubated on ice for 15 min. The *E. coli* cells are heat shocked for 2 min at 42°C and incubated at room temperature for 10 min. One ml of SOC media is added to the tube and incubated at 37°C in a shaker incubator at 200 rpm for 1 h. Two hundred micro liters of the culture media were transferred to Luria-Bertani bacterial culture plates containing 4 µl of IPTG (200 mg/ml) and 40 µl of X-Gal (20 mg/ml) and

spread uniformly using a sterile bent glass rod to grow transformed *E. coli*. The plates were incubated at 37°C for approximately 14 h. Blue-white color selection resulting from the expression of the β-galactosidase gene was used to identify non recombinants from recombinants. Recombinants will not produce β- galactosidase, thus forming white colonies. Several white colonies were selected to isolate plasmid DNA by growing in 3 ml of LB media containing appropriate antibiotic selection (72). In the case of plasmids where blue/white selection cannot be employed, *E. coli* cells were plated onto LB plates with only antibiotics and several white colonies were selected to isolate plasmid DNA and further analysis.

Ligation reactions: About twenty five ng of a linearized plasmid was added to 3 molar excess concentration of the insert DNA segment and ligated using 5 units of T4 DNA ligase in a 20 µl reaction containing 1X concentration of ligation buffer (Promega Corporation, Madison, WI). The ligation reaction is typically carried out by incubating at 16°C for 16 h. The ligated DNAs were purified by ethanol precipitation (72) and used in transformation experiments.

Phenol purification of DNA: DNA was purified free of proteins by using a phenol purification technique. To the tube containing the DNA, 20 µl of 3 M sodium acetate was added and volume of the solution was adjusted to 200 µl by adding nuclease free water. To this mixture, 200 µl of phenol (pH=8.0) was added, vortexed and centrifuged at 15,000 g for 15 min. Supernatant was transferred to a new tube and 200 µl of freshly prepared phenol:chloroform:isoamylalcohol (25:24:1) mixture was added, vortexed and centrifuged at 15,000g for 15 min. The supernatant was transferred to a new tube and 200 µl of chloroform:isoamylalcohol (24:1) mixture is added, vortexed and centrifuged at 15,000 g for 10 min. The supernatant was transferred to a new tube and 500 µl of 100% alcohol was added and incubated at -20°C for 15 min and centrifuged at 15,000g for 15 min followed by 70%

ethanol wash. The final DNA pellet was resuspended in 20 µl of nuclease free water or TE buffer and stored at -20°C until use.

Agarose gel electrophoresis: DNA was resolved on a 0.9% agarose gel by electrophoresis. The agarose gel was prepared by dissolving agarose powder in 1X TAE buffer containing 0.1 µg/ml of ethidium bromide using heat. About 5 µl of each of the plasmid DNA or PCR product or restriction digestion products were loaded onto the wells of a polymerized agarose gel. Appropriate DNA markers were also loaded onto the gel as a control. The gel was subjected to electrophoresis at a voltage setting of 90 V for about 1 h in an electrophoresis chamber containing 1X TAE buffer containing 0.1 µg/ml of ethidium bromide. The size and integrity of the resolved DNA was verified by visualizing the predicted size DNA bands under UV light and by comparing their migration patterns with the DNA markers.

DNAse treatment of RNA samples: RNA was treated with DNase to remove contaminating genomic DNA from the solution. The reaction was typically performed in a 20 µl volume containing 5 µl of RNA sample, 1X concentration of RQ1DNase buffer and 1 µl of RQ1DNase (Promega Corporation, Madison, WI). The reaction mixture was incubated at 37°C for 1 hour and later the enzyme was heat inactivated at 75°C for 10 min.

Preparation of LB agar plates and liquid media: The LB liquid medium is prepared by dissolving 15 g of Tryptone, 10 g of sodium chloride and 10 g of yeast extract in nanopure water to a final volume of 1 liter. The pH is adjusted is 7.0 using 5N NaOH and the liquid medium is autoclaved on liquid cycle. For preparing LB agar plates, the medium is prepared as described above but 15 g of agar powder was added before autoclaving. After autoclaving when the

temperature of the agar media is about 60°C the agar media is pored into sterile plates and stored at 4°C.

