STRUCTURAL PREDICTION ANALYSIS OF *EHRLICHIA CHAFFEENSIS* OUTER MEMBRANE PROTEINS, P28 OMP-14 AND P28 OMP-19 ASSESSED BY CIRCULAR DICHROSIM AND PORIN ASSAYS

by

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B.S., ANDHRA UNIVERSITY, VISAKHAPATNAM, A.P, INDIA, 2007

A THESIS

Submitted in partial fulfillment of the requirements for the degree

MASTER OF SCIENCE

Department of Diagnostic Medicine/ Pathobiology

College of Veterinary Medicine

KANSAS STATE UNIVERSITY

Manhattan, Kansas

2011

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ABSTRACT

Ehrlichia chaffeensis, a Gram-negative organism belonging to the order Rickettsiales, is responsible for an emerging infectious disease in humans, the human monocytic ehrlichiosis. *E. chaffeensis* also infects several other vertebrate hosts including dogs, goats, coyotes and white tailed deers. This organism is transmitted by an infected tick, *Amblyomma americanum*. The exact pathogenic mechanisms involved for the persistence of the pathogen in vertebrate hosts are still unclear. *E. chaffeensis* protein expression varies significantly in vertebrate and tick hosts. Differentially expressed proteins include the immunodominant outer membrane proteins encoded by the p28-Omp multigene locus. The p28-Omp 14 is expressed primarily in tick cells and the p28-Omp 19 is the major expressed protein in macrophages both under *in vitro* and *in vivo* conditions. The objective of this study is to prepare recombinant proteins and use them to assess the secondary structures and protein functions. The protein sequences were analyzed with the aid of bioinformatics programs to make structural predictions. The analysis suggested the presence of eight β barrel structures for both the p28-Omp proteins. The coding sequence of the p28-Omp genes were cloned and over expressions of proteins in in *E. coli* was accomplished by using the plasmid expression construct, pET28. The proteins were purified to near homogeneity and used to refold using detergents to mimic native protein structure in the bacterial outer membrane. Refolding of proteins was analyzed by two methods; SDS-PAGE and Circular Dichroism. The Circular dichroism spectroscopy analysis suggested the formation of β-sheet structures of proteins in micelles formed with the detergents. β-sheet structures may have been

formed with the hydrophobic domains of the protein imbedded in the micelles. The hydrophilic segments (predicted by bio informatics analysis) may be exposed to the aqueous phase. The recombinant proteins were also used to prepare proteoliposomes and tested for the porin activity. The analysis demonstrated the porin activity for both p28-Omp 14 and 19 recombinant proteins by using mono-, di- and tetra- saccharides as well as for amino acid L-glutamine. This study forms the basis for initiating studies to compare the structural difference between the two differentially expressed proteins of *E. chaffeensis.*

ACKNOWLEDGEMENTS

I would like to express my deepest appreciation to my major professor, Dr. Roman R. Ganta, who supported me in my research with his patience and knowledge. His assistance and guidance provides me with an excellent atmosphere for doing research. I want to say special thanks to Dr. Roman R. Ganta for introducing MoU between Andhra University and Kansas State University, which provided a great platform for me to learn and explore the knowledge.

I would also like to show my sincere gratitude to all members of my advisory committee: Dr. Om Prakash and Dr Dr. Kyeong-Ok Chang for their kind assistance and extensive evaluation of my M.S program. They were very beneficial in providing me with ideas to better my overall project. I truly appreciate the time and effort they dedicated to helping me in the completion of my program.

I would like to thank Dr. Daisuke (NMR Lab) for his guidance and providing help for completion of my research.

I would especially like to thank Chuanmin Cheng for her guidance as well as friendship. A special thanks also goes to my lab members: Huitao Liu, Chakravarthy Katragadda, Dr. Latitha Peddireddi and Bonto Faburay. To Dr. Bonto, I would like to thank for your encouragement and support all the time.

I would also like to thank faculty members in Andhra University, Biochemistry department for giving opportunity to join in the MoU program

Dedication

I dedicate this dissertation to my family members, teachers, and friends.

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CHAPTER I: LITERATURE REVIEW

ABSTRACT

Ehrlichia chaffeensis, a Gram-negative organism belonging to the order Rickettsiales, is responsible for an emerging infectious disease in humans, the human monocytic ehrlichiosis. *E. chaffeensis* also infects several other vertebrate hosts including dogs, goats, coyotes and white tailed deers. This organism is transmitted by an infected tick, *Amblyomma americanum*. The exact pathogenic mechanisms involved for the persistence of the pathogen in vertebrate hosts are still unclear. *E. chaffeensis* protein expression varies significantly in vertebrate and tick hosts. Differentially expressed proteins include the immunodominant outer membrane proteins encoded by the p28-Omp multigene locus. The p28-Omp 14 is expressed primarily in tick cells and the p28-Omp 19 is the major expressed protein in macrophages both under *in vitro* and *in vivo* conditions. The objective of this study is to prepare recombinant proteins and use them to assess the secondary structures and protein functions. The protein sequences were analyzed with the aid of bioinformatics programs to make structural predictions. The analysis suggested the presence of eight β barrel structures for both the p28-Omp proteins. The coding sequence of the p28-Omp genes were cloned and over expressions of proteins in in *E. coli* was accomplished by using the plasmid expression construct, pET28. The proteins were purified to near homogeneity and used to refold using detergents to mimic native protein structure in the bacterial outer membrane. Refolding of proteins was analyzed by two methods; SDS-PAGE and Circular Dichroism. The Circular dichroism spectroscopy analysis suggested the formation of β-sheet structures of proteins in micelles formed with the detergents. β-sheet structures may have been formed with the hydrophobic domains of the protein imbedded in the micelles. The hydrophilic segments (predicted by bio informatics analysis) may be exposed to the

