Molecular characterization of five predicted DNA-binding proteins in *Ehrlichia chaffeensis*

by

Kimia Alizadeh

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Approved by:

Major Professor
Dr. Roman Ganta

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Abstract

Tick-borne diseases are considered as a major concern threatening the health of people and many other vertebrate hosts. Ticks can transmit a variety of pathogens including viruses, bacteria and parasites from one host to another while acquiring their blood meal. *Ehrlichia chaffeensis* is an important tick-borne pathogen responsible for the disease, monocytic ehrlichiosis. This Gram-negative intracellular pathogen is able to survive in a variety of host species including ticks and vertebrate hosts. Understanding how this pathogen is able to maintain its infectivity in multiple hosts is critical for development of methods of control. We have studied how *E. chaffeensis* overcomes the clearance by both tick and canine hosts. This research was conducted through multiple molecular manipulation experiments to further our understanding of bacterial gene regulation. We determined the transcription variations for five predicted DNA-binding proteins in *E. chaffeensis*: MerR, EcxR, CtrA, Hu and Tr1, while transitioning from reticulate cell (RC) form to dense core cell (DC) form and vice versa, and variations resulting from type of host cell to investigate how such differences impact gene regulation. We defined the expression of all five predicted transcription regulators at the RNA level and demonstrated unique RNA expression patterns in vertebrate and tick host cells and in the RC and DC forms. Additionally, we identified the protein expression for all five predicted transcription regulators for DC and RC forms of *E. chaffeensis*, purified from both host cell backgrounds using protein-specific antibody. This research will help advancing our knowledge on gene regulation in bacteria, providing means for more efficient prevention and control.

Table of Contents

List of Figures

List of Tables

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Dedication

I dedicate my work to my incredible parents who are the backbone of who I am today, and my amazing brother who has always encouraged me to work hard and pursue my dreams.

Chapter 1 - Review of Literature

Vector-borne diseases

Vector-borne diseases are among the major public health concerns worldwide, posing a huge burden of morbidity and mortality around the globe. In human populations, more than one billion cases have been reported annually, with around one million cases leading to death [1]. Vector-borne diseases also account for impactful economic losses concerning food animal and companion animal health across the globe. A biological vector is an organism that serves to acquire, harbor, support replication, and transmit the causative agent of a disease from one vertebrate to another. Many of these vectors are arthropods that feed on vertebrate hosts acquiring blood meals. They include, but not limited to, mosquitoes, fleas, sandflies and ticks. Vectors ingest the causative microorganism during a blood meal, and once infected, they can transmit the pathogen during each subsequent blood feeding times [2]. Viruses, bacteria, and parasites are known to be transmitted from vectors. Vector-borne diseases include many widely known diseases, such as malaria, Dengue fever, human African trypanosomiasis, leishmaniasis, yellow fever, schistosomiasis, Japanese encephalitis, West Nile virus, Lyme disease, Ehrlichiosis, Anaplasmosis, and heart worm disease, in humans and veterinary species. A number of factors such as environmental, demographic and social factors affect the geographical distribution of vectors and vector-borne diseases. Mosquitoes are known as the most threatening vectors in public health, transmitting the causative agents of many deadly diseases. Malaria, which is the most important vector-borne disease, responsible for 228 million cases worldwide annually is transmitted by *Anopheline* mosquitoes. Other mosquito-borne diseases include Dengue fever, Chikungunya, Japanese encephalitis, Rift valley fever, West Nile fever, Zika fever etc. [3]. Different types of flies such as sandflies, blackflies and tsetse flies also contribute to

number of infectious diseases including leishmaniosis, onchocerciasis, and African trypanosomiasis, also known as sleeping sickness. Fleas and lice are two other important vectors, responsible for causing important diseases such as plague and typhus respectively [1]. Ticks continue to emerge as the major vectors in causing multiple diseases in people, livestock and companion animals [1].

Tick-borne diseases:

Ticks are considered to be the second most important vectors of human infectious diseases [4]. Ticks are blood-feeding arthropods that can act as vectors for transmitting causative agents of a variety of infectious diseases to animals and humans. Tick populations have been increasing over time and the geographical distribution of these arthropods is expanding due to climate changes and human activities. Ticks belong to the subphylum *Chelicerata*, the class *Arachnida*, the subclass *Acari*, the order *acarine* and the suborder *Ixodida* [5]. Ticks are generally classified into two types: soft ticks belong to *Argasidae* family, and hard ticks representing from *Ixodidae* family. The hard ticks possess a scutum on the dorsal surface of theirs bodies, which is a tough sclerotized plate, while soft ticks have a leathery integument, lacking a dorsal scutum [6]. Soft ticks are mostly parasites of avian species, and preferably inhabit covered areas including caves and nests of their hosts and animal housings [7]. Hard ticks generally inhabit tropical and sub-tropical areas and are able to parasitize a broader range of vertebrate hosts including humans [8].

There are four stages in the life cycle of hard ticks: egg, larvae, nymph, and adults (Figure 1.1). The life cycle begins with mating of female and male ticks after a blood meal. Subsequently, the female ticks lay many eggs. Tick eggs hatch into the six legged larval stage. The larvae typically feed on small vertebrates, such as rodents, and once their blood meal is

completed, they detach and molt into nymphs. The nymphal stage is characterized by the presence of eight legs. Nymphs typically remain unfed during winter and blood feeding may occur in the spring [9]. Once blood acquisition is complete for nymphs which takes place on any host including mammals, birds, and reptiles. Fed nymphs molt to adult stage by transforming into female and male ticks. Adult female and male ticks feed typically on larger hosts, most commonly of mammalian species where mating occurs following which a female takes considerably a large blood meal. Fully fed female produces a large mass of eggs (typically about 5000 or larger). Completion of all the life stages of ticks can last up to two to three years [6]. Pathogens are generally acquired while a tick is feeding on a host at any motile stage. Pathogens can be transferred to the next stage during molting (transstadially) or transovarially from infected eggs to larvae, although not all pathogens are transovarially transmitted. The newly infected ticks serve as a transmitting vectors [10]. In nature, the reservoir hosts are responsible for the continuous maintenance of a given pathogen. Incidental hosts harbor and provide sustenance for pathogens, but do not necessarily facilitate the spread of the pathogen to new uninfected hosts [6].

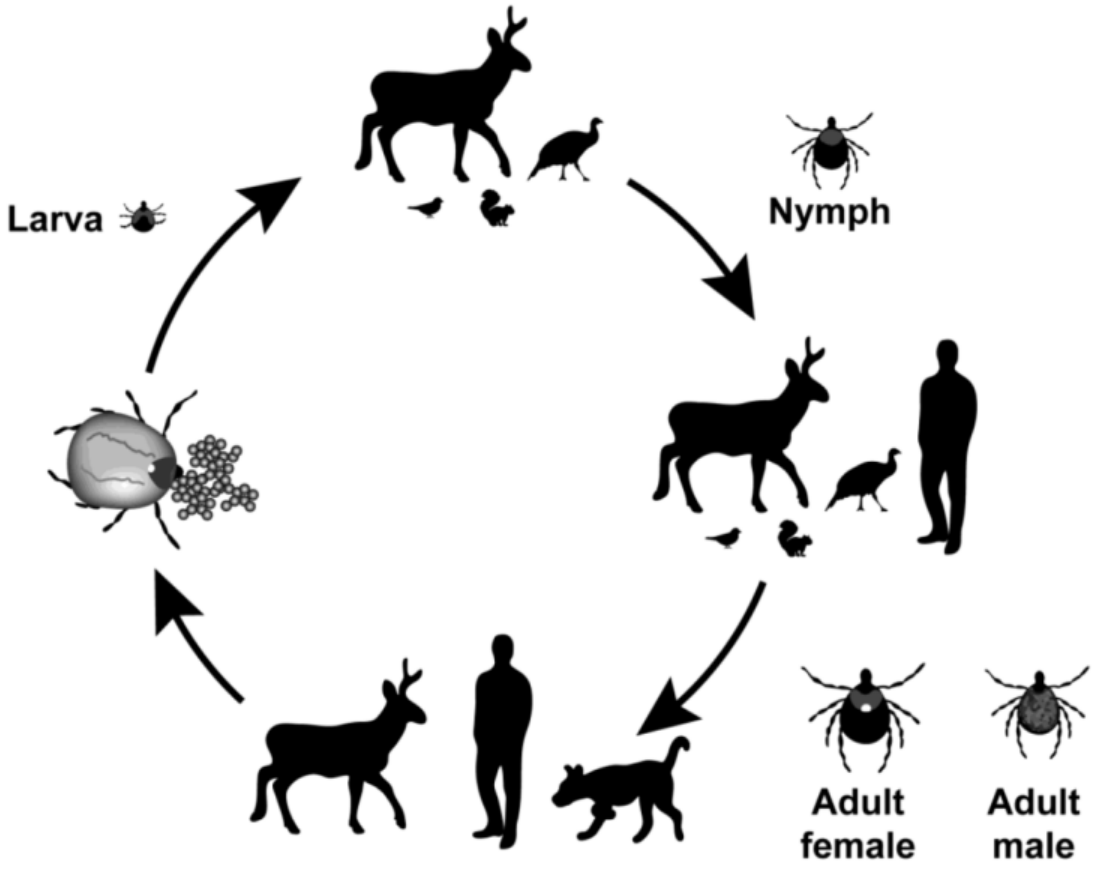

Figure 1.1: Life cycle of an *Amblyomma americanum* tick (Figure created by Dr. Roman Ganta)

Hard ticks transmit a variety of pathogens to humans and various vertebrate species resulting in causing many tick-borne diseases. Thus, they continue to impact the health of people, companion and livestock animals and also inflecting major economic losses throughout worldwide [6]. Until mid 1970s when Lyme disease is first identified, tick-borne diseases are not regarded as a major concern for the human health. Lyme disease is caused by *Borrelia burgdorferi* in the United States and by several related *Borrelia* species across the globe [11]. It is the most prevalent tick-borne disease in the United States. Rickettsial diseases caused by the

pathogens of genera *Ehrlichia, Anaplasma* and *Rickettsia* are emerging as the second most important tick-borne diseases.

Rickettsia rickettsii

Rickettsia rickettsii is a Gram-negative intracellular bacterium and the causative agent of Rocky Mountain Spotted Fever (RMSF) in people and dogs. RMSF is first identified over 100 years ago in Rocky Mountains, and is currently more prevalent in all parts of North, South and Central Americas. RMSF is a life-threatening disease characterized by a high fatal fever in humans. A case fatality rate of 69% was reported in a 1902 investigation [12]. The incubation period of RMSF typically ranges from 2 to 14 days following tick exposure. Diagnosis is generally based on the clinical examination of the patient and history of tick exposure. Diagnosis can be challenging due to the similarity of the symptoms observed in *R. rickettsii* infected patients with those observed because of several viral infections. Delay in the timely diagnosis and treatment can result in increasing the mortality rate in affected patients [13]. The RMSF symptoms include fever, lethargy, myalgia, headache and gastrointestinal problems, as well as skin rash occurring 2 to 3 days after the onset of clinical signs [14]. Pathogenesis of *R. rickettsii* involves introduction of the pathogen through the skin, followed by dissemination via lymphatics and blood vessels. This pathogen uses surface-exposed proteins and rickettsial phospholipase to attach to the vascular endothelium and smooth muscle cells and starts multiplying within these cells. As a result, generalized vascular injury and extravasation of fluid occur, which in turn can lead to edema, hypovolemia, hypotension, and hypoalbuminemia [12, 15].

Dermacentor tick species are initially identified as responsible for the spread of RMSF. *Dermacentor variabilis*, commonly known as the American dog tick is regarded as the RMSF

vector in western costal and eastern parts of the USA, while *Dermacentor andersoni*, also known as the Rocky Mountain wood tick, is more prevalent in Rocky Mountain region and Canada [13]. *Amblyomma americanum* (the lone star tick) , *Amblyomma cajennense* (the Cayenne tick) and *Rhipicephalus sanguineous* (the brown dog tick) are also identified as RMSF vectors in South America and Brazil [16, 17]. Doxycycline and chloramphenicol are the antibiotics used to treat RMSF and are generally efficient if administered early on during the course of disease [12]. Due to lack of adequate studies on *R. rickettsii*, there is currently no vaccine available against this pathogen. A recent study by our team investigated the efficacies of two experimental vaccines, a subunit vaccine containing two recombinant outer membrane proteins (RCA) and a whole-cell inactivated antigen vaccine (WCA). In this study, our team looked over the immunogenicity of these vaccines against virulent *R. rickettsii* in a recently established canine model for RMSF. This study demonstrated that WCA-vaccinated dogs are protected from virulent RMSF disease, with the clearance of the pathogen to nearly undetected levels in the blood, lungs, liver, spleen, and brain, while dogs receiving RCA develop the disease similar to the unvaccinated dogs challenged with *R. rickettsii* [18].