Competent cell preparation: A colony of XL1Blue MRF' strain of *E. coli* was grown in 3 ml of LB media containing tetracycline (50 µg/ml) overnight in 37°C incubator shaker. The following morning, the liquid culture was inoculated into a 500 ml flask containing 100 ml of LB medium with tetracycline (50 µg/ml). When the optical density measured at 660 nm reached between 0.2-0.3, the culture was harvested by centrifugation at 2,500 g for 5 min. The supernatant was discarded and the pellet suspended in 10 ml of Tris buffer and incubated on ice for 30 min. The culture was centrifuged again at 2,500 g for 5 min and the pellet suspended in 2 ml of buffer and stored at 4°C.

Filling-in reaction: The PCR or restriction enzyme digestion products were made blunt ended by using T4 DNA polymerase enzyme. Typically, eighty nanograms of DNA was treated with 10 units of T4 DNA polymerase (Promega Corporation, Madison, WI,) for 5 min in the presence of 0.2 mM dNTPs to create a blunt end. The blunt ended products were purified by phenol-chloroform, ethanol precipitated and suspended in 10 µl of TE buffer pH=8.0 (10 mM Tris-HCl and 1 mM EDTA).

CHAPTER 6 - References

1. 2007. Vector-borne Diseases -- Understanding the Environmental, Human Health, and Ecological Connections, *Internet*.

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

3. **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.

4. **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(12)**:2838-2842.

5. **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.

6. **Andrew, H. R. and R. A. Norval**. 1989. The carrier status of sheep, cattle and African buffalo recovered from heartwater. Vet. Parasitol. **34**:261-266.

7. **Bakken, J. S. and J. S. Dumler**. 2000. Human granulocytic ehrlichiosis. Clin. *i*nfect. Dis. **31**:554-560.

8. **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.

9. **Banfield, J. F.** 1966. Tick bites in man. Med. J. Aust. **2**:600-601.

10. **Bishop, R., B. Sohanpal, D. P. Kariuki, A. S. Young, V. Nene, H. Baylis, B. A. Allsopp, P. R. Spooner, T. T. Dolan, and S. P. Morzaria**. 1992. Detection of carrier state in *Theileria parva*-infected cattle by polymerase chain reaction. Parasitology **104**:215-232.

11. **Bock, R., L. Jackson, V. A. de, and W. Jorgensen**. 2004. Babesiosis of cattle. Parasitology **129 Suppl**:S247-S269.

12. **Boone, S. A. and C. P. Gerba**. 2007. Significance of fomites in the spread of respiratory and enteric viral disease. Appl. Environ. Microbiol. **73**:1687-1696.

13. **Brouqui, P. and D. Raoult**. 1992. *i*n vitro antibiotic susceptibility of the newly recognized agent of ehrlichiosis in humans, *Ehrlichia* chaffeensis. Antimicrob. Agents Chemother. **36**:2799-2803.

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

15. **Buller, R. S., M. Arens, S. P. Hmiel, C. D. Paddock, J. W. Sumner, Y. Rikhisa, 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.

16. **Campbell, G. L., A. A. Marfin, R. S. Lanciotti, and D. J. Gubler**. 2002. West Nile virus. Lancet infect. Dis. **2**:519-529.

17. **Chapes, S. K. and R. R. Ganta**. 2005. Mouse infection models for space flight immunology. Adv. Space Biol. Med. **10**:81-104.

18. **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.

19. **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.

20. **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.

21. **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.

22. **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.

23. **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.

24. **Dawson, J. E., B. E. Anderson, D. B. Fishbein, J. L. Sanchez, C. S. 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.