aqueous phase. The recombinant proteins were also used to prepare proteoliposomes and tested for the porin activity. The analysis demonstrated the porin activity for both p28-Omp 14 and 19 recombinant proteins by using mono-, di- and tetra- saccharides as well as for amino acid L-glutamine. This study forms the basis for initiating studies to compare the structural difference between the two differentially expressed proteins of *E. chaffeensis.*

Introduction

Vector borne diseases

Three major factors contribute to the spread of vector-borne diseases; infectious microorganisms (eg: viruses, bacteria, parasites), vectors (eg: mosquitoes, ticks, fleas) and reservoir hosts [1, 2]. Vectors-borne disease agents are mostly transmitted by arthropod vectors. Vectors act as vehicles by which infectious agents are transmitted from an infected host to another susceptible host [2]. Major vector-borne infectious agents are maintained in nature by persisting in reservoir hosts and arthropod vectors [3, 4]. Some of the important infectious diseases in humans are the result of infections caused by vectors such as mosquitoes and ticks [5, 6]. For example, malaria is caused by a mosquito-borne infection with *Plasmodium* species. Human malaria is still considered the most important disease because it is responsible for significant morbidity and mortality [7]. Nearly 350-400 million human cases of malaria are reported annually caused by *Plasmodium falciparum* and *Plasmodium vivax* [8]. About half a million of these result in fatalities [8-11]. Similarly, tick-borne diseases are a significant health concern to humans and domestic animals [2, 3]. For example, the Lyme disease is a major problem for humans and various vertebrate animals. It has a widespread distribution throughout the world. It is discovered in 1975 as a human disease and is responsible for 1000s of human cases in the USA each year [12, 13]. Louse-borne epidemic typhus, caused by *Rickettsia prowozakii,* is another vector-borne disease responsible for significant fatalities in people [14, 15]. Among populations concentrated at places such as concentration camps during wars or civil disturbances, *R. prowozakii*

infections prevail and cause significant mortality (up to 30%). For example, deaths resulting from this disease are often more in wars than war casualties. For example in 1997 at refugee camps in Burundi, Africa, nearly 30,000 people died due to *R. prowozakii* infections [6, 16].

Rocky Mountain spotted fever, African sleeping sickness, sandfly fever, Chages disease, and louse borne typhus are among the vector borne disease indentified in early 1900s [5]. From 1984 to 2004, nine new tick borne diseases caused by rickettsial agents have been reported [17]. Human Ehrlichiosis and Anaplasmosis are among the rickettsial diseases, discovered during the last three decades [17-21]

Vector-borne diseases also have a high an impact on the economy of the world due to the diseases caused to agriculture animals. Every year, millions of dollars of economic losses occur in the world as a result of the vector-borne diseases [22, 23]. For example, outbreak of babesiosis and anaplasmosis in the early 1990s in Latin America reported an estimated annual economic loss between 875 to 1,365 million dollars [24].

Tick borne diseases

Ticks are the second major vectors after mosquitoes for spreading infectious diseases to animals and people [25-27]. Ticks are blood sucking parasitic arthropods (obligate, hematophagous) and are found in every region of the world [5, 28]. Ancient Greeks mentioned about ticks, but an actual demonstration of ticks as the pathogen transmitting vectors is not reported until the 19th century where Smith and Kilbourne

demonstrated the *Babesia bigemina*, a protozoan parasite transmission by *Boophilus* ticks to cattle [29, 30]. Ticks act as vectors for transmitting various infectious agents including protozoans, bacteria and viruses. For example, protozoan parasite *Cytauxzoonosis felis* is transmitted by *Dermocentar variabilis* to cats in South and Southeast regions of the U.S.A [31]. This parasite has a wide spread prevalence in sub Saharan Africa and is transmitted by ticks to various *felidea* species [31, 32]. *Babesia microti* is another protozoan parasite transmitted by ticks (*Ixodes scapularis*) and causes infections in humans [33]. Similarly, several *Babesia* species transmitted by ticks are responsible for babosiosis in cattle, horses, and dogs. Rickettsial bacteria, *Ehrlichia chaffeensis, Ehrlichia ewingii,* and *Anaplasma phagocytophilum* are transmitted by Ixodid ticks and cause Ehrlichiosis and Anaplasmaosis, respectively in people and in domestic animals [34-37]. *Borrelia burgdorferi* (another bacterial pathogen), transmitted by Ixodes species, is responsible for the Lyme disease in people and vertebrate animals [13]. Rocky Mountain spotted fever agent (*Rickettsia rickettsii*) is transmitted by three different species of ticks (*Dermacentor variabilis, Dermacentor andersoni and Amblyomma cajennense*) and is a major health problem in people and dogs around the world, including the U.S.A [27, 38, 39]. An example for tick-borne viral infection is Colorado tick fever caused by Colorado tick fever virus transmitted by *D. andersoni* ticks to humans [40].