Anaplasma marginale

Anaplasma marginale is another rickettsial tick-borne pathogen and the causative agent of anaplasmosis in cattle [19]. Bovine anaplasmosis is distributed globally, although more prevalent in countries in tropical and subtropical climates, including South and Central Americas and nearly all parts of the United States, southern Europe, Asia, Africa and Australia (https://www.merckvetmanual.com/circulatory-system/blood-parasites/anaplasmosis-in-ruminants?autoredirectid=17931&query=bovine%20anaplasmosis)

Cattle and tick vectors also serve as reservoirs of infection facilitating the infection spread to susceptible hosts [20]. This pathogen mainly infects cattle, leading to a hemolytic disease, accompanied by wight loss, lowered milk production, abortion, and mortality, and therefore, is of a meaningful impact economically [21]. Sheep and wild ruminants including deer, antelopes, elk, water buffalo, bighorn and pronghorn can also be affected by this pathogen [22]. Erythrocyte is the only known site of replication for *A. marginale* in cattle where this pathogen forms membrane-bound inclusion bodies, each surrounding about 4 to 8 microorganisms. Acute infection with *A. marginale* can involve up to 70% or more of the red blood cells. Attempts of the bovine reticuloendothelial system to remove the infected cells eventually lead to different degrees of anemia and icterus [23]. *A. marginale* can be transmitted mechanically, besides the tick transmission. Susceptible cattle can be infected by fomites such as infected needles or tattooing and surgical instruments and biting flies. Transplacental transmission occurs across the placenta to the offspring [24]. Ticks are responsible for the biological transmission of *A. marginale*. Around 20 different species of ticks have been identified as vectors for *A. marginale* around the world. These include *Boophilus* spp., *Rhipicephalus* spp., *Ixodes Ricinus*, and several *Dermacentor* spp. such as *D. andersoni*, *D. variabilis*, *D. occidentalis* (Pacific Coast tick), and *D. albipictus* (Winder/Moose tick) [23]. Cattle are susceptible to *A. marginale* infection at all ages, but the severity of the clinical signs vary based on the age of an animal. Calves less than 6 months of age are generally less susceptible. Cattle may develop a mild disease if infected around 6 months to one year of age. Infection in cattle above one year old, the disease generally has the poorest prognosis and is often fatal. Regardless of the age, infected cattle maintain the pathogen and serve as a carrier for the rest of their lives [25]. Diagnosis is usually made by detection of the intraerythrocytic

inclusions of *A. marginale* in the blood smear prepared from samples of clinically infected animals during the acute phase of the infection. Blood smears should be stained using Giemsa or polychromatic stain [25]. However, this method does not provide a definitive diagnosis in the case of carrier animals or prior to the onset of clinical sings. Alternative diagnostic methods include molecular techniques such as PCR and real-time quantitative PCR, and serological techniques including enzyme linked immunosorbent assays (competitive ELISA), indirect fluorescent antibody testing and complement fixation assays [25]. Preventive procedures include treatment of herds in endemic areas, controlling vectors, and strict control on the imported animals from endemic areas. Preimmunizing cattle with *Anaplasma centrale* in several parts of the world aids in diminishing the severity of the disease resulting from the highly virulent *A. marginale* infection [26]. Parenteral therapy with oxytetracycline and chlortetracycline therapy as a food additive is approved in dairy and beef cattle, although animals continue to maintain infections similar to non-treated animals. Chlortetracycline can be used continuously as a treatment plan, while oxytetracycline should not be administered more than four days [27].

Anaplasma phagocytophilum

Anaplasma phagocytophilum is an obligate intracellular bacterium that primarily infects neutrophils [28]. This rickettsial tick-borne pathogen is responsible for causing human granulocytic anaplasmosis (HGA) which is the second most important tick-borne disease in the United States [28] and is typically transmitted by infected ticks from *Ixodes* species [29]. This pathogen is first identified in ovine leukocytes in 1910. Sheep, goats, cattle and deer are the recognized hosts for this pathogen in Europe [30]. Later on, equine and canine infections with *A. phaghocytophilum* are reported from parts of California [31, 32]. The first case of human

infection with *A. phagocytophilum* in the United States is reported in 1992 [33]. *A. phagocytophilum* is more prevalent in northern California, mid-Atlantic states, upper Midwest, New England, and many European countries [34, 35]. Different ticks from the *Ixodes* species transmit this pathogen globally. *I. scapularis*, *I. pacificus*, *I. ricinus*, and *I. persulcatus* are known to transmit *A. phagocytophilum* primarily in the eastern United States, western United States, Europe and parts of Asia respectively [36]. The white-footed mouse is recognized as the mammalian reservoir in the eastern United States, but other small mammals and white-tailed deer are also known to acquire *A. phagocytophilum* infections. The pathogen infections cause varying clinical signs in different hosts, which range from a transient bacteriemia with around 1-4 weeks of duration in white-footed mice to a persistent and subclinical infection in deer [37, 38]. Similarly, HGA can be clinically variable, but typically involves a moderately severe fever, myalgia, headache, and malaise. HGA is more fatal among elderly and immunocompromised individuals [39]. Gastrointestinal signs e.g., diarrhea, vomiting and nausea, respiratory signs such as cough, acute respiratory distress syndrome (ARDS) and pulmonary infiltrates, and central nervous system disorders are also seen in patients to a lesser extent. The common laboratory findings in HGA patients generally include leukopenia, anemia, thrombocytopenia, and increased hepatic transaminase concentrations [28]. Diagnosis is generally made based on detection of the microorganism in blood smears, serological testing, immunohistochemistry, PCR, and sometimes culture isolation [40]. The common antibiotic therapy against *A. phagocytophilum* is the doxycycline treatment, which should be administered as quickly as possible [41]. Vaccines against *A. phagocytophilum* are still being investigated [42].

Ehrlichia canis

Ehrlichia canis is a tick-borne pathogen responsible for canine monocytic ehrlichiosis (CME). Domestic dogs serve as the main host for this pathogen, but it can also cause infection in humans to a lesser degree [43-46]. *E. canis* is first described in 1935 in Algeria, but it is now prevalent throughout the world except in Australia and New Zealand [45]. *Rhipicephalus sanguineus* or commonly known as brown dog tick is the primary vector for this pathogen and is able to transmit this pathogen both transstadially and intrastadially [47]. Three phases are described throughout the course of infection with *E. canis* including acute, subclinical and chronic [47]. The acute stage usually consists of a mild illness that begins around 1-3 weeks following exposure to an infected tick [45]. The majority of the infected dogs in acute and subclinical phases are treatable with doxycycline or other tetracyclines when administered at the appropriate dose and frequency. Nevertheless, pathogen may not be completely cleared and can persist throughout the life of an infected dog [48, 49]. A grave prognosis is reported for dogs having the chronic phase of the disease [47]. Major symptoms reported in dogs with the infection include fever, lethargy, depression, anorexia, weight loss, generalized lymph adenomegaly, splenomegaly, spontaneous bleeding due to thrombocytopenia and vascular endothelial inflammation and ocular abnormalities such as anterior and posterior uveitis [47]. Moreover, oral lesions e.g., ulcerative stomatitis and necrotic glossitis, edema in hind limb and/or scrotal area, and central nervous system signs including ataxia, seizures, vestibular dysfunction and cervical pain have been commonly noticed in patients infected chronically [47]. Diagnosis is generally based on hematological and serological methods such as ELISA, PCR targeting 16S rRNA gene [50], but the most commonly used test and the gold standard for detection of *E. canis* is immunofluorescent assay [51]. Additionally, clinicians should always consider the possibility of

concurrent infection with other tick-borne infections in dogs when an abnormal or atypical representation of the disease is noted [47].

Ehrlichia ewingii

Ehrlichia ewingii is another tick-borne pathogen that is responsible for causing canine granulocytic ehrlichiosis (CGE) and human ewingii ehrlichiosis (HEE) [50]. This pathogen is first identified in 1971, and it used to be considered as different strain of *E. canis* strain associated with diarrhea and vomiting [50]. Later in 1992, it is confirmed as a new *Ehrlichia* species; *E. ewingii* [52].

The only confirmed vector for this pathogen is *Amblyomma americanum*, although the bacterial DNA's presence in *Rhipicephalus sanguineus* and *Dermacentor variabilis* is documented by PCR analysis later [53]. *E. ewingii* is primarily reported in the south-eastern or central regions of the United States where *A. Americanum* is widely distributed. Moreover, there are several reports indicating *E. ewingii* infection in Brazil and Africa [54, 55]. In dogs, *E. ewingii* generally develops an acute disease manifested by fever, anorexia, weight loss, lethargy, and lameness associated with neutrophilic arthritis and joint swelling. Gastrointestinal signs e.g., diarrhea and vomiting, and neurological signs including head tilt, tremor, and anisocoria are also reported [56]. In the patients coinfected with *E. canis*, CGE manifestations are usually more severe [57]. The first case on *E. ewingii* infection in people is reported in 1999 [58]. The clinical presentation of HEE resembles that of HME, and generally includes fever, headache and thrombocytopenia [58]. To date, no fatal cases have been reported resulting from *E. ewingii* infection in people [59, 60]. Peripheral blood smear stained with Giemsa, immunofluorescent assay, and PCR can be used to diagnose HEE. However, it is not possible to differentiate

between *E. ewingii* and *A. pahgocytophilum* by examining a blood smear because of the resemblance in morphological features of their morulae and their similar cell tropism to neutrophils. Additionally, antibodies against *E. ewingii* cross react with *E. chaffeensis* in serological assays which makes these tests less precise [61].

Ehrlichia chaffeensis

Ehrlichia chaffeensis is another Gram-negative obligate intracellular bacterium that is also transmitted by a hard tick [62]. *E. chaffeensis* has cell tropism primarily to monocytes and macrophages [10]. Within these cells, this pathogen forms a cytoplasmic vacuole originating from an early endosome that surrounds the microcolonies of bacteria called morulae [10]. Under light microscope, the morulae represent as dark blue mulberry-like inclusions when stained with Romanowsky (polychromatic) stains [65] (Figure 1.2). Morulae typically contain many morphologically uniform or highly variable organisms [66]. When examined through electron microscopy, the two separate morphologies of *E. chaffeensis* can be recognized. The first form is called dense-core cell (DC) which is the smaller infectious form, measured around 0.4 to 0.6 μm in diameter and characterized by a dense nucleoid [65]. DCs are able to enter the naïve host cells through phagocytosis and then transform into larger non-infectious and replicating form called reticulate cell (RC). RCs are pleomorphic in shape and measured around 0.4 - 0.6 μm by 0.7 - 1.9 μm in diameter [65]. RCs grow and replicate within the cytoplasmic phagosomes by binary fission and subsequently transform back into the DC form before being released from the infected cells [67, 68].

E. chaffeensis infection is first reported in 1986 in a human patient with a history of tick bites, and the infection is initially confused as an *E. canis* infection [65]. Further molecular

studies performed during the following year confirmed that it is a new species, namely *E. chaffeensis*. It is the causative agent of monocytic ehrlichiosis in people and several different vertebrate hosts, including dogs, goats, coyotes, and white tailed deer (a reservoir of the pathogen) [69] [71]. Infections with this pathogen in people cause human monocytic ehrlichiosis (HME) and it is considered as a major public health concern since 1990s [69]. HME is a well-known emerging disease throughout the United State, especially in the south-easters states and west-central Texas and north to Iowa, where the transmitting tick vector, *A. americanum* is most prevalent [70]. This pathogen is preserved in white-tailed deer which is known as the reservoir host [65]. Reported HME cases have continuously been increasing over the past years [65]. This pathogen infects people of all ages, but elderly and children are more susceptible. Immunocompromised individuals and patients receiving organ transplantation are also at a greater risk for developing HME [72, 73]. Common clinical manifestation in infected people includes fever, headache, myalgias, arthralgias, malaise and nausea, but lymphadenopathies, gastrointestinal signs e.g., diarrhea, and vomiting, and respiratory signs e.g., cough have been also reported [65]. Thrombocytopenia, anemia, and mild increase in hepatic transaminase levels are among the hematological abnormalities commonly observed in infected patients [74]. Severity of the clinical manifestations typically correlates with the degree of bacteriemia [61]. Several confirmatory testing methods exist for diagnosis of HME. The most common diagnostic test is serology performed to detect and quantify the anti-*Ehrlichia* antibodies. Other diagnostic methods include immunofluorescence assay (IFA), ELISA, Western blot, PCR, and direct microscopy of blood smear [50, 74]. Treatment of HME typically entails antibiotic therapy with tetracyclines. Doxycycline is often considered as the treatment of choice [75]. In the cases when doxycycline is contraindicated (as in children under 12 due to teeth discoloration, women who

are pregnant or breast-feeding, and people allergic to tetracycline antibiotics), rifampicin can be safely administered [76].

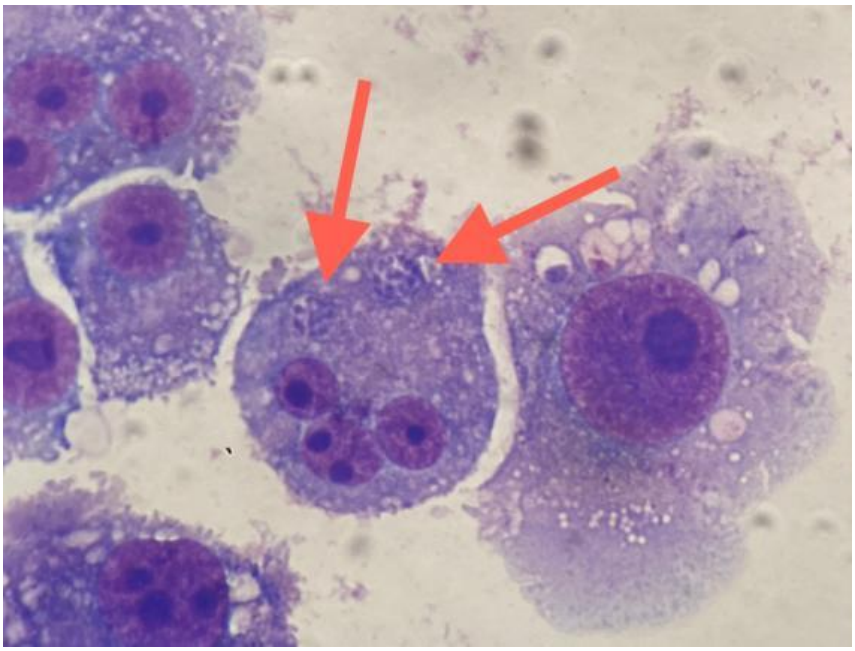

Figure 1.2: Cytospin preparation of cultured canine macrophages demonstrating variably sized basophilic inclusions (morulae) within the cytoplasm

Despite the recent discovery of several *Anaplasma, Ehrlichia* and various rickettsial pathogens since 1980s, and the continuous emergence of reported tick-borne rickettsial disease cases [12, 77], very little scientific knowledge exists in understanding how these pathogens cause disease in people and other vertebrate hosts and the strategies they employ to survive in tick hosts for completion of their life cycle. The fundamental knowledge can be better understood by

investigating how bacteria senses its host environment and subsequently alters its gene expression for further adaptation.