25. **Dawson, J. E., K. L. Biggie, C. K. Warner, K. Cookson, S. Jenkins, J. F. Levine, and J. G. Olson**. 1996. Polymerase chain reaction evidence of *Ehrlichia chaffeensis*, an

etiologic agent of human erlichiosis, in dogs from southeast Virginia. Am. J. Vet. Res. **57**:1175-1179.

26. **Dawson, J. E., J. E. Childs, K. L. Biggie, C. Moore, D. Stallknecht, J. Shaddock, J. Bouseman, E. Hofmeister, and J. G. Olson**. 1994. White-tailed deer as a potential reservoir of *Ehrlichia* spp. J. Wildl. Dis. **30**:162-168.

27. **Dourado, H. V.** 1992. Malaria parasites, vectors and biologic cycle. Rev. *i*nst. Med. Trop. Sao Paulo **34 Suppl 9**:S6-S9.

28. **Doyle, C. K., A. M. Cardenas, D. M. Aguiar, M. B. Labruna, L. M. Ndip, X. J. Yu, and J. W. McBride**. 2005. Molecular characterization of E. canis gp36 and *E. chaffeensis* gp47 tandem repeats among isolates from different geographic locations. Ann. N. Y. Acad. Sci. **1063**:433-435.

29. **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.

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

31. **Dumler, J. S. and J. S. Bakken**. 1998. Human ehrlichioses: newly recognized infections transmitted by ticks. Annu. Rev. Med. **49:201-13**:201-213.

32. **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 *Anaplasma*taceae 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. *i*nt. J. Syst. Evol. Microbiol. **51**:2145-2165.

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

34. **Dumler, J. S., W. L. Sutker, and D. H. Walker**. 1993. Persistent *i*nfection with *Ehrlichia chaffeensis*. Clin. *i*nfect. Dis. **17**:903-905.

35. **Eguchi, Y., T. *i*toh, and J. Tomizawa**. 1991. Antisense RNA. Annu. Rev. Biochem. **60**:631-652.

36. **Ewing, S. A., J. E. Dawson, A. A. Kocan, R. W. Barker, C. K. Warner, R. J. Panciera, J. C. Fox, K. M. Kocan, and E. F. Blouin**. 1995. Experimental transmission of *Ehrlichia chaffeensis* (Rickettsiales: Ehrlichieae) among White-tailed deer by *Amblyomma americanum* (Acari: *i*xodidae). J. Med. Entomol. **32**:368-374.

37. **Felsheim, R. F., M. J. Herron, C. M. Nelson, N. Y. Burkhardt, A. F. Barbet, T. J. Kurtti, and U. G. Munderloh**. 2006. Transformation of *Anaplasma* phagocytophilum. BMC. Biotechnol. **6:42.**:42.

38. **Felsheim, R. F., G. H. Palmer, A. Barbet, and U. G. Munderloh**. 2007. An unexpected *Anaplasma* marginale transformant.

39. **Feng, H. M. and D. H. Walker**. 2004. Mechanisms of immunity to *Ehrlichia* muris: a model of monocytotropic ehrlichiosis. *i*nfect. *i*mmun. **72**:966-971.

40. **Fishbein, D. B. and D. T. Dennis**. 1995. Tick-borne diseases--a growing risk [editorial; comment]. N. Engl. J. Med. **333**:452-453.

41. **Fotedar, R., U. Banerjee, S. Singh, Shriniwas, and A. K. Verma**. 1992. The housefly (Musca domestica) as a carrier of pathogenic microorganisms in a hospital environment. J. Hosp. *i*nfect. **20**:209-215.

42. **French, T. W. and J. W. Harvey**. 1983. Serologic diagnosis of infectious cyclic thrombocytopenia in dogs using an indirect flourescent antibody test. Am. J. Vet. Res. **44**:2407-2411.

43. **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. *i*nfect. *i*mmun. **75**:135-145.

44. **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. *i*nfect. *i*mmun. **72**:159-167.

45. **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. *i*nfect. *i*mmun. **70**:380-388.

46. **Ge, Y. and Y. Rikihisa**. 2007. Surface-exposed proteins of *Ehrlichia* chaffeensis. *i*nfect. *i*mmun. **75**:3833-3841.