Ticks belong to the class Arachnida. There are three tick families: Ixodidae (hard ticks), Argasidae (soft ticks) and Nuttalliellidae (morphologically intermediate between hard and soft ticks). The hard tick family has a list of 694 identified species, whereas the soft tick family has 177 recognized species and Nuttalliellidae family included only one species [5, 41, 42]. Larva, nymph or adult ticks can acquire a

pathogen from an infected host during their blood feeding. Pathogens acquired in larval or nymphal stages progress to nymphal or adult stages, respectively [28, 43]. Pathogens may then be transmitted to a niave host during subsequent blood feeding cycles [5]. Some disease causing agents may be transovarially passed on from an adult female to larvae via infected eggs, while other disease causing agents are maintained only transtadially (from larva to nymphs and nymph to adult). For example, *Rickettsia* species are transovarially maintained while *Ehrlichia* and *Anaplasma* species are only maintained transtadially [44]. Generally, ticks have three host life cycles. Ticks seek a host for attachment to feed for several days. Once replete, ticks detach from a host and use the blood meal to progress to its next stage of life cycle. The life cycle of Ixodid ticks is typically completed in 2-3 years, which depends on the environment and the availability of hosts. Importantly, ticks quest for a host when seeking a blood meal and may wait in the environment for a long period until a suitable host is found [5, 42]. Humans are accidental hosts for tick feeding and pathogen transmission. Few species of ticks are host-specific for seeking a blood meal, whereas most others can take a blood meal from a wide range of vertebrates [5].

Ticks serve as the major vectors for rickettsial pathogens belonging to the rickettsiaceae and anaplasmatace family organisms. Ixodid ticks are first considered as the vectors for rickettsial organisms when Rocky Mountain wood tick (*Dermacentor andersoni*) is identified as the vector for transmitting *Rickettsia rickettsii*, the agent of Rocky Mountain spotted fever in the U.S.A in 1906 [45]. Ticks are responsible for causing several important rickettsials diseases to people. They include Rocky Mountain spotted fever (Western hemisphere), Rickettsial pox (USA and former Soviet Union), Boutonneuse fever (Mediterranean countries, Africa,

Southwest Asia and India), Siberian tick typhus (Siberia, Mongolia, northern China), Australian tick typhus (Australia), Oriental spotted fever (Japan), and African tick-bite fever (South Africa) [45-47]. Tick-borne rickettsial diseases in people caused by Anaplasmataceae family pathogens include human granulocytic anaplasmosis, human ewingii ehrlichiosis, and human monocytic ehrlichiosis [35, 48-50]. Ticks are also the major vectors for the Anaplasmataceae family pathogen diseases in various vertebrates including dogs, cattles and sheeps [37, 50, 51]. A list of Anaplasmataceae tick-borne human and animal infections disease is presented in Table 1.1.

Classification

Members of the genera *Ehrlichia* and *Anaplasma* are obligate intracellular bacteria currently placed in the proteobacteria phylum, secition alpha; order Rickettsials and family Anaplasmataceae, while other closely related genera are *Rickettsia* and *Orientia* belonging to the Rickettsiacae family within the order Rickettsials [52, 53].

The Anaplasmataceae family includes four genera, *Anaplasama, Ehrlichia, Wolbachia* and *Neorickettsia* [52][54]. *Anaplasama* and *Ehrlichia* species are transmitted by ticks [37, 52]. *Neorecketisia* species are also vector-borne pathogens, but are harbored by trematodes [52]. *Wolbachia* species are non-pathogenic endosymbionts in various arthropods and nematodes [55, 56]*. Anaplasma* genus includes several identified pathogens of people and animals: *A. phagocytophilum, A. mrginale, A. centrala, A. ovis* and *Anaplasma platys* [52]*.* Similarly, *Ehrlichia* genus also includes several pathogens causing diseases in people and animals. They

include *E. chaffeensis, E. ewingii, E. canis, E. muris and E. ruminantium* [19, 48, 50, 52]. *Neorickettsia* species included *N. sennestsu* (human pathogen)*, N.risticii* (horse pathogen), and *N. helmentheca (*canine pathogen) [57, 58]*.*

Ehrlichia species infect predominantly leukocytes of vertebrate hosts. The only exception is *E. ruminantium* that infects endothelial cells. *Anaplasma* species infect bone marrow derived cells in different animal hosts including leukocytes, erythrocytes, and platelets*. Neorickettsia* species infect predominantly mononuclear phagocytes and occasionally enterocytes in the mammalian hosts [54].