Bacterial Gene Regulation:

Gene regulation in bacteria is primarily the control over transcription of a gene and is initiated with an RNA polymerase core enzyme. Bacterial RNA polymerases holoenzymes containing a sigma subunit assure prompt and efficient transcription in bacteria facilitating adapting to changes in the environment promoting their better survival [78, 79]. An RNA polymerase core enzyme is a multi-subunit protein complex consisting of two alpha (α) subunits, a beta (β) subunit, a beta′ (β′) subunit and an omega (ω) subunit [80]. RNA polymerase is generally not specific in transcribing a gene unless a sigma subunit is bound to it. Once a sigma factor is bound to the core enzyme, the enzyme complex is referred to as a holoenzyme. Subsequently, the RNA polymerase holoenzyme binds to a specific gene promoter region located upstream to a gene's protein coding region [81]. The RNA polymerase binding to the promoter region is primarily facilitated by sigma factor. Thereafter, transcription of a DNA strand to produce a messenger RNA can only be initiated following the binding of a sigma factor. The mRNA, once produced, is used to generate a protein following going through the protein translation machinery, which is the final product of a gene [82]. Sigma factors paly a key role in the regulation of a gene expression in response to environmental alternations, and are therefore, critical for gene regulation. Each bacterium possesses different numbers of sigma factors. The quantity of sigma factors present in a bacterium primarily relies on its environmental heterogenicity [83]. For example, 7 sigma factors exist in *E. coli,* whereas 109 sigma factors are present in *Sorangium cellulosum* [84]. Obligate intracellular bacteria typically have less number of sigma factors

which is probably due to their smaller genome size resulting from the reductive genome evolution [85]. *E. chaffeensis*, as an example, possesses only two sigma factors. Sigma factor 70 (σ^{70}) which is a housekeeping factor that operates under normal conditions to activate transcription, and sigma factor 32 (σ^{32}) which is an alternative factor that is likely engaged in responding to the stressed host-cell environments. These two sigma factors in *E. chaffeensis* are encoded by *rpoD* (ECH_0760) and *rpoH* (ECH_0655) genes, respectively (GenBank # NC_007799.1) [86].

The biggest challenge in studying gene regulation in *E. chaffeensis* is the lack of a transformation system. Our research team has previously developed an *in vitro* transcription and a surrogate transcription system based on *E. coli* to study gene regulation in *E. chaffeensis* [87-89]. In the *E. coli* surrogate system, a specific *E. coli* strain is used in which its native σ^{32} or 70 are inactivated. Instead, *E. chaffeensis* σ factors are being expressed from a plasmid in the mutated *E. coli* to complement the *E. coli* RNAP function [89]. Our team also mapped the promoter regions of several genes, including DnaK (ECH-0471) gene (a stress response regulatory protein coding gene) and demonstrated that the chaperon protein encoded from the genes is primarily transcribed by σ^{32} [89]. Our research group also demonstrated that *E. chaffeensis* gene regulation is unique in having the ability to transcribe a gene from either one of the two sigma factors; σ^{32} and σ^{70}, although specificities of genes vary [89]. Genes that are regulated by σ^{32} are thought to be crucial for *E. chaffeensis* survival under the stressful host cell environment since they react to cellular responses at different stress conditions. This has been previously reported in other Gram-negative bacteria as well [90-94].

Further investigations should be made to better understand the gene regulation in *E. chaffeensis* and other intracellular pathogens. Acknowledging how gene expression is differently regulated in various host cells to facilitate a pathogen's adaptation to the host cell microenvironment is very challenging and complex. Our research team has performed investigations on the function of *E. chaffeensis* RNAP holoenzyme containing σ^{32} or σ^{70} to further understand how various genes are regulated in this pathogen. We utilized the previously developed *E. coli* surrogate system in the strain, CAG57101 to show recognition of the DnaK gene promoter mapping by *E. chaffeensis* RNAP containing σ^{32} [89]. We used the *E. coli* strain; CAG57101 where its native σ32 is inactivated. The native *rpoH* gene, normally coding for σ^{32} is inactivated in this *E. coli* strain and instead, an *E. chaffeensis* plasmid containing σ^{32} was expressed. This study is the first detailed description of gene mapping in pathogens belonging to the *Anaplasmataceae* family [71].

Chapter 2 - Scope of Thesis

Scope of the research:

Despite the substantial progress made by our research team, little is known about the role of transcriptional regulators which bind to the promoter regions of a gene and regulate gene expression. It has been previously reported by our team and other investigators that *E. chaffeensis* gene expression alters in a host cell-specific manner [95-98]. Additionally, previous studies from our team and others have shown that there is a remarkable difference between pathogen gene expression in tick and- macrophage- derived *E. chaffeensis* [95, 99-102]. It has also been reported that certain proteins are differentially expressed in dense core cell (DC) and reticulate cell (RC) forms of *E. chaffeensis* [103-105]. DC and RC are the two different forms maintained by *E. chaffeensis* to promote its infection and replication in both tick and vertebrate host cell intracellular life cycle [106]. This pathogen as the DC form enters a host cell through phagocytosis, and then transforms into the larger RC which replicates within a phagosome. RCs eventually transform into DC form prior to releasing from the infected cell [98]. Our knowledge of how the phenotypic transformation of *E. chaffeensis* occurs is very limited. Transcriptional regulators, also commonly referred as DNA-binding proteins (DBPs), play a major role in controlling the transcription of a gene through their interactions with the gene promoter to regulate transcriptional regulation of a gene [67, 107-109]. DBPs are also important for inciting morphological alterations and also in regulating the gene expression during host-cell induced environmental stresses.

Previous studies indicate that *E. chaffeensis* genome contains a limited number of predicted transcription regulators. The known transcription regulators include MerR

(ECH_0163), CtrA (ECH_1012), EcxR (ECH_0795), HU (ECH_0804), and Tr1 (ECH_1118)] [86]. In *E. chaffeensis*, CtrA, EcxR and Tr1 are described as active transcriptional regulators [110, 111].

ApxR and ErxR are two homologs of EcxR in *Anaplasma phagocytophilum* and *Ehrlichia ruminantium* respectively, which similarly play a role in transcriptional regulation [112, 113]. EcxR homologs have also been observed in *Anaplasma marginale* and *Ehrlichia canis* [111]. EcxR is a global DNA regulator, known to control type IV secretion system genes. EcxR has also been shown to autoregulate its gene expression [111]. In *A. phagocytophilum*, ApxR regulates the expression of Tr1 and the p44E genes [111]. P44E gene in turn activates the transcription of several 44-kDa immunodominant pleomorphic major surface proteins. EcxR in *E. chaffeensis* might similarly be regulating the expression of Tr1 and p28-Omp genes (homologs of p44 gene family in *Anaplasma* species) [111].

CtrA is identified in *Caulobacter crescentus* as a global regulator, controlling many cell cycle events such as cell division, methylation and initiation of DNA replication [114]. Since homologues of CtrA have been found in a number of α-proteobacteria, this DBP is considered as an evolutionary conserved protein [115]. Previous findings indicate that CtrA contributes to regulating the initiation of stress resistance during transitioning from the current to the next host cell through its regulon [108]. CtrA is also identified as a regulator in *E. chaffeensis* contributing to its transition to the infectious DC form [108]. In *E. chaffeensis*, CtrA is shown to bind to the promoter regions of various genes, however, the cell functions controlled by this gene regulator are yet to be further understood.

Tr1 is another transcriptional regulator that has been shown as the transcriptional regulator for the differential expression of p28-outer membrane proteins; omp-1B and p28 in *E.*

chaffeensis along with EcxR [110]. Tr1 harbors a winged helix-turn-helix and a DNA-binding motif and this protein is highly conserved in several *Ehrlichia* and *Anaplasma* species [116]. Further investigations on how Tr1 contributes to adaptation of *Ehrlichia* and *Anaplasma* species when encountering various host cell environments is valuable for furthering our understanding of its role for the bacterial gene regulation.

MerR family of transcriptional regulators are mercury resistance (mer) operons located on the transposable elements; Tn21 and Tn501 [107]. These DBPs are present in a variety of bacterial genera. All the regulators in this family share a similar sequence within their first 100 amino acids which contain a helix-turn-helix motif followed by a coiled-coil region [107]. Most of the MerR transcriptional regulators react to environmental alterations e.g., antibiotics, oxidative stress, and heavy metals. They exert their function by binding to the spacer region between the -35 and -10 promoter elements and up or down regulate the activity of *mer* genes [117]. In the presence of Hg(II) salts, MerR typically activates *mer* genes, while in the absence of Hg(II) these genes are usually repressed [118, 119]. Activation of regulation by MerR generally takes place through protein-dependent DNA distortion [107]. MerR is also known to autoregulate its own synthesis [120]. To date, there are no studies investigated the role of MerR in *E. chaffeensis*.

HU is a histone-like binding protein (HBP) and is likely another global regulator, as its homologs exhibit binding to multiple regions within the genome of a bacterium [121, 122]. Histones are highly basic proteins containing copious amounts of arginine and lysine residues. In the cells, histones act as spools that DNA strands can wrap around and form a structured component known as nucleosome [123]. HU is a small protein of about 10 kDa with varying

lengths spanning from 90 to 99 amino acids. In *E. coli*, HU contributes to DNA recombination and repair. The role of MerR in *E. chaffeensis* gene regulation is yet to be further understood.

Considering the limited knowledge, much remains to be understood regarding how *E. chaffeensis* transforms from DC to RC and vice versa. Understanding how DBPs are differentially expressed, how their transcription is altered when transitioning between the two morphological forms and in response to microenvironment of various host cells allow a more comprehensive perception of how genes are regulated in *E. chaffeensis*. This current research aims to investigate the existing knowledge gaps.

Chapter 3 - Materials and Methods

E. chaffeensis cultivation

Arkansas isolate of *E. chaffeensis* (ATCC # CRL-10389) was cultured in either canine macrophage cell line DH82 or embryonic tick cell line, ISE6 as previously described [111]. Five T150 cell culture flasks (Invitrogen Life Technologies, Carlsbad, CA) of DH82 or five T75 cell culture flasks of ISE6 (Invitrogen Life Technologies, Carlsbad, CA) were infected with wild-type *E. chaffeensis* and incubated until >90% of infectivity was observed and at which point, they were harvested and used for the subsequent experiments. Infected cell cultures were continuously monitored throughout the course of infection using Cytopro® 7620 Cytocentrifuge Rotor and stained using Hema 3 solutions [111].

Purification of RC and DC fractions of *E. chaffeensis*

Purification of RC and DC fractions from *E. chaffeensis*-infected DH82 and ISE6 cells was performed by subjecting the cell lysates to renografin density gradient and ultracentrifugation. Briefly, infected host cells were harvested at an infectivity rate of >90% and pelleted down at 18,000 x g for 15 minutes at 4°C. The supernatant was then discarded, and the cell pellet was resuspended in 10 mL of 1x DPBS (Dulbecco's phosphate buffered saline). The cells were homogenized using Fisher Scientific 60 Sonic Cell Dismembrator (Fisher Scientific, Hampton, NH) with 2 pulses, each for 30 seconds at a power setting of 6.5 on ice. Post sonication, the disrupted cell suspension was spun down at 700 x for 10 minutes 4°C to remove the cell debris and remaining cell-free bacteria containing supernatant was collected. The supernatant was then filtered using a syringe and a 2 μm filter to separate cell-free bacteria. Subsequently, the filtrate was centrifuged at 18,000 x g for 15 minutes at 4°C to pellet down the

cell-free bacteria which include both RC and DC forms. The pellet was resuspended in 3 mL of fresh 1x DPBS and used for density gradient purification.

For renografin density gradient method, different concentrations of renografin (25%, 35% and 45%) were prepared using 1x DPBS, then 2 mL each was carefully overlayed in an ultra-centrifuge tube with the highest concentration at the bottom and lowest at the top. The cell free bacterial suspension was then overlayed on top of the 25% renografin carefully. The tubes containing renografin and cell suspension layers were placed in the ultracentrifuge buckets (S50-ST swinging bucket rotor) and centrifuged at 100,000 x g for 1 hour at 4°C. After the centrifugation, the tubes were carefully removed and the presence of the three turbid layers consisting of the cell junk layer, RC layer (located on the top of DC layer, but below the cell debris) and DC layer (located below RC fraction). Each layer was carefully collected in a fresh ultracentrifuge tube and washed with 5 mL of 1x DPBS 3 times. Suspensions were centrifuged each time at 100,000 x g for 15 minutes at 4°C during the wash. After the third wash, the supernatant was discarded and the final pellets consisting of purified DCs or RCs were either resuspended in 1 mL TRIzol™ reagent or 300 µL of NP40 (50 mM Tris-HCl pH 8.0, 150 mM NaCl, 1% NP-40) containing 1% protease inhibitor. Samples resuspended in the TRIzol™ reagent and NP40 buffer were stored in -80°C and -20°C for RNA or protein purification respectively.

RNA isolation and purification

Purified *E. chaffeensis* DC and RC pellets from infected DH82 or ISE6 cells that were previously resuspended in 1 mL TRIzol™ reagent were used for RNA isolation. The frozen samples stored at -80° were first thawed and allowed to stand for 5 minutes at room temperature.