47. **Gibbs, E. P., E. J. Homan, C. L. Mo, E. C. Greiner, J. Gonzalez, L. H. Thompson, M. T. Oveido, T. E. Walton, and T. M. Yuill**. 1992. Epidemiology of bluetongue viruses in the American tropics. Regional Bluetongue Team. Ann. N. Y. Acad. Sci. **653**:243-250.

48. **Goonaratna, C.** 2007. Epidemic of chikungunya fever. Natl. Med. J. *i*ndia **20**:96.

49. **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.

50. **Gothe, R., K. Kunze, and H. Hoogstraal**. 1979. The mechanisms of pathogenicity in the tick paralyses. J. Med. Entomol. **16**:357-369.

51. **Gray, S. M. and N. Banerjee**. 1999. Mechanisms of arthropod transmission of plant and animal viruses. Microbiol. Mol. Biol. Rev. **63**:128-148.

52. **Gupta, V. and V. Gautam**. 2004. Scrub Typhus--A short review. J. Commun. Dis. **36**:284-289.

53. **Heeb, H. L., M. J. Wilkerson, R. Chun, and R. R. Ganta**. 2003. Large granular lymphocytosis, lymphocyte subset inversion, thrombocytopenia, dysproteinemia, and positive *Ehrlichia* serology in a dog. J. Am. Anim Hosp. Assoc. **39**:379-384.

54. **Heinzen, R. A.** 2003. Rickettsial actin-based motility: behavior and involvement of cytoskeletal regulators. Ann. N. Y. Acad. Sci. **990**:535-547.

55. **Hill, C. A., F. C. Kafatos, S. K. Stansfield, and F. H. Collins**. 2005. Arthropod-borne diseases: vector control in the genomics era. Nat. Rev. Microbiol. **3**:262-268.

56. **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.

57. **Houhamdi, L., P. Parola, and D. Raoult**. 2005. [Lice and lice-borne diseases in humans]. Med. Trop. (Mars.) **65**:13-23.

58. ***i*jdo, J. W., C. Wu, L. A. Magnarelli, K. C. Stafford, *iii*, J. F. Anderson, and E. Fikrig**. 2000. Detection of *Ehrlichia* chaffeensis DNA in *Amblyomma americanum* ticks in Connecticut and Rhode *i*sland. J. Clin. Microbiol. **38**:4655-4656.

59. **Jongejan, F. and G. Uilenberg**. 2004. The global importance of ticks. Parasitology **129 Suppl**:S3-14.

60. **Kivaria, F. M.** 2006. Estimated direct economic costs associated with tick-borne diseases on cattle in Tanzania. Trop. Anim Health Prod. **38**:291-299.

61. **Kong, P., L. Rubio, M. Polek, and B. W. Falk**. 2000. Population structure and genetic diversity within California Citrus tristeza virus (CTV) isolates. Virus Genes **21**:139-145.

62. **Kramer, A., *i*. Schwebke, and G. Kampf**. 2006. How long do nosocomial pathogens persist on inanimate surfaces? A systematic review. BMC. *i*nfect. Dis. **6**:130.

63. **Kuttler, K. L.** 1988. World-wide impact of babesiosis, p. 1-22. *in* M. Ristic (ed.), Babesiosis of Domestic Animals and Man. CRC Press, Boca Raton, Florida.

64. **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 SC*i*D mice from fatal infection by the obligate intracellular bacterial pathogen *Ehrlichia chaffeensis.* J *i*mmunol. **166**:1855.

65. **Lin, M. and Y. Rikihisa**. 2003. *Ehrlichia* chaffeensis and *Anaplasma* phagocytophilum lack genes for lipid A biosynthesis and incorporate cholesterol for their survival. *i*nfect. *i*mmun. **71**:5324-5331.

66. **Lloyd, C. M. and A. R. Walker**. 1993. The effect of inflammatory and hypersensitive reactions, in response to the feeding of the tick *Amblyomma* variegatum, on the progression of experimental dermatophilosis infections. Exp. Appl. Acarol. **17**:345-356.