E. chaffeensis is an emerging human infectious agent which causes human monocytotropic ehrlichiosis (HME) [49, 59, 60]. *E. chaffeensis* is first discovered in 1986 in Arkansas in a human patient with flu like clinical symptoms [59]. Peripheral blood smears analysis of the patient lead to the identification of *E. canis* like organism [61]. The identity of the organism causing the disease is established in 1991 following the molecular genetics analysis [61]. The organism is closely related to *E. cains*, and is named as *E. chaffeensis* [49]. Subsequent studies resulted in culturing the organism recovered from a patient in canine macrophage cell line, DH82.

E. chaffeensis is transmitted by the lone star tick, *Amblyomma americanum*. This bacterium is maintained in nature primarily in white-tailed deer (*Odocoileus virginianus*) population and in lone-star ticks [25, 62-64] . *A. americanum* ticks feed on white-tailed deer*,* and thus provide an ample opportunity for *E. chaffeensis* to be acquired and transmitted between ticks and deer population (Figure 1 and 2). *E. chaffeensis* also infects other vertebrates including, dogs, goats, rodents and mule

deer [20, 65-68]. White tailed deer is considered the primary reservoir host for the transmission cycle of this organism [64].

Reports of HME cases have increased during the last three decades. The higher incidence of HME is reported in immune compromised and elderly people, particularly with high morbidity and mortality rates [60]. An average number of 600 human cases of HME are reported each year in the United States (Figure 3) [60, 69]. Most of the reported HME cases are from south central and southeastern regions of the United States where the vector tick population is more prevalent [60, 70-73](Figure 4).

E. chaffeensis is an obligate parasitic pathogen that infects and multiplies within monocytes and macrophages of blood and various organs, including spleen, liver, lung and bone marrow of a host [19, 74, 75]. This pathogen also infects lymphocytes, atypical lymphocytes; promyelocytes, metamyelocytes, and in segmented neutrophils, but major infected cells are monocytes and macrophages [19, 76].

E. chaffeensis grows within the cytoplasmic vacuoles derived from the formation of early endosomes. Light microscopic examination of the polychromatic stained infected cells reveal *E. chaffeensis* organisms as mulberry-like structures within intracytoplasmic inclusions [77]. The organisms replicating within the cytoplasmic vacuoles are commonly referred to as morula. *E. chaffeensis* replicates within the vacuoles by binary fission [78]. Electron microscopic examination reveals two morphological forms; the dense core bodies (DCs) and reticulate bodies (RCs). Dense core bodies are electron dense bodies, considered as the metabolically inactive forms (Figure 6). The DCs are infectious forms released from infected cells

by lysis or by exocytosis. The RCs are metabolically active replicating forms mostly seen within the phagosomes.

Clinical signs

The clinical signs of *E. chaffeensis* infection causing HME disease may appear in patients within one to two weeks following blood feeding by an infected *A. americanum* tick and the pathogen transmission [79]. The early clinical signs vary from asymptomatic to mild flu-like symptoms [60, 79]. Most common symptoms include malaise, headache, lower back pain, muscle aches, chills, nausea, gastrointestinal symptoms, and development of sudden onset fever ~102˚F [80, 81]. Sometimes, the symptoms may include cough, pharyngitis, swollen lymph nodes, and vomiting [73, 82]. Some patients also develop rashes (30-40%). The infection may progress to severe illness with multiorgans failure. Greater than 50% patients are reported to exhibit moderate leucopenia and a sharp decrease in the white blood cell count [73, 82]. Nearly 90% of patients reported a decrease in the number of platelets. Along with abnormalities in the blood, elevated levels of liver transaminase are often noted because of infection [80, 81]. Approximately 20% of infected patients develop signs and symptoms of central nervous system of meningitis syndrome [49]. Although people of all age groups are susceptible, but most severe cases are observed in elderly age people with compromised immunity. The fatality of an individual may occur due to hemorrhage, organ failure, or development of secondary bacterial infections [49, 83].

Diagnosis for *E. chaffeensis* infection is more often based on patient's history (such as exposure to tick bites) [60], a blood smear analysis revealing the presence

of infected monocytes with other laboratory techniques, such as the indirect immunofluroscence assay for *E. chaffeensis* antibodies and PCR amplification of a specific genomic region of the pathogen. Studying blood smears is a common diagnosing practice, but it is considered as insensitive in detecting the pathogen. The pathogen is identified by staining blood smears by Romanovsky type polychromatic stains [84]. Indirect immunoflorscence assay (IFA) is used for the detection of antibodies against *E. chaffe*ensis antigens, but due to cross reactivity with other closely related organisms, the test can lead to false positives [85]. In patients at early stages of infection, the low amount of antibodies may result in false negatives. PCR assays are used for identifying DNA recovered from a whole blood sample or a serum sample. The PCR assays are rapid, sensitive, and specific in diagnosing a patient sample, but are not routinely used for human clinical diagnosis. The targets used for PCR assays include GroESL gene, Variable Length PCR Target gene (VLPT) or species-specific segment of a 16S rRNA gene [60, 61, 80, 86, 87].