Subsequently, 200 µL chloroform was added to each tube, followed by vigorous agitation for 15 seconds. The samples were then allowed to sand for 15 minutes at room temperature. To allow phase separation, samples were centrifuged at 12,000 x g for 15 minutes using the Avanti J-26 XPI high speed centrifuge (Beckman Coulter Life Sciences, Indianapolis, IN) at 4°C. The colorless aqueous phase layered on top of the samples was collected and transferred to a fresh tube and 500 µL of isopropanol was added. The samples were mixed by pipetting and then allowed to stand for 10 minutes at room temperature. Subsequently, the samples were centrifuged at 12,000 x g for 10 minutes at 4°C. After centrifugation, the supernatant was discarded, and the RNA pellets were mixed with 1 mL of 75% ethanol and vortexed for 15 seconds. The samples were then centrifuged at 7,500 x g for 5 minutes at 4°C and the supernatant was discarded. The RNA pellets were allowed to air dry for 5-10 minutes, then resuspended in 50 µL of nuclease-free water and stored at -80°C until needed.

Isolated RNA samples were further purified using the Monarch® RNA Cleanup Kit (New England Biolabs, Ipswich, MA) as per manufacturer specifications. In brief, frozen RNA samples were allowed to thaw, then 100 µL of RNA Cleanup Binding Buffer was added to each sample. The samples were then resuspended with 150 µL of 100% ethanol and loaded onto a column containing a collection tube. The samples were subsequently centrifuged at 16,000 x g for 1 minute at room temperature. The flow-through from each tube was discarded and 500 µL of RNA Cleanup wash buffer was added to wash the samples. The samples were then centrifuged at 16,000 x g for 1 minute at room temperature and washed a second time, followed by another centrifugation at the same setting to remove the excess traces of ethanol and salt. The columns were transferred to a fresh tube and the purified RNA samples were eluted using 20 µL of nuclease-free water and stored at -80°C until needed.

DNase treatment of purified RNA samples

Concentrations of the purified RNA samples were measured using a Nanodrop 8000 (NanoDrop Technologies, Inc., Wilmington, DE). RNA samples were then treated with TURBO DNA-freeTM Kit (Invitrogen Life Technologies, Carlsbad, CA) to remove the residual DNA. DNase treatment of RNA samples was performed using 50 µL reaction mixture consisting of 1 µL of TURBO DNase™ enzyme, 10X TURBO DNase™ buffer, and nuclease-free water. The mixture was then incubated at 37°C for one hour. After incubation, the samples were resuspended using 5 µL of DNase inactivation reagent followed by another incubation at room temperature for 5 minutes, along with gentle mixing for 2-3 times during the incubation. Samples were subsequently centrifuged at 10,000 x g for 2 minutes at 4°C and the supernatant was collected in a fresh tube and stored at -80°C until needed for the following experiments.

PCR to check DNA contamination in the purified RNA samples from RC and DC fractions

The RNAs purified from *E. chaffeensis* RC and DC forms recovered from DH82 and ISE6 cells were assessed by DNA polymerase chain reaction (PCR) to rule out the presence of any DNA contamination. Primers used for the PCR experiments are listed in Table 1. The PCR analysis was performed in a 25 µL reaction mixture using Invitrogen™ Platinum™ Taq DNA Polymerase (Invitrogen Life Technologies, Carlsbad, CA) shown in Table 2. The reaction was carried out under the following reaction conditions: initial denaturation at 94°C for 20 minutes, followed by 40 cycles of 94°C for 30 seconds, 51°C for 30 seconds, 72°C for 1 minutes and a

final extension at 72°C for 5 minutes and kept on hold at 12°C until the PCR samples were recovered.

Table 1. Primers used in PCR to check for DNA contamination in the purified RNA samples

Primers	Sequence (5' to 3')	Orientation
RG1472	CCT GGA TGT TGA ACA ATA TGT AC	Forward
RG1470	CCC TTA AGC TTA ACA TAT CAT CTA G	Reverse

Table 2. PCR Reaction Mixture

Reagents	Volume (µL)
10X PCR Reaction Buffer (-MgCl2)	2.5
10 mM dNTPs	0.5
50 mM MgCl2 DNA free	0.75
10 µM Forward Primer	0.5
10 µM Reverse Primer	0.5
Platinum™ Taq DNA Polymerase	0.75
Template	1
Nuclease-free water	19

qRT-PCR for determination of relative gene expression

Purified RNA samples from *E. chaffeensis* RC and DC isolates of both DH82 and ISE6 cells were assessed by one-step quantitative reverse transcriptase PCR (qRT-PCR) to detect the relative gene expression for five genes, including ecxR (ECH_0795), tr1 (ECH_1118), ctrA (ECH_1012), merR (ECH_0163) and hup (ECH_0804). For this purpose, the SuperScript™ III Platinum™ One-Step qRT-PCR Kit (Invitrogen Life Technologies, Carlsbad, CA) was used according to the previously described method [50]. Primers and probes utilized in these experiments are listed in Tables 3 and 4. The qRT-PCR analysis was carried out in a 20 μL reaction mixture using the SuperScript™ III RT/Platinum™ Taq Mix (Invitrogen Life Technologies, Carlsbad, CA). The following temperature cycles were used to conduct the qRT-PCR analysis: reverse transcription at 50°C for 30 minutes, 95°C for 3 minutes, followed by 40 amplification cycles of 95°C for 15 seconds, 50°C for 30 seconds and 60°C for 1 minute. The reaction mixture is further elaborated in Table 5.

Table 3. Primers used in qRT-PCR for detecting the expression of the five gene regulators in *E. chaffeensis*

Primers	Sequence (5' to 3')	Orientation	Target Gene
RG2162	GCA CTA GAA ACT TCT CAT CTA A	Forward	*hup*
RG2163	GAG CTA GTG TTT CTA ACT TCC	Reverse	*hup*

RG2165	GTG ATG ATA GAG GAG GAT ATA GA	Forward	*ctrA*
RG2166	TG CTT CCT CAA CAT ACT TT	Reverse	*ctrA*
RG2168	GGA ATC ACT TTC CAA CAA GTA	Forward	*tr1*
RG2169	AAC GTT AAG TAC GCT TGC	Reverse	*tr1*
RG2171	AAT GAT TAC GGC ACT AAG TAT AA	Forward	*ecxR*
RG2172	GGT CTA CGC CCA GTA TC	Reverse	*ecxR*
RG2174	TTT CCT CAG GTT AAT CCA AT	Forward	*merR*
RG2175	TGC TGC ACA CCT TTA ATC	Reverse	*merR*
RG2180	CAA GTC GAA CGG ACA AT	Forward	*16s*
RG2182	T TCT AAT GGC TAT TCC ATA CTA C	Reverse	*16s*

Table 4. Probes used in qRT-PCR for detecting the expression of the five gene regulators in *E. chaffeensis*

Probes	**Sequence (5'- 3')**	**Target Gene**
RG2164	/56-FAM/TG GAA CAC T/ZEN/A CAT GTT ATC CAA TGT GCA GA/3IABkFQ/	*hup*

RG2167	/56-FAM/CA ACA TTG G/ZEN/C ACC ACC ATG ATC CC/3IABkFQ/	*ctrA*
RG2170	/56-FAM/AG CCT GCT A/ZEN/A TCA CTA TAC GGT TTG TTC C/3IABkFQ/	*tr1*
RG2173	/56-FAM/TC ACT GGA A/ZEN/C CAA GTA ACC ACA GCA /3IABkFQ/	*ecxR*
RG2176	/56-FAM/AC GTC GTG G/ZEN/T AGA AGA TTG TAT TCA CAA GT/3IABkFQ/	*merR*
RG2182	/56-FAM/CC CGT CTG C/ZEN/C ACT AAC AAT TAT TTA TAA CC/3IABkFQ/	*16s*

Table 5. One-Step qRT-PCR Reaction Mixture

Reagents	**Volume (µL)**
SuperScriptTM III RT/PlatinumTM *Taq* Mix	0.4
2X Reaction Mix	10
Forward Primer, 10 µM	0.4
Reverse Primer, 10 µM	0.4
Fluorogenic Probe, 10 µM	0.2
Template	4
Nuclease-free water	4.6

Preparation of purified RC and DC lysates using dry ice-ethanol bath

The purified *E. chaffeensis* RC and DC pellets resuspended in 300 µL of ST3D buffer were homogenized using dry ice-ethanol bath, as previously described with minor modifications [124]. In brief, frozen samples were allowed to thaw at room temperature, then placed in the dry

ice-ethanol bath for 3 minuets. The samples were then allowed to thaw thoroughly at room temperature. Lysozyme was subsequently added to each sample to reach a final concentration of 0.2 mg/mL. The samples were placed on a rotator for 10 minutes at room temperature for lysozyme to exert its lysis function. Subsequently, the samples were placed in the dry ice-ethanol bath for 3 minutes and then thawed at room temperature. The freeze-thaw cycle was repeated 4 times, while the samples were gently mixed before placing back in the dry ice-ethanol bath. The samples were finally centrifuged at 18,000 x g for 20 minutes at 4°C and the supernatant was collected in a fresh tube and stored at -20°C until needed for SDS-PAGE and Western blot experiments.

Purification of the 5 predicted DNA-binding proteins

E. coli system was used to express the five predicted *E. chaffeensis* DBPs as described previously by our research team [88]. In brief, overnight *E. coli* cultures of the strain BL21(DE3) (Novagen, San Diego, CA) containing the specific DNA binding protein gene-containing recombinant plasmid of pET32a (Novagen) were diluted 1:100 into a fresh Luria-Bertani (LB) medium containing 100 μg/mL ampicillin to support the strain's growth and grown to an optical density of 0.4-0.5. The cultures were then induced with 1 mM IPTG and incubated overnight at 30°C. Subsequently, bacterial cells were harvested by centrifugation at 5,000 x g for 15 minutes at 4°C. The supernatant was discarded and the pellets were stored in -20°C until further needed for protein purification [125].

HiTrap™ TALON® crude, 5 mL TALON Superflow™ cloumns were used to purify the recombinant proteins produced in *E. coli* as per the manufacturer's protocol. In brief, each gram of the *E. coli* pellet was resuspended in 5 to 10 mL of binding buffer. Then, enzymatic lysis was

conducted by addition of 0.2 mg/mL lysozyme, 20 µg/mL DNase, 1 mM pefabloc SC (MilliporeSigma, Burlington, MS, 1 mM MgCl2, and protease inhibitor (Thermo Fisher Scientific, Waltham, MA) to the binding buffer. Tubes containing the samples were placed on the rotator and stirred for 30 minutes at room temperature. Subsequently, the samples were homogenized using Fisher Scientific 60 Sonic Cell Dismembrator (Fisher Scientific, Hampton, NH) with 10 pulses, each for 30 seconds (total duration of 10 minutes) at a power setting of 10-12 on ice. The samples were then centrifuged at 18,000 x g for 15 minutes at 4°C and the supernatant was collected. In order to purify the protein, the columns were first washed out 3-5 times with distilled water an equilibrated with 5 column volumes of binding buffer, with a flow rate of 5 mL/min. Next, the supernatant was applied to the column using a syringe at a similar flow rate. The columns were subsequently washed 4 times using wash buffer consisting of 50 mM sodium phosphate, 300 mM NaCl, 5 mM imidazole at a pH of 7.4 and the flow through was collected in a fresh tube. Finally, the protein was eluted using 10 mL elution buffer consisting of 50 mM sodium phosphate, 300 mM NaCl, 150 mM imidazole at a pH of 7.4 in 10 separate microtubes, each containing 1 mL of the purified protein. For purification of insoluble proteins, all three buffers used during the purification process including binding, washing and elution buffers were supplemented with 8 M of urea. The purified proteins were stored at -20°C until needed for further experiments.

Purification of antibody using Pierce Protein A IgG Purification and AminoLink™ Plus Micro Immobilization Kits

The polyclonal sera were raised in rabbits against ECH-0163 (MerR), ECH_1012 (CtrA), ECH_0795 (EcxR), ECH_0804 (HU), and ECH_1118 (Tr1) recombinant proteins using a fee-

for-service facility (Thermo Fisher, Waltham, MA, USA). The sera were used to purify the antibody using Pierce™ Protein A IgG Purification Kit (Thermo Fisher, Waltham, MA, USA) as per the manufacturer's protocol. In brief, the storage solution existing in the protein A columns containing 0.02% sodium azide was discarded and the columns were equilibrated by applying 3-5 mL of Protein A IgG Binding buffer. The Binding buffer was then allowed to drain through the columns and the samples were subsequently applied. After the antibody samples were thoroughly flown through, the columns were washed 3 times using 5-15 mL of Binding buffer. The Binding buffer was allowed to drain through the columns during each wash. Finally, the bound antibodies were eluted using the IgG Elution buffer, and collected as separate 1 mL fractions of the elute that drains from the column. The purified antibodies were stored at 4°C until further purification.