68. **Loftis, A. D., W. K. Reeves, J. P. Spurlock, S. M. Mahan, D. R. Troughton, G. A. Dasch, and M. L. Levin**. 2006. *I*nfection of a goat with a tick-transmitted *Ehrlichia* from Georgia, U.S.A., that is closely related to *Ehrlichia* ruminantium. J. Vector. Ecol. **31**:213-223.

69. **Long, S. W., T. J. Whitworth, D. H. Walker, and X. J. Yu**. 2005. Overcoming barriers to the transformation of the genus *Ehrlichia*. Ann. N. Y. Acad. Sci. **1063:403-10.**:403-410.

70. **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.

71. **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.

72. **Maniatis, T., E. F. Fritsch, and J. Sambrook**. 1982. Molecular Cloning: A Laboratory Manual. Cold Spring Harbor Laboratory, Cold Spring Harbor, NY.

73. **McCosker, D. J.** 1981. The global importance of babesiosis, *in* M. Ristic and J. P. Kreier (ed.), Babesiosis. Academic Press, New York.

74. **McDade, J. E. and V. F. Newhouse**. 1986. Natural history of Rickettsia rickettsii. Annu. Rev. Microbiol. **40:287-309.**:287-309.

75. **McLean, R. G., S. R. Ubico, D. Bourne, and N. Komar**. 2002. West Nile virus in livestock and wildlife. Curr. Top. Microbiol. *I*mmunol. **267**:271-308.

76. **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.

77. **Moody, E. K., R. W. Barker, J. L. White, and J. M. Crutcher**. 1998. Ticks and tick-borne diseases in Oklahoma. J. Okla. State. Med. Assoc. **91**:438-445.

78. **Munderloh, U. G., E. F. Blouin, K. M. Kocan, N. L. Ge, W. L. Edwards, and T. J. Kurtti**. 1996. Establishment of the tick (Acari:*I*xodidae)-borne cattle pathogen *Anaplasma* marginale (Rickettsiales:*Anaplasma*taceae) in tick cell culture. J. Med. Entomol. **33**:656-664.

79. **Munderloh, U. G., 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. *I*nvasion and intracellular development of the human granulocytic ehrlichiosis agent in tick cell culture. J. Clin. Microbiol. **37**:2518-2524.

80. **Munderloh, U. G. and T. J. Kurtti**. 1989. Formulation of medium for tick cell culture. Exp. Appl. Acarol. **7**:219-229.

81. **Murray, E. S. and S. B. Torrey**. 1975. Virulence of Rickettsia prowazeki for head lice. Ann. N. Y. Acad. Sci. **266**:25-34.

82. **Murray, S. and E. Weir**. 2005. West Nile virus. CMAJ. **173**:484.

83. **Obonyo, M., U. G. Munderloh, V. Fingerle, B. Wilske, and T. J. Kurtti**. 1999. Borrelia burgdorferi in tick cell culture modulates expression of outer surface proteins A and C in response to temperature. J. Clin. Microbiol. **37**:2137-2141.

84. **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*. *i*nfect. *i*mmun. **69**:2083-2091.

85. **Ohasi, N., N. Zhi, Y. Zhang, and Y. Rikihisa**. 1998. *i*mmunodominant major outer membrane proteins of *Ehrlichia chaffeensis* are encoded by a polymorphic multigene family. *i*nfect. *i*mmun. **66**:132-139.

86. **Olano, J. P., G. Wen, H. M. Feng, J. W. McBride, and D. H. Walker**. 2004. Histologic, serologic, and molecular analysis of persistent ehrlichiosis in a murine model. Am. J. Pathol. **165**:997-1006.

87. **Paddock, C. D. and J. E. Childs**. *Ehrlichia* chaffeensis: a prototypical emerging pathogen. Clin. Microbiol. Rev. 2003. Jan. ;16. (1.):37. -64. **16**:37-64.