Treatment

E. chaffeensis appears to be resistant to most of the antibiotics, including ciprofloxacin and penicillin. Tetracycline and its derivatives, broad-spectrum antimicrobials which inhibit protein synthesis in various bacterial species have proven very effective in treating the infection. Doxycycline in particular is considered as an effective drug of choice for the HME cases [19, 79].

Molecular biology:

The genome of *E. chaffeensis* is 1.18 mb and includes 1115 open reading frames [88, 89]. The *E. chaffeensis* genome is considerably smaller than *E. coli* genome. It is about one quarter in size when compared to *E. coli* genome. Many genes in *E. chaffeensis* have been lost in the course of evolution, possibly for its adaptation to obligate parasitic life [89]. They include genes required for the biosynthesis of lipopolysaccharides and peptidoglycans [90, 91]. This organism is an auxotroph and depends on the host for amino acids and other metabolites.

Several studies reported the molecular characterization of various genes in this organism. They include various unnamed genes for p19, p22, p28, p32, p44, p106, p120, p200 kDa protein coding genes and quinolate synthatase gene [91-94]. In addition, Type VI secretin system and two components regulator protein system also have been partially characterized [95, 96].

Several immune reactive proteins of *E. chaffeensis* were identified from this organism. They include, p28 kDa, p47 kDa, p120 kDa proteins are expressed on the outer membrane of *E. chaffeensis* and appear to interact with the host cells [77, 92]. The p120 kDa and p47 kDa proteins are differentially expressed in dense core bodies in infected monocytes, while the p28 proteins are shown to be expressed differentially in the tick cells and macrophages [77, 85]. Several genes of *E. chaffeensis* reported in literature contain tandem repeat sequence within the protein coding sequences. They are p32 (previously known as variable length PCR tandem repeat), p47, and p120 and p200. The tandem repeat sequences include amino acids serine and threonine. These tandem repeat sequences of proteins are strongly

recognized by the immune sera of *E. chaffeensis* infected hosts, including humans and dogs [85, 97, 98] .

The p47 is 285 amino acids longer, immunoreactive protein and includes 19 amino acids tandem repeats. Approximately half of the protein is represented by the repeated sequences [98]. The p47 protein shows homology with renin receptor/ATP6AP2/CAPER protein and with DNA III polymerase subunit gamma. These proteins were expressed only on the surface of the dense core forms of *E chaffeensis* [98, 99].

The p120 kDa protein is another immunoreactive differentially expressed protein of this organism [97]. Its expression is higher in the dense core bodies. This protein is also composed of multiple repeat sequences, which includes serine residues. This protein may act as an adhesion and interacts with the host cell to facilitate pathogen survival [87]. The immunogenic p140 kDa protein of *E. canis* is homologues to p120 kDa protein of *E chaffeensis* [87, 97].

The p200-kDa protein, previously considered as glycoprotein, is also a tandem repeat protein. The tandem repeats in this protein are 19 ankyrin sequences [98]. This protein is similar to p200 and AnKA of *E. canis* and *A. phagocytophilum,* respectively [100]. (Which also contain ankyrine repeats). These proteins are translocated into the nuclei of infected host cells. They interact with the adenine – rich motif of host gene promoters and intergenic Alu repeat sequence [98, 101]. The p200-kDa protein may also play a role in inhibiting apoptosis of the infected cells. The implications of these properties are that the bacterium may alter the host gene expression in support of its survival [101].

The p28 proteins are the major expressed proteins on the outer membrane of the organism [102-105]. The homologues of these proteins are identified in other *Ehrlichia* species, Such as *E. rumantium*, *E. muris*, *E. canis* [103, 104]. These proteins also share considerable homology to other surface proteins of *A. phagocytophilum* and *A. marginala*. *A. phagocytophilum* and *A. marginala* proteins have been shown to be involved in antigenic variation in support of the pathogen to escape from host immunity [106-110]. The p28 proteins in *E. chaffeensis* are differentially expressed in macrophages of vertebrate hosts and infected tick cells under both *in vitro* and *in vivo* conditions [111-115]. The differentially expressed p28 proteins in macrophages and tick cells are p28-Omp14 and 19, respectively [112-114].

Several other proteins are also expressed in the intercellular development of the pathogen during its replication and maturation. They include type four secretin system (T4SS) apparatus proteins and two-component regulator system proteins. The T4SS is an ATP dependent bacterial transport system which transports macromolecules (such as proteins and DNA) across the bacterial and host cell membrane [95, 116]. The T4SS may be used by Gram negative bacteria to deliver virulence factors to modulate the host genomes in support of their survival. The T4SS proteins expression is observed in the organism present in infected host cells. The T4SS proteins are constitute of a complex assembly of proteins. They include proteins made from four virB2, one virB3, two virB4, four virB6, two virB8, two virB9, one virB10, one virB11and one virD4 [95, 117].

Recent studies suggest that T4SS is used to deliver AnkA repeat proteins from *E. chaffeensis* and *A. phagocytophilum* to infected host cells [95, 118, 119]. The

AnkA a protein of *A. phagocytophilum* has also been shown to be transported into host cell nucleus and interact with promoters and various repeat sequences in the genome. The significance of these interactions remains to be established [118-120]. However, like other Gram-negative bacteria, the proteins secreted by T4SS may be important in altering the host gene expression in support of the pathogen for its survival.