To purify the antibodies further, AminoLink™ Plus Micro Immobilization Kit (Thermo Fisher, Waltham, MA, USA) was used as per the manufacturer's protocol. In brief, AminoLink Plus Resin was gently suspended in the columns by end-over-end mixing. Next, 2mL of pH 10 Coupling Buffer (0.1 M sodium phosphate, 0.15 M NaCl; pH 7.2 reconstituted with 500 ml of ultrapure water) was added to each column and the columns were centrifuged at 1,000 x g for 1 minute at 4°C while placed in a 15 mL tube. Then, an additional 2 mL of Coupling buffer was added to the columns and they were centrifuged again at the same setting. Subsequently, 1 mL of the first elution of the respective previously purified recombinant protein produced in *E. coli* was dissolved in 3 mL of pH 10 Coupling buffer. After placing the bottom cap, the mixture of recombinant protein dissolved in Coupling buffer was added to the columns and the top cap was similarly placed on the top. The columns were placed on a rotor and mixed by end-over-end

mixing overnight at room temperature. Subsequently, the top and bottom caps were removed, and the columns were placed in fresh 15 mL tubes. The tubes were then centrifuged at 1,000 x g for 1 minute at 4°C. Next, 2 mL of the Quenching Buffer containing 1 M Tris-HCl and 0.05% NaN3 at a pH of 7.4 was added to each column placed in a 15 mL tube and centrifuged using the same setting. This process was repeated an additional time and the flow through was discarded. In a separate fresh 5 mL tube, 2 mL of Quenching Buffer and 40 µL of 5M sodium cyanoborohydride solution (0.5 ml, dissolved in 0.01 M NaOH) were mixed and then added to the column after the bottom cap was replaced. The top cap was similarly replaced, and the columns were located on the rotor for 30 minutes at room temperature. Following the incubation, the caps were removed, and the columns were placed in 15 mL tubes and centrifuged at 1,000 x g for 1 minute at 4°C. Subsequently, the columns were washed 4 times using 2 mL of Wash solution (1 M NaCl and 0.05% NaN3) each time and centrifuged at 1,000 x g for 1 minute at 4°C after each wash. The flow through was discarded after each wash and 2 mL of the 0.02% sodium azide storage solution was added. The columns were centrifuged at 1,000 x g for 1 minute at 4°C and this process was repeated with an additional 2 mL of the storage solution. After discarding the flow through, the columns were placed on a holder and 6 mL of Wash solution containing 1M NaCl and 0.05% NaN3 were added to each column. The Wash solution was allowed to drain through the column drop by drop. In a separate fresh 15 mL tube, 3 mL of Wash solution and 2 mL of the respective IgG purified antibody (elution 1) were added and mixed gently by vortexing. The mixture of the IgG purified antibody and Wash solution was added to the column and mixed further on a rotor for 60 minutes at room temperature. Next, the columns were placed in fresh 15 mL tubes and centrifuged at 1,000 x g for 1 minute at 4°C. The columns were then washed 4 times with 2 mL of Wash solution each time and centrifuged using the similar settings.

Subsequently, 2 mL of IgG Elution buffer mixed with 100 µL 1M sodium phosphate was added to each column and the columns were centrifuged at 1,000 x g for 1 minute at 4°C. The flow through containing the purified antibody was collected in a fresh 5 mL tube as elution 1. The elution process was repeated 4 times collecting the flow through every time as elutions 1-4. The purified antibodies were kept at -20°C until further used as the primary antibody in the Western blot experiments.

SDS-PAGE and Western blot

Frozen lysates of purified *E. chaffeensis* DC and RC fractions recovered from DH82 and ISE6 cell cultures were allowed to thaw at room temperature. After mixing the samples gently by pipetting, the protein concentration of each sample was measured using PierceTM BCA Protein Assay Kit (Thermo Fisher Scientific, Waltham, MA). In brief, a standard curve was prepared using dilutions of BSA stock solution in 1x PBS with various protein concentrations. The serially diluted BSA as well as RC and DC samples were loaded into a 96-well plate and incubated at 37°C for 30 minutes. The optical density was then measured using the Epoch microplate spectrophotometer (BioTek Instruments, Winooski, VT) at 562 nm wavelength. The protein concentration of DC and RC samples was determined by comparing the measured values and the known BSA standards plotted using MS Excel graph. Subsequently, the protein samples were resuspended with appropriate amounts of 5X SDS loading buffer and boiled for 5 minutes. The samples were then, loaded into pre-made NovexTM 10-20% tricine gels (Invitrogen Life Technologies, Carlsbad, CA) and separated by SDS-PAGE at 125 V for 45 minutes to 1 hour.

Similar procedures were performed to measure the concentration of the five purified recombinant DNA-binding proteins produced in *E. coli,* and SDS-PAGE was similarly carried out to confirm the presence of the purified proteins at the expected size.

Following the electrophoresis, the resolved protein gels were either stained using a Colloidal Blue Staining Kit (Invitrogen Life Technologies, Carlsbad, CA) as per the manufacturer's protocol, or used for Western blotting analysis.

For Western blot analysis, resolved proteins in the gels were transferred to a PVDF 0.2 μm membrane (Thermo Fisher Scientific, Rockford, IL) by subjecting them to a voltage of 90 V for 1 hour. The membranes were then blocked for 1 hour at room temperature using 5% blocking buffer (1XTBST with 5% nonfat dry milk). The previously purified primary antibodies were diluted 1:10 in 5% blocking buffer and added to the membranes and the membranes were incubated at 4°C overnight. Subsequently, the membranes were washed five times (5-minutes each time) with 1X TBST (20 mM Tris, 150 mM NaCl, 0.1% Tween® 20 detergent). Then, the membranes were placed in the secondary antibody: goat anti-rabbit lgG conjugated to horseradish peroxidase (HRP) (Invitrogen Life Technologies, Carlsbad, CA) diluted 1:5000 in 5% blocking buffer and incubated for 1 hour at room temperature. Next, the membranes were washed five times (5-minutes each time) using 1X TBST. The HRP on immunoblots was detected using Pierce™ ECL Western Blotting Substrate (Thermo Fisher Scientific, Rockford, IL) as per the manufacturer's protocol and visualized using iBrightTM CL1500 Imaging System (Thermo Fisher Scientific, Rockford, IL).

Chapter 4 - Results

Evaluation of *E. chaffeensis* lysates prepared from tick cells (ISE6) and canine macrophages (DH82) culture-derived RC and DC fractions for RNA and protein analysis to map variations in DBP expressions

Renografin density gradient ultracentrifugation was employed to purify RC and DC fractions from the bacteria cultured in DH82 and ISE6 cell lines (Figure 3.1). The RC and DC fractions were subsequently used for RNA and protein analysis to map variations in the expression of the 5 predicted transcription regulators.

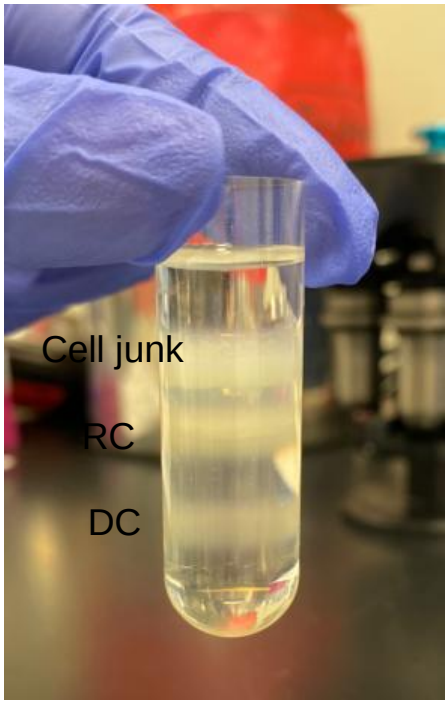

Figure 4.1: Cell lysate of ISE6 cells infected with wild-type *E. chafeensis* subjected to Renografin density gradient ultracentrifugation

PCR to check DNA contamination in the purified RNA samples from RC and DC fractions

The RNA samples purified from *E. chaffeensis* RC and DC isolates grown in both DH82 and ISE6 cells were assessed by polymerase chain reaction (PCR) assay to verify that the RNAs are free from contaminated bacterial genomic DNA.

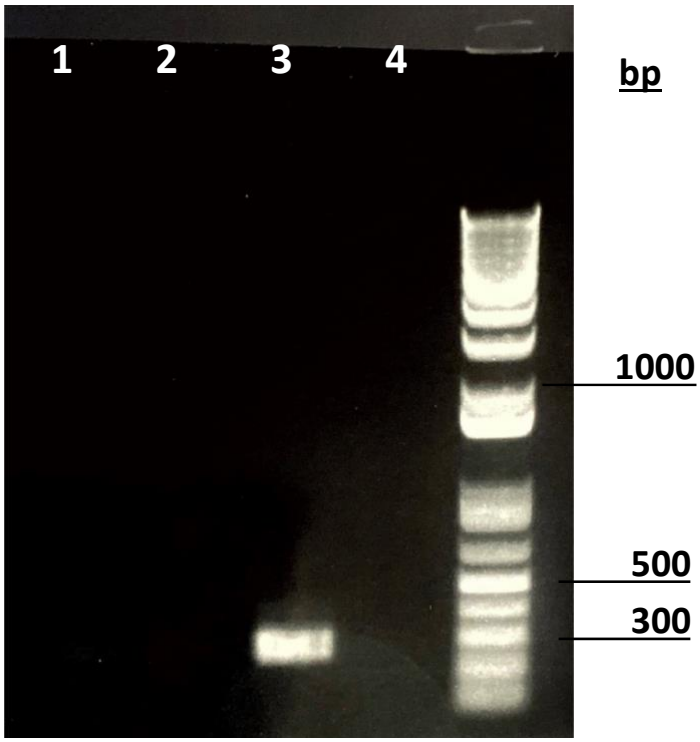

Figure 4.2: PCR to check DNA contamination in the purified RNA samples from RC and DC fractions

The RNA samples purified from *E. chaffeensis* RC and DC isolates grown in both DH82 and ISE6 cells were assessed by PCR to exclude the presence of any DNA contamination. Lane 1 and 2 represent the RNA purified from DH82 derived, RC and DC isolates respectively. Lane 3

and 4 represent DNA purified from E 7000 wild-type *E. chaffeensis* and nuclease free water used as the template to serve as positive and negative control respectively.

RNA expression variation for the five predicted transcription regulators (CtrA, EcxR, MerR, Hup, and Tr1) in RC and DC fractions purified from wild-type *E. chaffeensis* cultivated in DH82 and ISE6 cells assessed by qRT-PCR

We then assessed the RNA expression of five predicted transcription regulators of *E. chaffeensis* by quantitative reverse transcription-PCR (qRT-PCR). The experiment was performed using RNA recovered from RC and DC fractions that were purified from wild-type *E. chaffeensis* cultivated in DH82 and ISE6 cell lines. Assessment of the expression levels of all five predicted transcription regulators revealed higher expression levels in the RC form compared to DC when the *E. chaffeensis* was cultivated in the DH82 cell line. Conversely, expression levels of all five predicted transcription regulators were higher in the DC form compared to RC form when grown in the ISE6 cell line. The Tr1 expression was observed to be significantly higher for the RC form compared to the DC for bacteria grown in DH82 cell line. In ISE6 cells, Tr1 expression is slightly higher in DC, but the difference is not remarkable (Figure 4.3). The CtrA results similarly exhibit a significantly higher level of expression in RC than DC for DH82 cell culture-derived *E. chaffeensis*, while a lower expression level was observed in the RC form when the organism was cultivated in ISE6 cell line (Figure 4.4). The same pattern exists for the expression levels of MerR, however, the difference between expression level witnessed in RC and DC forms is not statistically significant in either of the cell lines (Figure

4.5). The most significant difference in the expression level of transcription regulators between the two forms was noted in the Hup expression when *E. chaffeensis* was cultivated in DH82 cell line (Figure 4.6). Similarly, in ISE6 cell line, fold expression change of EcxR in DC is almost twice as of RC, while in DH82 the expression level of this transcription regulator in DC is significantly lower (Figure 4.7).

While much remains to be explained regarding differential RNA expression for the five predicted transcription regulators and their impact on *E. chaffeensis* replication, our findings suggest the presence of unique gene expression patterns that are potentially important for *E. chaffeensis* transition between the two different morphologies and its growth and replication in various host cells.

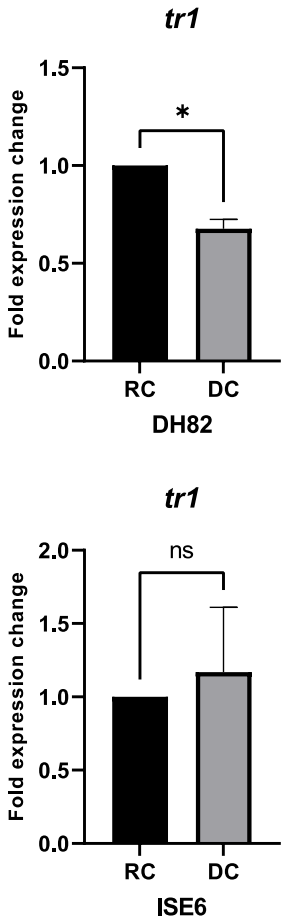

Figure 4.3: qRT-PCR expression levels of gene regulator *tr1*

The *tr1* expression was observed to be significantly higher in the RC form compared to DC grown in DH82 cell line. In ISE6 cells, *tr1* expression is slightly higher in DC, but the difference is not remarkable. Significant changes in transcript expression were identified with a single asterisk where the P- values were <0.05.

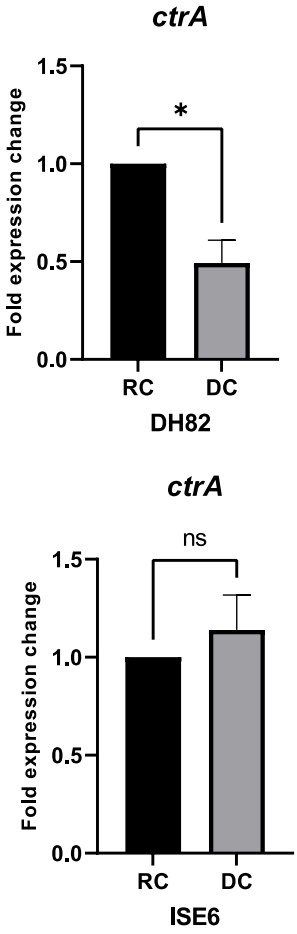

Figure 4.4: qRT-PCR expression levels of gene regulator *ctrA*

The *ctrA* results exhibit a significantly higher level of expression in RC than DC in the case of DH82 derived *E. chaffeensis*, while a lower expression level was noticed in the RC form when the organism was cultivated in ISE6 cell line. Significant changes in transcript expression were identified with a single asterisk where the P- values were <0.05.