88. **Paddock, C. D., S. M. Folk, G. M. Shore, L. J. Machado, M. M. Huycke, L. N. Slater, A. M. Liddell, R. S. Buller, G. A. Storch, T. P. Monson, D. Rimland, J. W. Sumner, J. Singleton, K. C. Bloch, Y. W. Tang, S. M. Standaert, and J. E. Childs**. 2001. *i*nfections with *Ehrlichia* chaffeensis and *Ehrlichia* ewingii in persons coinfected with human immunodeficiency virus. Clin. *i*nfect. Dis. **33**:1586-1594.

89. **Pal, U., A. M. de Silva, R. R. Montgomery, D. Fish, J. Anguita, J. F. Anderson, Y. Lobet, and E. Fikrig**. 2000. Attachment of Borrelia burgdorferi within *i*xodes scapularis mediated by outer surface protein A. J. Clin. *i*nvest **106**:561-569.

90. **Pal, U., X. Yang, M. Chen, L. K. Bockenstedt, J. F. Anderson, R. A. Flavell, M. V. Norgard, and E. Fikrig**. 2004. OspC facilitates Borrelia burgdorferi invasion of *i*xodes scapularis salivary glands. J. Clin. *i*nvest **113**:220-230.

91. **Parola, P., C. D. Paddock, and D. Raoult**. 2005. Tick-borne rickettsioses around the world: emerging diseases challenging old concepts. Clin. Microbiol. Rev. **18**:719-756.

92. **Parola, P. and D. Raoult**. 2001. Ticks and tickborne bacterial diseases in humans: an emerging infectious threat. Clin. *i*nfect. Dis. **32**:897-928.

93. **Peter, T. F., A. F. Barbet, A. R. Alleman, B. H. Simbi, M. J. Burridge, and S. M. Mahan**. 2000. Detection of the agent of heartwater, Cowdria ruminantium, in *Amblyomma* ticks by PCR: validation and application of the assay to field ticks. J. Clin. Microbiol. **38**:1539-1544.

94. **Qin, A., A. M. Tucker, A. Hines, and D. O. Wood**. 2004. Transposon mutagenesis of the obligate intracellular pathogen Rickettsia prowazekii. Appl. Environ. Microbiol. **70**:2816-2822.

95. **Rachek, L. *i*., A. Hines, A. M. Tucker, H. H. Winkler, and D. O. Wood**. 2000. Transformation of Rickettsia prowazekii to erythromycin resistance encoded by the Escherichia coli ereB gene. J. Bacteriol. **182**:3289-3291.

97. **Rachek, L. *i*., A. M. Tucker, H. H. Winkler, and D. O. Wood**. 1998. Transformation of Rickettsia prowazekii to rifampin resistance. J. Bacteriol. **180**:2118-2124.

98. **Radley, D. E., C. G. Brown, M. J. Burridge, M. P. Cunningham, M. A. Peirce, and R. E. Purnell**. 1974. East Coast fever: quantitative studies of Theileria parva in cattle. Exp. Parasitol. **36**:278-287.

99. **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.

100. **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 *Ehrlichia*e. Biochem. Biophys. Res. Commun. **247**:636-643.

101. **Renesto, P., P. Dehoux, E. Gouin, L. Touqui, P. Cossart, and D. Raoult**. 2003. *i*dentification and characterization of a phospholipase D-superfamily gene in rickettsiae. J. *i*nfect. Dis. **188**:1276-1283.

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

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

105. **Ristic, M. and D. L. Huxsoll**. 1984. Tribe *ii*. *Ehrlichieae* Philip 1957, 948[AL], p. 704-711. *in* N. R. Krieg and J. G. Holt (ed.), Bergey's Manual of Systematic Bacteriology, Vol.1. Williams and Wilkins, Baltimore.

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

107. **Sampson, B. A., C. Ambrosi, A. Charlot, K. Reiber, J. F. Veress, and V. Armbrustmacher**. 2000. The pathology of human West Nile Virus infection. Hum. Pathol. **31**:527-531.