E. chaffeensis genome includes genes required for the expression of two component regulatory system [121, 122]. Recent studies demonstrate that three histidine kinase proteins and three response regulators are expressed by *E. chaffeesnsis* when it replicates in human leucocytes [121]. Furthermore, inhibition of the histidine kinase function by the drug closantel (a known inhibitor of histidine kinase) resulted in the complete blocking of infection with this organism to host cells. These results suggest that the two component regulatory system is needed for the pathogen's survival in vertebrate host cells [54, 116]. The two component regulatory system is also functional in the *A. pahagocytophilum* [89].

E. chaffeensis obtains cholesterol or related sterols form the hosts or its environment to stabilize the cytoplasm membrane as it lacks genes for the synthesizing sterols. *E. chaffeensis* also lacks genes for synthesizing lipopolysaccharides (LPS) [90, 123]. In the absence of LPS on the cell surface, the organism is only protected with the membrane containing cholesterols [90, 124].

The host response to the *E. chaffeensis* infection

More severe cases caused by *E. chaffeensis* are reported in immune compromised individuals and elderly people. In recent times, severe cases of HME are also reported in children [60, 125, 126]. Several studies have been carried out to understand the immune response by host during infection with *E. chaffeensis* by performing experimental infections using the murine host. The murine studies suggest that the immunocompentent mice are able to clear the infection within 10-14 days [127-129]. In severe combined immune deficient (SCID) mice, which lack B and T-cells, the pathogen causes severe fatal disease [130, 131]. Persistence infection is observed in MHC II deficient mice infected with *E. chaffeensis* [129]. $CD4^+$ helper T-cells deficient in mice clears the infection, but the clearance is delayed. The delayed clearance in $CD4^+$ T-cell in different mice suggests that in amongst these T-cells, alternate T-cells such as gamma, delta T-cells may contribute to the pathogen clearance [132]. Similarly, infection in toll receptor deficient mice results in delaying the clearance of the pathogen for up to 30 days [127, 132]. These observations suggest the importance of both B-cells and T-cells in clearing the infection by host. These studies also demonstrate the importance of MHC II and toll like receptors to generate immunity against *E. chaffeensis* infections [127].

Antibodies also play an important role in the clearance of the pathogen from a host. It has been reported that the immune serum from an infected immune competent mice can protect *E. chaffeensis* infected SCID mice [129, 133]. The immunsera includes antibodies which recognize proteins expressed on outer membrane of *E. chaffeensis* [133, 134]. The antibody assessment studies also demonstrate that the antibodies alone are not sufficient to clear the pathogen from

the infected host. Cytokines are also involved in clearing of the pathogen [135]. Cytokines have an important role in mediating *E. chaffeensis* clearance from a vertebrate host. Human monocytic cell line (THP I) infected with *E. chaffeensis* suppresses the expression of several cytokines including IL-1α, IL-4, IL-6, IL-12, IL-15, and IL-18 [135]. Cytokines IL 15 and IL18 usually serve as the activators of IFN which activates the macrophage cells to kill a pathogen [54, 135, 136].

Recent proteomic studies from our laboratory identified 278 expressed proteins of *E. chaffeensis* [137]. The expressed proteins included numerous differentially expressed proteins of the organism grown in macrophages and tick cells [138]. The differentially expressed proteins also included several outer membrane proteins. Our laboratory also reported differences in the host response against *E. chaffeensis* originating from tick cells and macrophages in the murine host. These studies suggest that the pathogen clearance is delayed in vertebrate host when *E. chaffeensis* originated from tick cells [132]. The antibody and cytokine responses in mice infected with tick cell derived bacteria differed considerably compared to mice infected with organisms originating from vertebrate macrophages. The antibody response also steadily increased in mice infected with tick cell derived bacteria [128, 139]. Together these data suggest that the host cell specific differential protein expression by *E. chaffeensis* aids in the pathogens adaptation and persistence in a vertebrate host. As differentially expressed proteins included p28-Omp multigene locus proteins (described details below), the secondary structure analysis (conducted in the current studies) will be important to assess the biological significance of these proteins to the pathogen.

p28- Outer membrane proteins (p28-Omps)

p28-Omps of *E. chaffeensis* are encoded by a multigene locus (p28-Omp locus). This multigene locus contains 22 tandemly arranged paralogos genes which are separated by intergenic sequence varying from 9 to 600 bp [88, 111, 113, 115, 137, 139-141]. As the estimated molecular weight of the proteins encoded from p28 locus is between 28 to 32 kDa, the genes are referred to as p28-Omps. The p28-Omp gene coding sequences share extensive homology, throughout the coding sequences, except for three hypervariable regions [140]. The hypervariable regions contain immunogenic B-cell epitopes and are recognized by sera from the *E. chaffeensis* infected people and animals [134, 142]. The p28-Omp locus also differs considerably in several *E. chaffeensis* isolates [115].