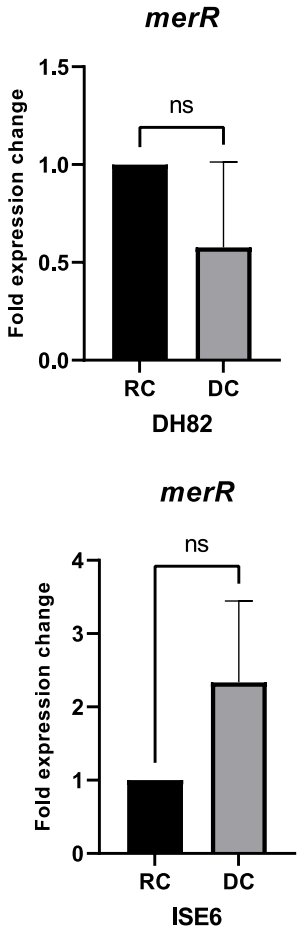

Figure 4.5: qRT-PCR expression levels of gene regulator *merR*

The *merR* expression was observed to be higher in the RC form compared to DC grown in DH82 cell line. In ISE6 cells, *tr1* expression is higher in DC, but the difference is not remarkable in either of the cell lines.

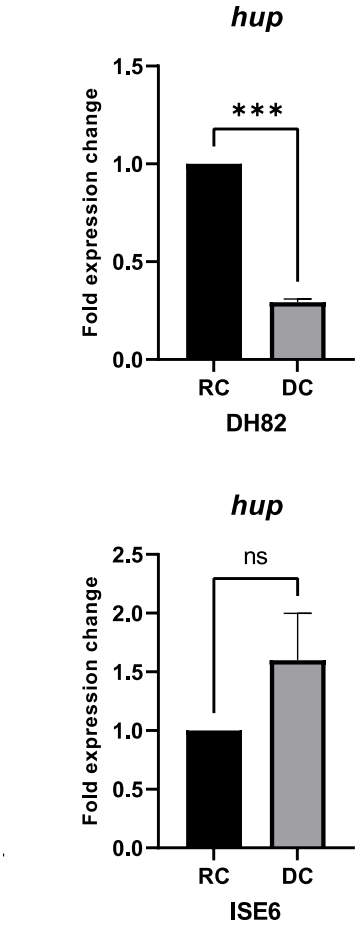

Figure 4.6: qRT-PCR expression levels of gene regulator *hup*

The *hup* results exhibit a significantly higher level of expression in RC than DC in the case of DH82 derived *E. chaffeensis* which is the most notable difference observed when comparing DC and RC forms among all five transcription regulators. A lower expression level was observed in the RC form when the bacteria was cultivated in ISE6 cell line.

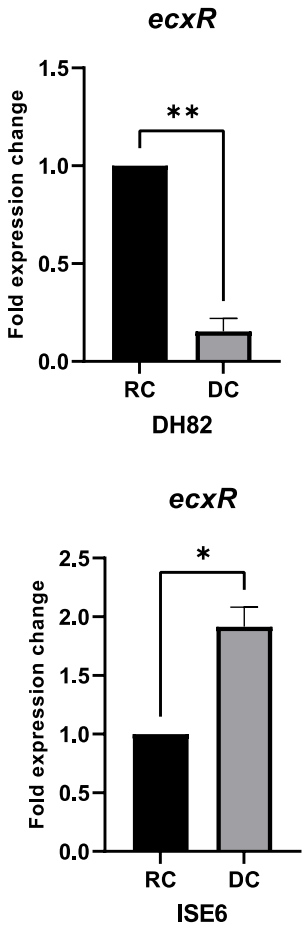

Figure 4.7: qRT-PCR expression levels of gene regulator *ecxR*

The *ecxR* expression was observed to be significantly higher in the RC form compared to DC grown in DH82 cell line. Additionally, in ISE6 cells *ecxR* expression is remarkably higher in DC. Significant changes in transcript expression were identified with a single asterisk where the P- values were <0.05 and a double asterisk where the P-values were <0.01.

Validating expression of the recombinant proteins by the *E. coli* surrogate system

Purified recombinant protein samples of all five transcription regulators were assessed by SDS-PAGE analysis to confirm the presence of the protein at the correct size. Protein bands consistent with the expected protein sizes were observed in the SDS-PAGE results assessing all five purified DNA-binding proteins, demonstrating the expression of these transcription regulators in the *E. coli* expression system (Figures 4.8-12). HU and EcxR recombinant proteins were expressed as soluble proteins in the *E. coli* expression system (Figure 4.8 and Figure 4.9), whereas MerR, CtrA and tr1 were expressed as insoluble proteins (Figures 4.10-12).

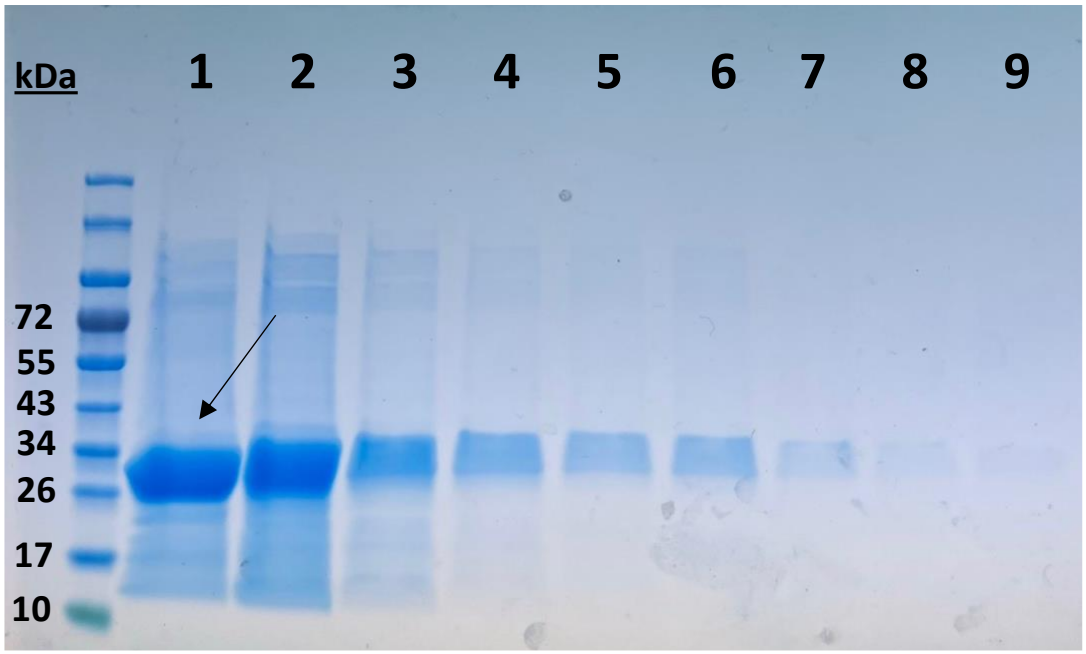

Figure 4.8: SDS-PAGE protein expression of HU in *E. coli* surrogate system

Proteins resolved on an SDS-PAGE tricine gel to confirm the expression of the HU recombinant protein in the *E. coli* surrogate system. Lanes 1 to 9 indicate elutions 1-9 containing the purified recombinant protein. HU protein (11.2 kDa) having a His tag (17 kDa) with total predicted size of 28 kDa is visible in the soluble protein fractions of *E. coli* (identified with an arrow).

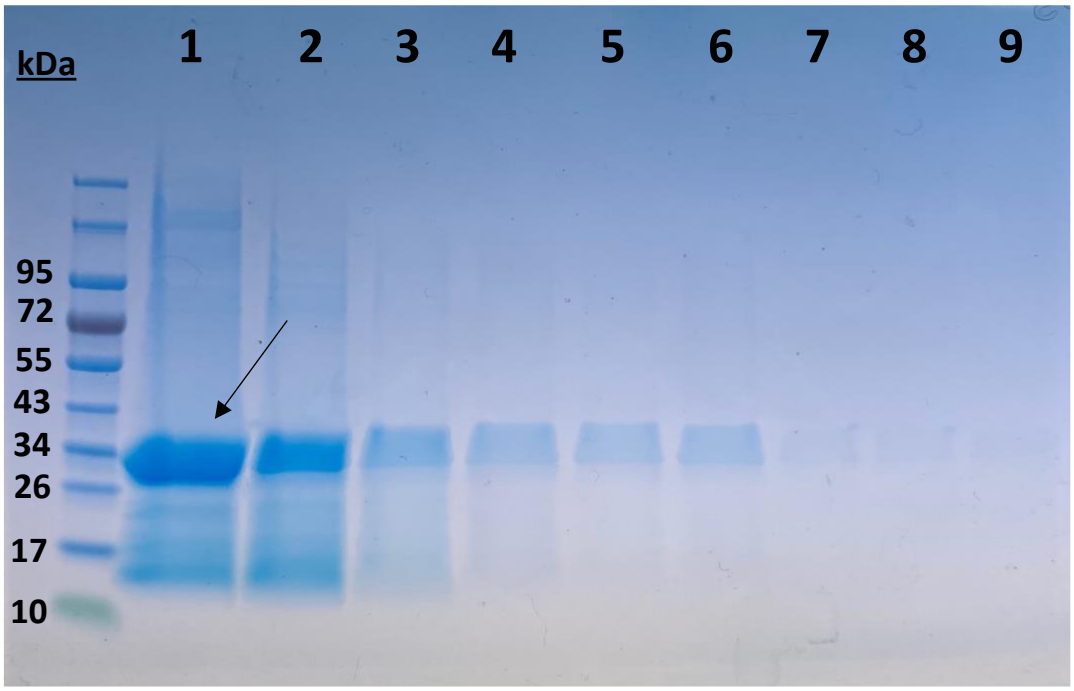

Figure 4.9: SDS-PAGE protein expression of EcxR in *E. coli* surrogate system

Proteins resolved on an SDS-PAGE tricine gel to observe the expression of the EcxR recombinant protein in the *E. coli* surrogate system. Lanes 1 to 9 indicate elutions 1-9 of the purified recombinant protein. EcxR protein (12.7 kDa) containing the His tag (17 kDa) with the total predicted protein size of ~30 kDa is observed (arrow) for the soluble protein fractions of *E. coli*.

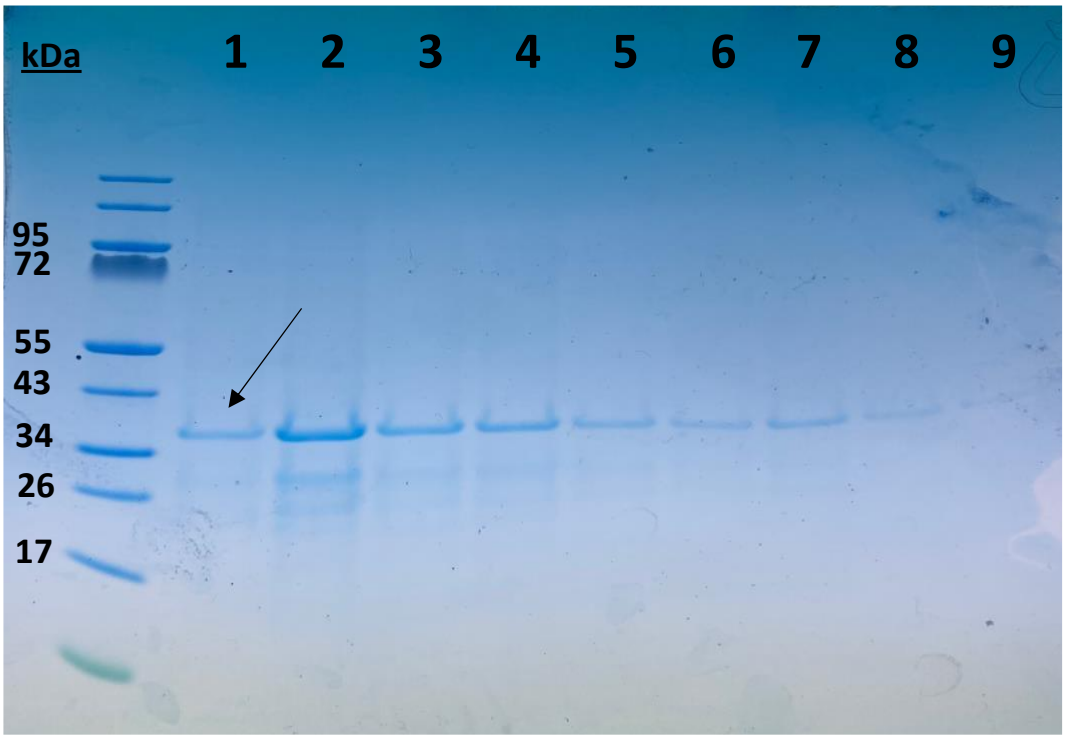

Figure 4.10: SDS-PAGE protein expression of MerR in *E. coli* surrogate system

Proteins resolved on an SDS-PAGE tricine gel to confirm the expression of the MerR recombinant protein in the *E.coli* surrogate system. Lanes 1 to 9 indicate elutions 1-9 of the purified recombinant protein. The estimated protein size of MerR (14.5 kDa) containing His tag (17 kDa) is 32 kDa which is observed (arrow) in the insoluble protein fractions of *E. coli*.