108. **Sattenspiel, L.** 2000. Tropical environments, human activities, and the transmission of infectious diseases. Am. J. Phys. Anthropol. **Suppl 31**:3-31.

109. **Schwan, T. G. and J. Piesman**. 2000. Temporal changes in outer surface proteins A and C of the lyme disease-associated spirochete, Borrelia burgdorferi, during the chain of infection in ticks and mice. J. Clin. Microbiol. **38**:382-388.

110. **Scott, T. W. and S. C. Weaver**. 1989. Eastern equine encephalomyelitis virus: epidemiology and evolution of mosquito transmission. Adv. Virus Res **37**:277-328.

111. **Seo, G. M. G. R. R.** 2007. Proteome analysis of *E. chaffeensis* in different host cell backgrounds, *in* .

112. **Shibata, S., M. Kawahara, Y. Rikihisa, H. Fujita, Y. Watanabe, C. Suto, and T. Ito**. 2000. New *Ehrlichia* species closely related to *Ehrlichia* chaffeensis isolated from Ixodes ovatus ticks in Japan. J Clin. Microbiol. **38**:1331-1338.

113. **Singh, R. K., H. P. Pandey, and S. Sundar**. 2006. Visceral leishmaniasis (kala-azar): challenges ahead. Indian J. Med. Res **123**:331-344.

114. **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.

115. **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. 2006 Sep;8(9):1475-87 .

116. **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.

117. **Sirigireddy, K. R., G. A. Kennedy, A. Broce, L. Zurek, and R. R. Ganta**. 2006. High prevalence of West Nile virus: a continuing risk in acquiring infection from a mosquito bite. Vector. Borne. Zoonotic. Dis. **6**:351-360.

118. **Solomon, T. and M. Mallewa**. 2001. Dengue and other emerging flaviviruses. J. Infect. **42**:104-115.

119. **Sonenshine, D. E.** 1991. Biology of Ticks, *in* . Oxford University Press.

120. **Stanislawska, J. and W. L. Olszewski**. 2005. RNA interference--significance and applications. Arch. Immunol. Ther. Exp. (Warsz.) **53**:39-46.

121. **Stephen K.Chapes and Roman R.Ganta**. 2007. Defining the Immune Response to *Ehrlichia species* Using Murine Models. Vet. Parasitol., in press.

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

123. **Stuen, S., E. O. Engvall, and K. Artursson**. 1998. Persistence of *Ehrlichia* phagocytophila infection in lambs in relation to clinical parameters and antibody responses [In Process Citation]. Vet. Rec. **143**:553-555.

124. **Taege, A. J.** 2000. Tick trouble: overview of tick-borne diseases. Cleve. Clin. J. Med. **67**:241, 245-241, 249.

125. **Tamura, A., N. Ohashi, H. Urakami, and S. Miyamura**. 1995. Classification of Rickettsia tsutsugamushi in a new genus, Orientia gen. nov., as Orientia tsutsugamushi comb. nov. *I*nt. J. Syst. Bacteriol. **45**:589-591.

126. **Telford, S. R. and J. E. Dawson**. 1996. Persistent infection of C3H/HeJ mice by *Ehrlichia chaffeensis.* Vet. Microbiol. **52**:103-112.

127. **Telford, S. R., *iii* and H. K. Goethert**. 2004. Emerging tick-borne infections: rediscovered and better characterized, or truly 'new' ? Parasitology **129 Suppl**:S301-S327.

128. **Terkeltaub, R. A.** 2000. Lyme disease 2000. Emerging zoonoses complicate patient work-up and treatment. Geriatrics. **55**:34-40, 43.

129. **Tijsterman, M. and R. H. Plasterk**. 2004. Dicers at R*i*SC; the mechanism of RNAi. Cell **117**:1-3.

130. **Titball, R. W., J. Hill, D. G. Lawton, and K. A. Brown**. 2003. Yersinia pestis and plague. Biochem. Soc. Trans. **31**:104-107.