The homologs of p28-Omp locus are also found in other *Ehrlichia* species. The p28-Omp locus (referred as p30-Omp locus) in *E. canis* also includes 22 tandemly arranged genes. In *E. ruminantium*, this locus is referred as MAP1 and it includes 19 genes [143]. *E. ewingii* and *E. muris* has 16 and 21 paralogous genes, respectively [104, 105]. (A cartoon representation of the p28-Omp multigene locus including the host specifically expressed genes of *E. chaffeensis, E. canis, E. ruminantum* is shown in Figure 7)

Several studies have been performed to map gene expression of the p28-Omp loci in *Ehrlichia* species. Based on the transcriptional analysis, multiple genes of the p28-Omp locus in *E. chaffeensis* are shown to be transcriptionally active in macrophages with maximum expression observed for p28-Omp 19 [105, 141, 143, 144]. In tick cells, p28 Omp 14 gene product is the only one detected. The differential expression is conformed in infected vertebrate hosts and ticks [104, 112,

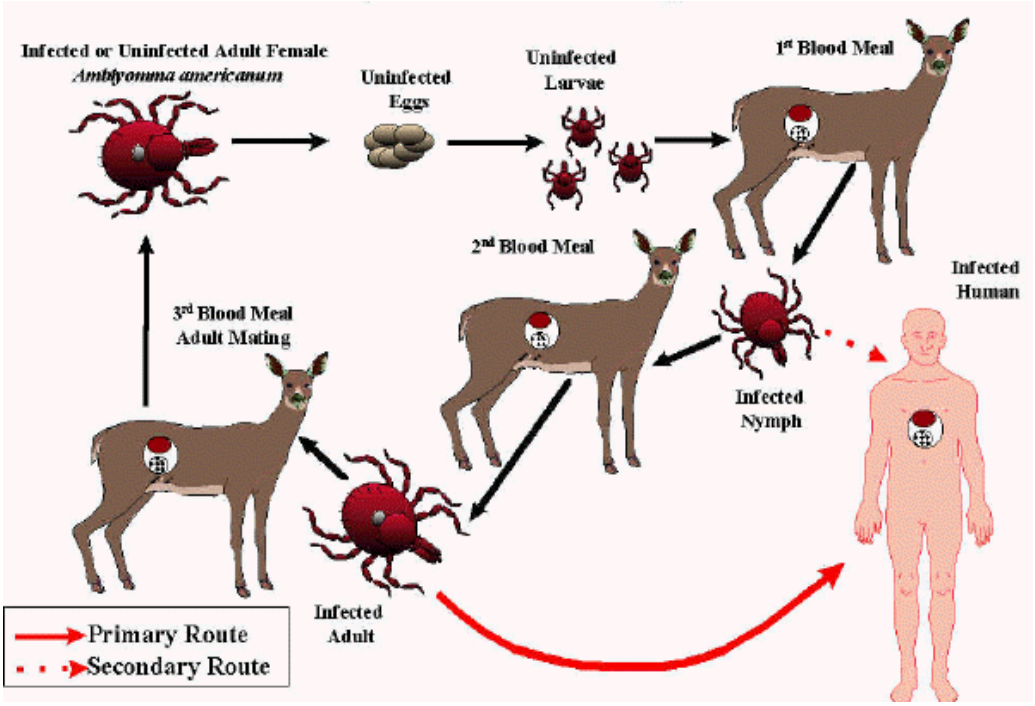
145-147]. The differential expression is also reported for homologs of the p28-Omp multigene loci *E. canis* and *E. rumianatium*. These differentially expressed proteins of *E. chaffeensis* and *E. canis* are also post transnationally modified to include glycosylation and phosphorylation moieties [138, 141]. The major surface protein (msp2) and outer membrane protein (p44) of *A. marginale* and *A. phagocytophilum,* respectively, *are* closely related to p28-Omp proteins [106-110]. The msp2 and p44 proteins are also made from multigene loci and include highly immunogenic variable regions [52, 145]. The antigenic variants generated from msp 2 and p44 genes of *A. marginala* and *A. phagocytophilum* are shown to play a role in immune evasion [51, 148-150].

Despite the detailed knowledge about the p28-Omp genes primary structure, their expression patterns, their recognitions by B-cells, very little is known about their secondary and tertiary structure in the outer membrane, similarly, their biological function is not well described. In an effort to understand the structure and functional relations, kumagai et al performed structure predication analysis and also examined the porin activity for two p28-Omp 18 and 19 proteins. The studies also suggest that two of the p28-Omp protein 18 and Omp 19 possess porin activity [151].