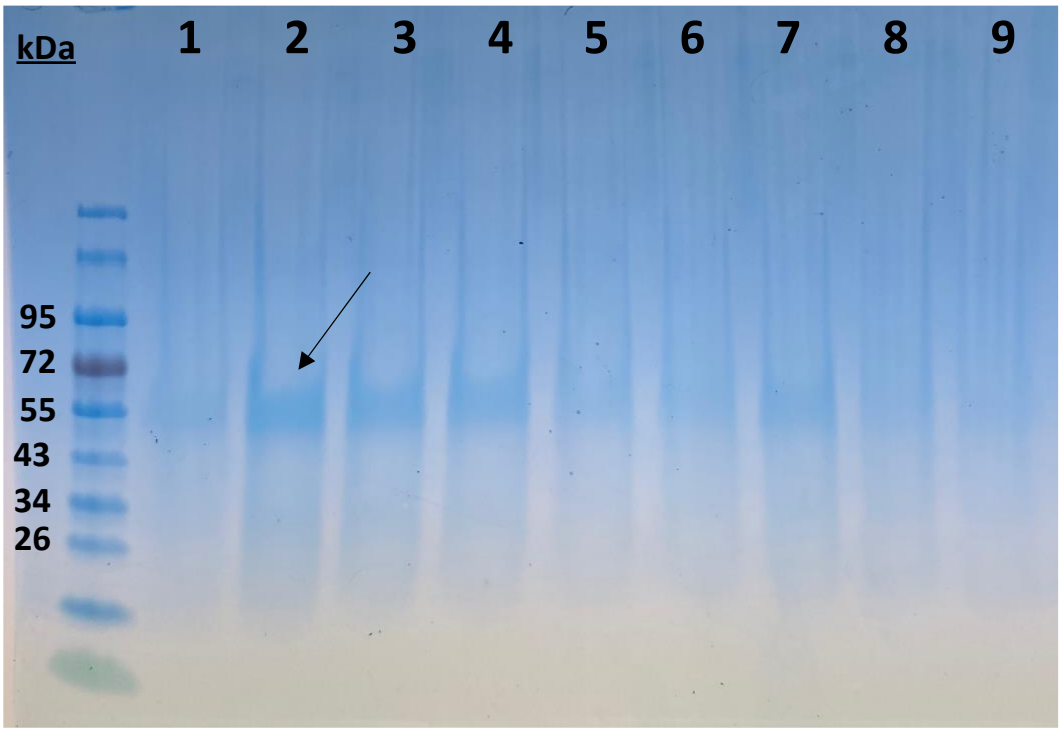

Figure 4.11: SDS-PAGE protein expression of CtrA in *E. coli* surrogate system

Proteins resolved on an SDS-PAGE tricine gel to observe the expression of the CtrA recombinant protein in the *E.coli* surrogate system. Lanes 1 to 9 indicate elutions 1-9 of the purified recombinant protein. The estimated protein size of CtrA (32 kDa) containing His tag (17 kDa) is 47 kDa which is observed (arrow) in the insoluble protein fractions of *E. coli*.

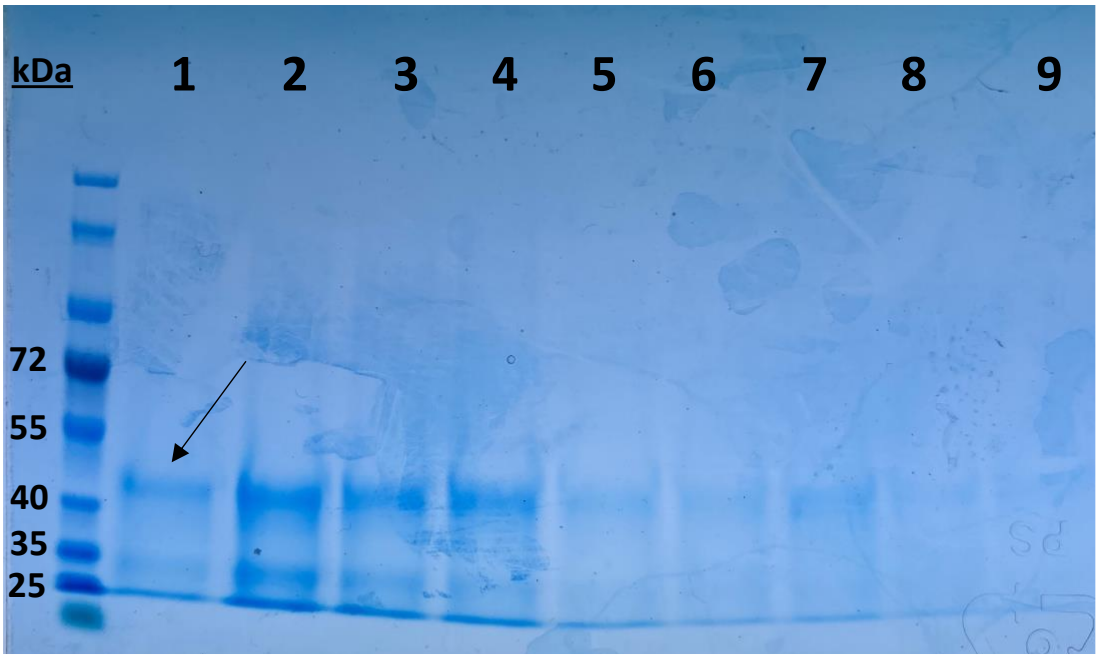

Figure 4.12: SDS-PAGE protein expression of tr1 in *E. coli* surrogate system

Proteins resolved on an SDS-PAGE tricine gel to confirm the expression of the tr1 recombinant protein in the *E. coli* surrogate system. Lanes 1 to 9 indicate elutions 1-9 of the purified recombinant protein. The estimated protein size of tr1 (24.2 kDa) containing His tag (17 kDa) is 41 kDa which is observed (arrow) in the insoluble protein fractions of *E. coli*.

Assessment of expression of the five predicted transcription regulators expressed in purified RC and DC fractions of *E. chaffeensis* cultivated in DH82 and ISE6 cell lines by Western blot analysis

Purified RC and DC protein fractions of *E. chaffeensis* recovered from infected DH82 and ISE6 cell cultures were assessed by Western blot analysis using the antibodies previously purified from the polyclonal sera raised in rabbits against MerR, CtrA, EcxR, HU, and Tr1 recombinant proteins. For each Western blot analysis, the respective recombinant proteins produced in the *E. coli* system were used to serve as positive controls, and uninfected DH82 cell lysates were used negative controls. Similar concentrations of protein samples from DC and RC lysates recovered from DH82 and ISE6 were measured by BCA and subjected to Western blot analysis to assess protein expression differences between the DC versus RC fractions (Figures 4.13-17).

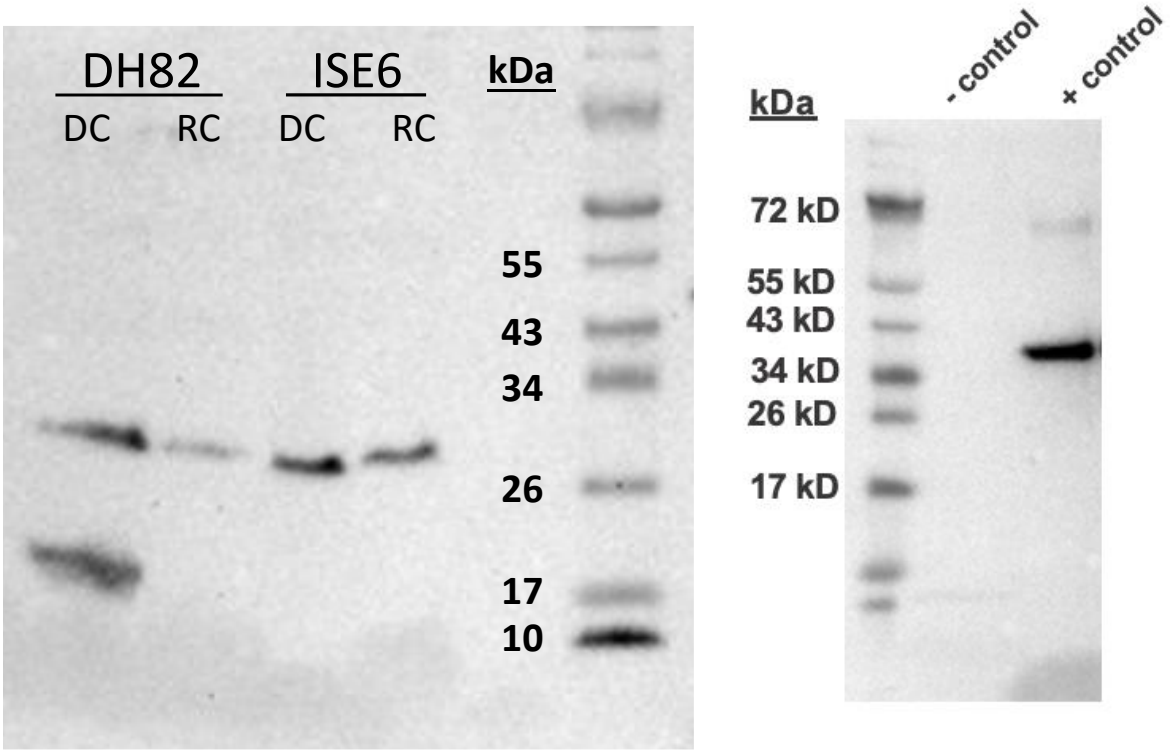

Figure 4.13. Western Blot protein expression of MerR

Assessment of MerR protein expression in DC and RC forms of *E. chaffeensis* purified from DH82 and ISE6 cell cultures. The right panel included a positive control recombinant protein blot which yielded two bands; a predicted ~32 kDa monomer size and a 74 kDa predicted dimer. MerR protein expression was observed in both DC and RC forms of *E. chaffeensis* purified from both DH82 and ISE6 cells. The protein detected in the Western blot was nearly twice the size of a monomer (~29 kDa) suggesting the presence of this protein within *E. chaffeensis* in the form of a dimer. The monomer of MerR was also observed, but only in the lysates prepared from DC fraction of the bacteria recovered from DH82 cultures.

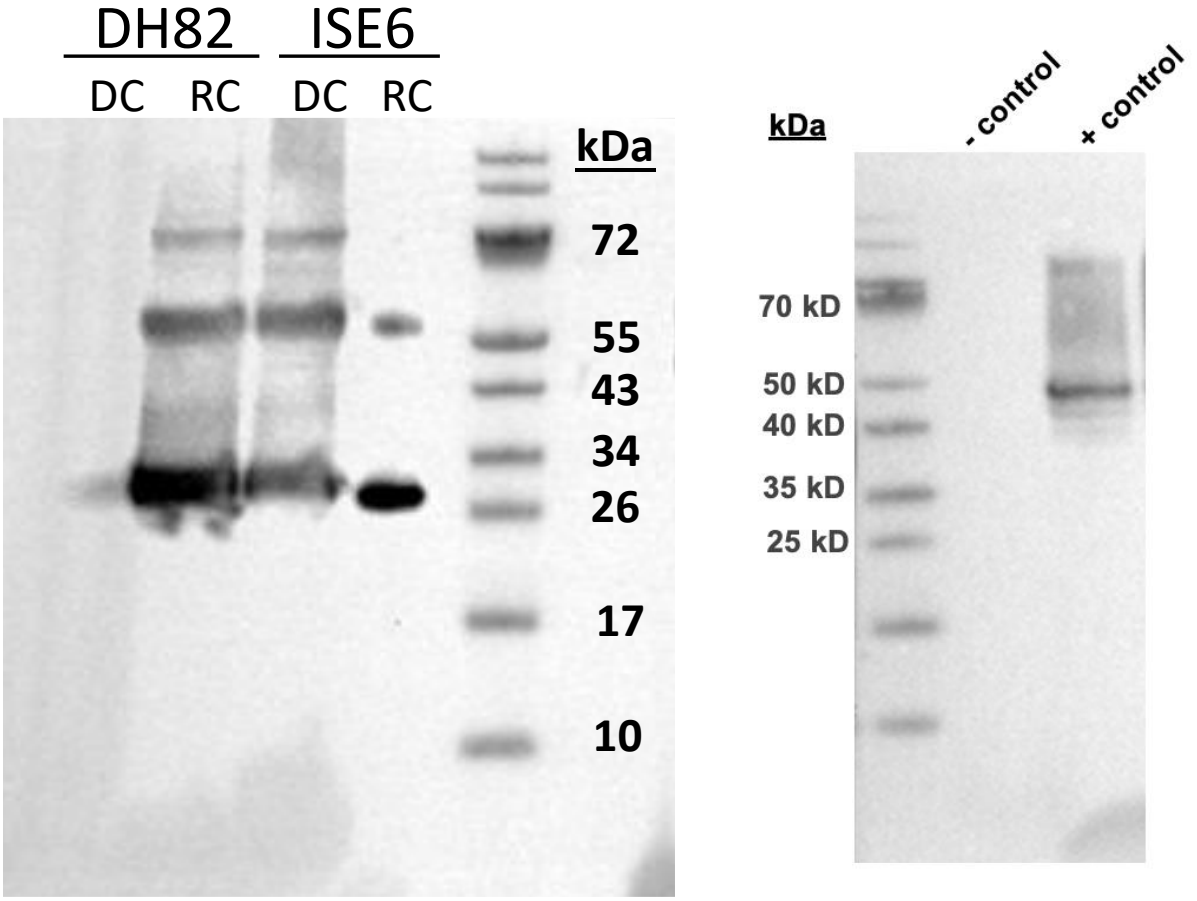

Figure 4.14. Western Blot protein expression of tr1

Assessment of tr1 protein expression in DCs and RCs purified from DH82 and ISE6 cell cultures. The image on the right side represents the positive control and demonstrates the validity of antibody to detect the his-tagged tr1 protein. On the left panel, tr1 expression was assessed in DC and RC fractions of *E. chaffeensis* purified from both DH82 and ISE6 cells. The predicted size protein (26.2 kDa) was observed in all fractions except in DC fraction derived from DH82 cultures. Additionally, we observed tr1 antibody detected proteins at double and triple the size of a tr1 monomer, suggesting that the protein may be expressed as dimer and trimer.