131. **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. *i*nfect. *i*mmun. **70**:4701-4704.

132. **Van, S. R. and J. Evans**. 2001. Lyme disease. Curr. Opin. Rheumatol. **13**:293-299.

133. **Walker, A. R. and C. M. Lloyd**. 1993. Experiments on the relationship between feeding of the tick *Amblyomma* variegatum (Acari: *i*xodidae) and dermatophilosis skin disease in sheep. J. Med. Entomol. **30**:136-143.

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

135. **Walker, D. H.** 1998. Tick-transmitted infectious diseases in the United States. Annu. Rev. Public Health **19**:237-269.

136. **Walker, D. H. and J. S. Dumler**. 1996. Emergence of the ehrlichiosis as human health problems. Emerg. *i*nfect. Dis. **2**:18-29.

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

138. **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: *i*xodidae) from the Georgia coast and Barrier *i*slands. J. Med. Entomol. **37**:276-280.

139. **Whitworth, T., V. L. Popov, X. J. Yu, D. H. Walker, and D. H. Bouyer**. 2005. Expression of the Rickettsia prowazekii pld or tlyC gene in Salmonella enterica serovar Typhimurium mediates phagosomal escape. *i*nfect. *i*mmun. **73**:6668-6673.

140. **Winslow, G. M., C. Bitsaktsis, and E. Yager**. 2005. Susceptibility and resistance to monocytic ehrlichiosis in the mouse. Ann. N. Y. Acad. Sci. **1063:395-402.**:395-402.

141. **Winslow, G. M., E. Yager, K. Shilo, D. N. Collins, and F. K. Chu**. 1998. *I*nfection of the Laboratory Mouse with the *I*ntracellular Pathogen *Ehrlichia chaffeensis.* *I*nfect. *I*mmun. **66**:3892-3899.

142. **Womble, D. D.** 2000. GCG: The Wisconsin Package of sequence analysis programs. Methods Mol. Biol. **132:3-22.**:3-22.

143. **Wood, D. O. and A. F. Azad**. 2000. Genetic manipulation of rickettsiae: a preview. *I*nfect. *I*mmun. **68**:6091-6093.

144. **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.

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

146. **Yager, E., C. Bitsaktsis, B. Nandi, J. W. McBride, and G. Winslow**. 2005. Essential role for humoral immunity during *Ehrlichia* infection in immunocompetent mice. *I*nfect. *I*mmun. **73**:8009-8016.

147. **Yu, X., J. W. McBride, X. Zhang, and D. H. Walker**. 2000. Characterization of the complete transcriptionally active *Ehrlichia chaffeensis* 28 kDa outer membrane protein multigene family. Gene **248**:59-68.

148. **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.

149. **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.

150. **Yunker, C. E.** 1996. Heartwater in sheep and goats: a review. Onderstepoort J. Vet. Res **63**:159-170.

151. **Zaugg, J. L., D. Stiller, M. E. Croan, and S. D. Lincoln**. 1986. Transmission of *Anaplasma marginale* Theiler by males of *Dermacentor andersoni* Stiles fed on an *I*daho field infected, chronic carrier cow. Am. J. Vet. Res. **47**:2269-2271.

152. **Zhang, J. Z., J. W. McBride, and X. J. Yu**. 2003. L-selectin and E-selectin expressed on monocytes mediating *Ehrlichia* chaffeensis attachment onto host cells. FEMS Microbiol. Lett. **227**:303-309.

153. **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.

154. **Zhang, J. Z., M. Sinha, B. A. Luxon, and X. J. Yu**. 2004. Survival strategy of obligately intracellular *Ehrlichia* chaffeensis: novel modulation of immune response and host cell cycles. *i*nfect. *i*mmun. **72**:498-507.

155. **Zhang, X. F., J. Z. Zhang, S. W. Long, R. P. Ruble, and X. J. Yu**. 2003. Experimental *Ehrlichia* chaffeensis infection in beagles. J. Med. Microbiol. **52**:1021-1026.