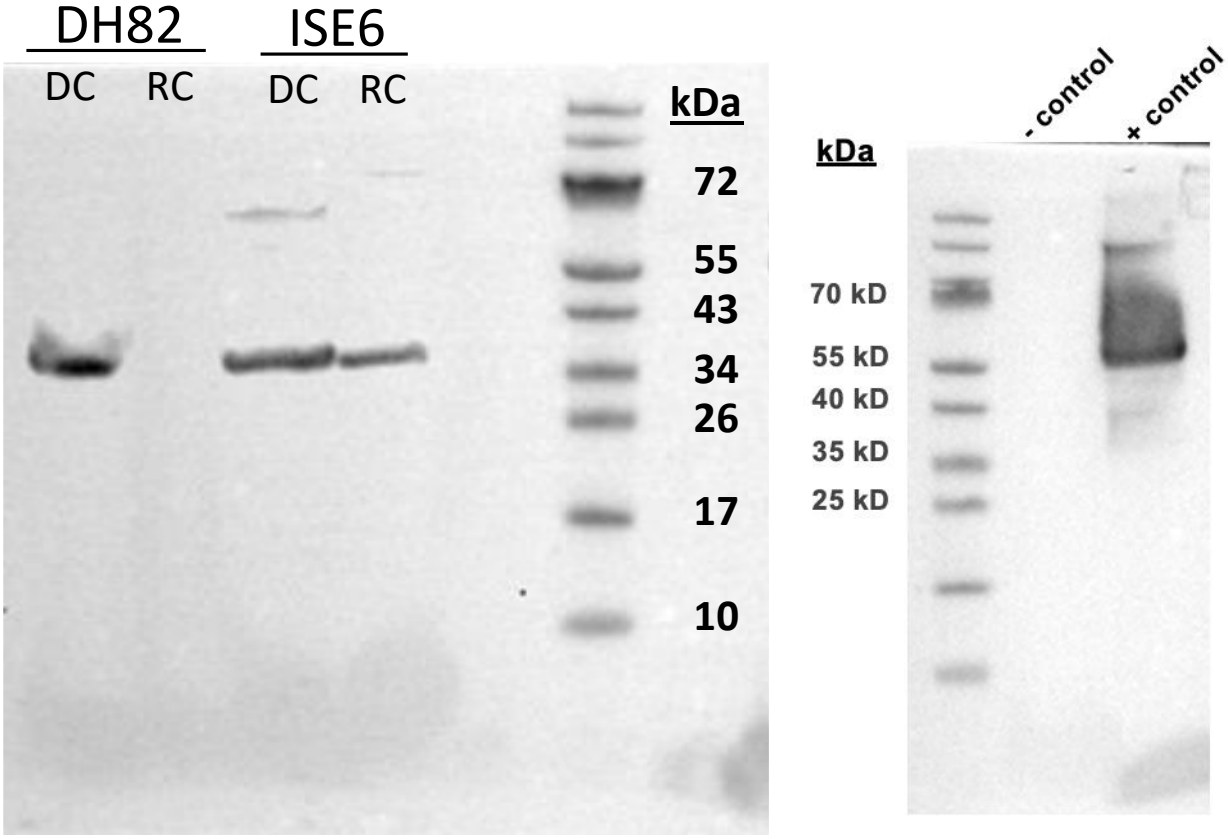

Figure 4.15. Western Blot protein expression of CtrA

Assessment of CtrA protein expression in DCs and RCs purified from DH82 and ISE6 cell cultures. The right panel of the blot included recombinant his-tagged CtrA protein hybridized with the protein-specific antibody to serve as the positive control. The experimental data were shown in the image on the left panel. CtrA expression (32 kDa) was observed in DC and RC fractions of *E. chaffeensis* recovered from ISE6 cells and in the DC fraction recovered from DH82 cells. There was no protein expression observed in the RC fraction of DH82 cultures. A predicted dimer was also observed in the DC form proteins recovered from ISE6 cell cultures.

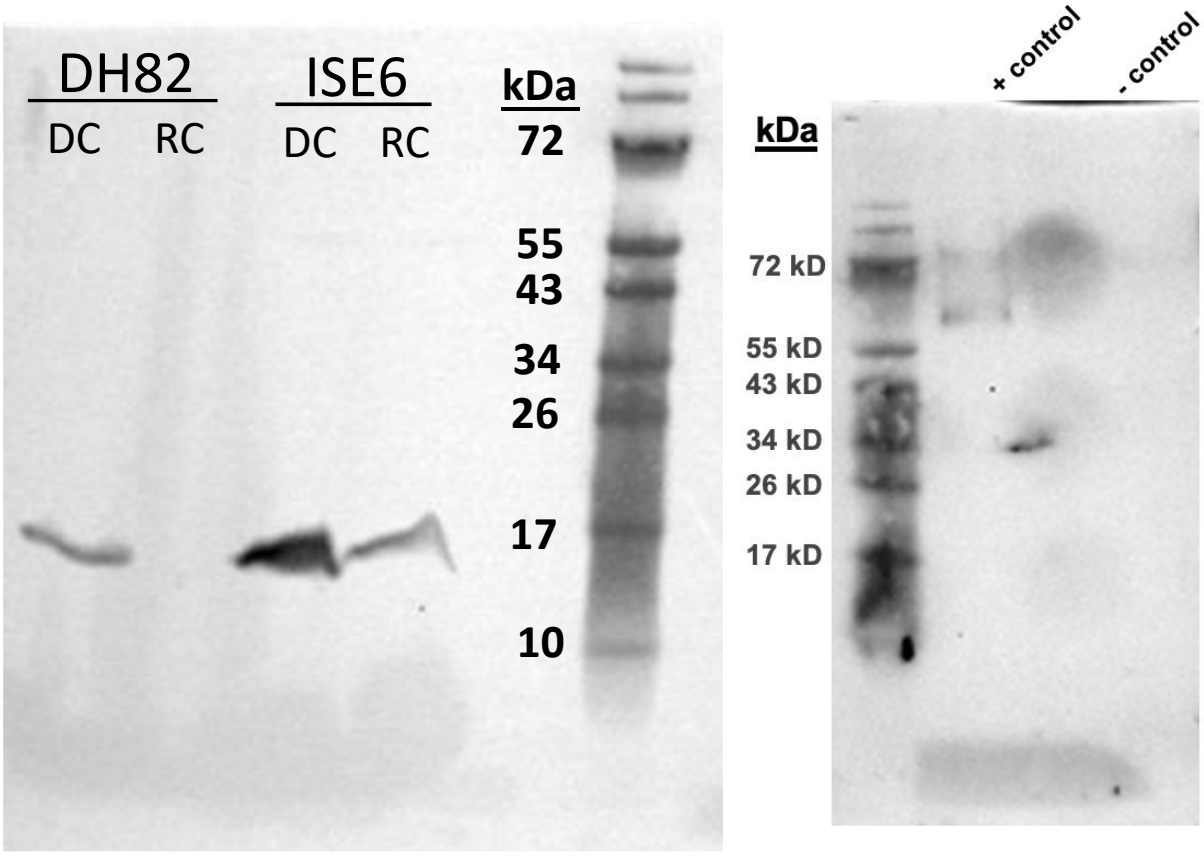

Figure 4.16. Western Blot protein expression of HU

Assessment of HU protein expression in DCs and RCs purified from DH82 and ISE6 cells. The right panel figure included the recombinant his-tagged hu protein serving as the positive control. HU expression was observed in DC and RC form proteins recovered from ISE6 cell cultures, while only the DC form from the DH82 cells had the protein that is slightly large (15 kDa) than the predicted size (11.2 kDa). ISE6 cell culture derived DC fraction had considerably more protein than RC fraction.

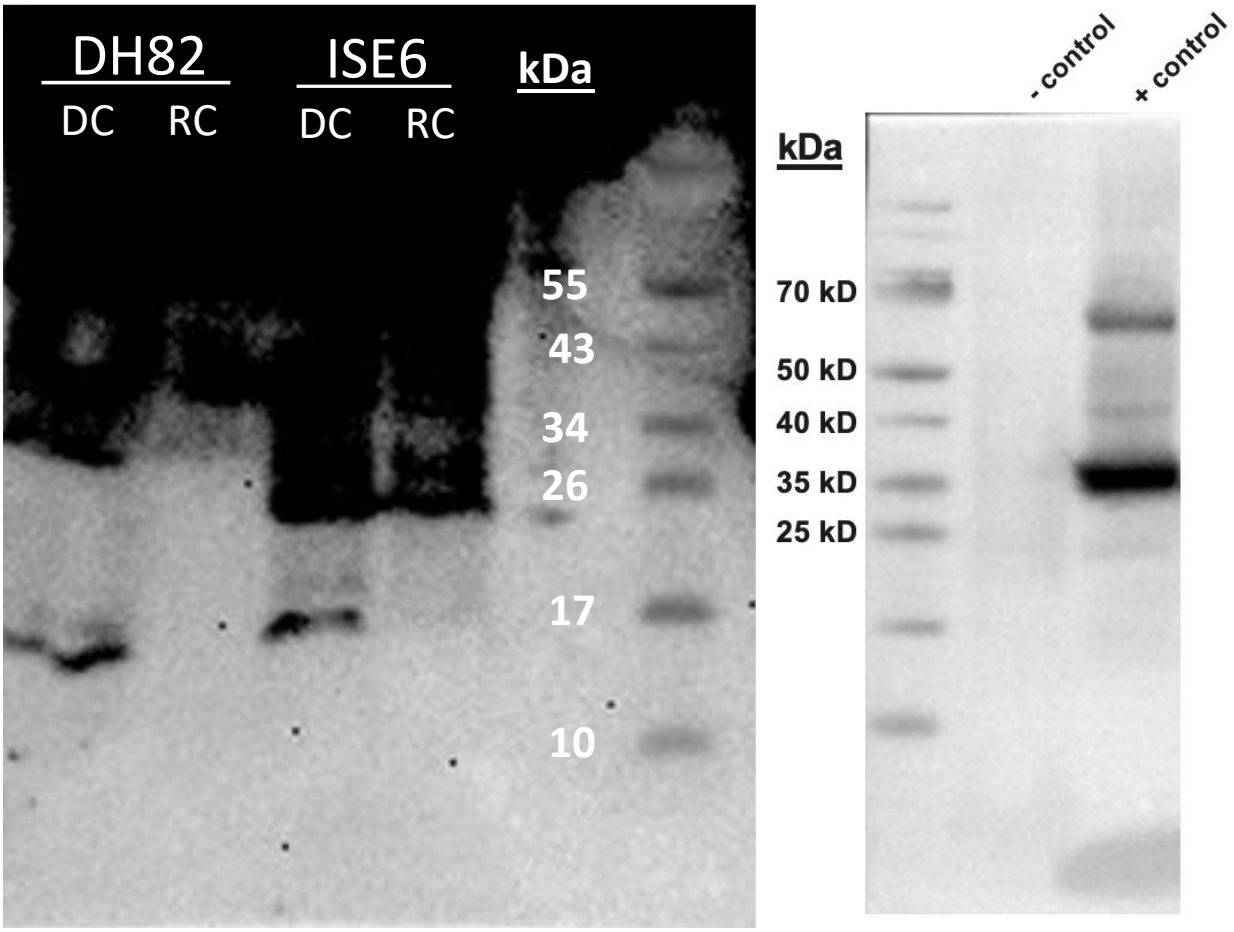

Figure 4.17. Western Blot protein expression of EcxR

Assessment of EcxR protein expression in DCs and RCs purified from DH82 and ISE6 cell lines. The right panel includes the recombinant his-tagged EcxR protein serving as the positive control; which was expressed as dimer and monomer, consistent with the predicted sizes. EcxR expression as a monomer was observed primarily in the DC forms of *E. chaffeensis* purified from the ISE6 and DH82 cultures, whereas the protein similar in size of dimer was observed in both DC and RC form lysates recovered from ISE6 cells.

Chapter 5 - Discussion

In this study, we determined the transcription for five predicted DNA-binding proteins in *E. chaffeensis* t from RC and DC forms of *E. chaffeensis*, and variations resulting from type of host cell the organism was cultured. We first defined the expression of all five predicted transcription regulators at the RNA level for *E. chaffeensis* and demonstrated unique RNA expression patterns in both host cells (DH82 macrophages, and ISE6 tick cells) and in the RC and DC forms of the pathogen. We then determined the protein expression differences for all five predicted transcription regulators in DC and RC forms of *E. chaffeensis* purified from DH82 and ISE6 cell cultured organisms using protein-specific polyclonal antibodies. The study revealed a significantly higher expression in RC than DC cultivated in macrophages at the RNA level for some of the DNA protein genes. We also showed that the purified recombinant DNA-binding proteins are often at the predicted size and are seen in the form of doublets or triplets for most of the studied proteins. DNA-binding proteins are traditionally known to form dimers or multimers [107, 117, 120], which is consistent with our findings. Even though we showed that all five predicted proteins are transcribed at the RNA level, there appears to be no direct correlation between RNA expression to that of the protein expression for some DBPs. In particular, we observed higher expression of the proteins mostly in the DC from of *E. chaffeensis* independent of the organism cultured in DH82 or ISE6 cell cultures. More investigations are needed to explain the differences that exist between the RNA and protein expression. This study helps in furthering our understanding for how *E. chaffeensis* regulates its gene expression in vertebrate and tick host cell growth.

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