Figures related to Chapter one

Figure 1 *Amblyomma Americanum*

Figure 2 White tailed deer



Source: http://www.cdc.gov/Ncidod/dvrd/ehrlichia/Natural_Hx/nathx1.htm

Figure 3 Proposed life cycles for *E. chaffeensis.*

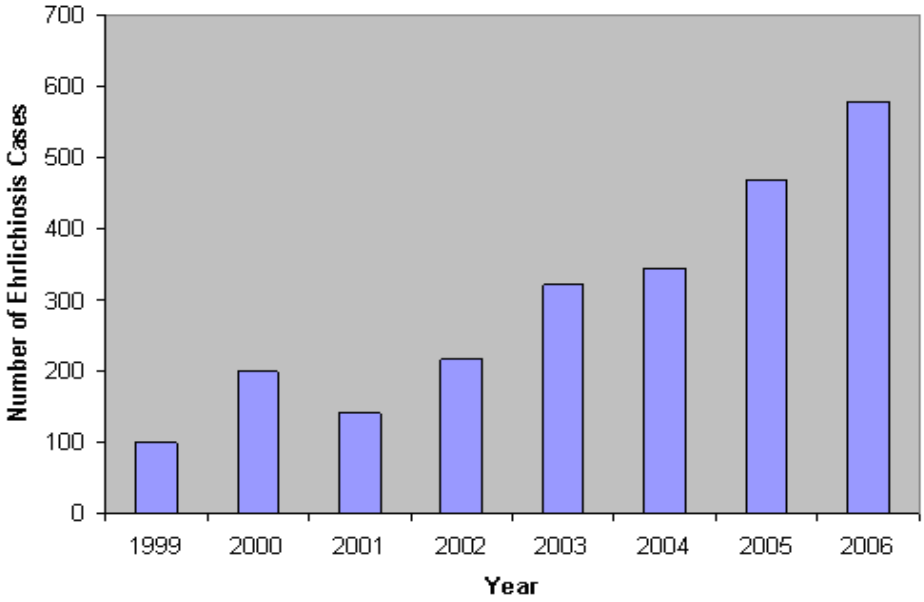


Figure 4 Number of Ehrlichiosis cases (caused by *Ehrlichia chaffeensis*) reported to CDC by state health departments, 1999-2006.

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

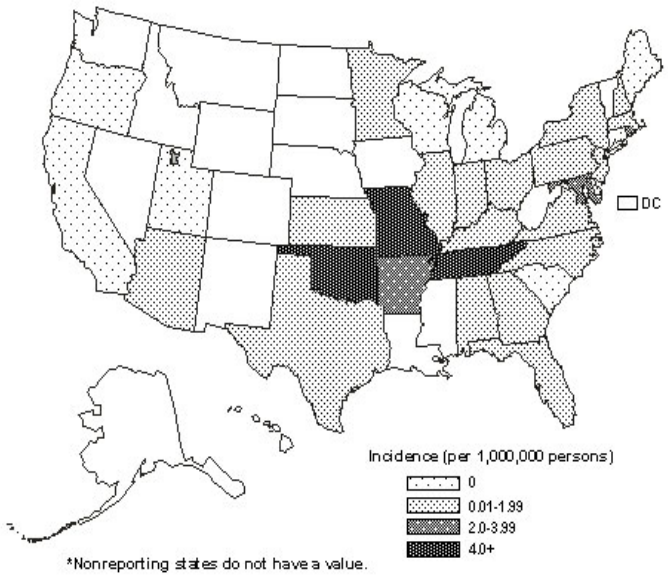


Figure 5 Distribution of *Ambloymma Americanum* USA.

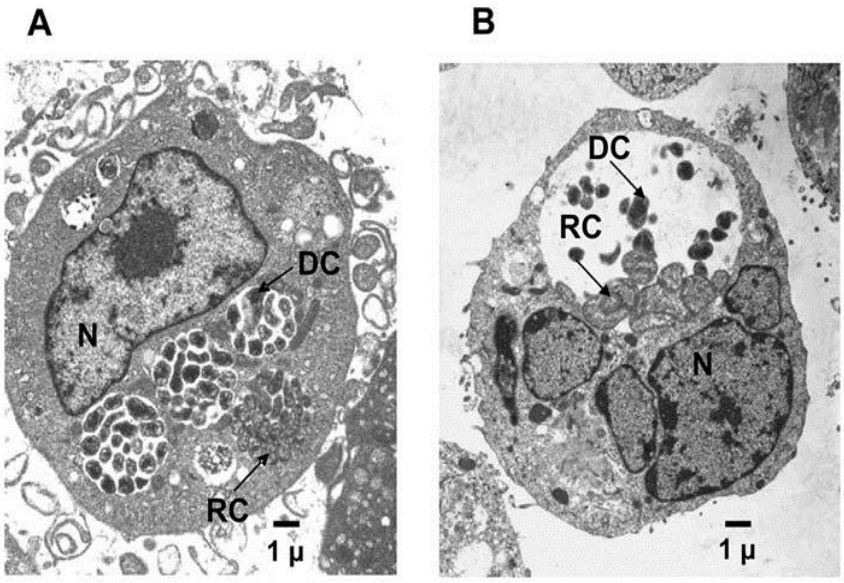


Figure 6 Transmission electron microscopy analysis was performed on E. chaffeensis-infected macrophages (A) and tick cells (ISE6) (B). (N, nucleus; DC, dense-cored bodies of Ehrlichia in phagosomes; RC, reticulate bodies of Ehrlichia in phagosome) (Reproduced with permission from Frontiers In Biosciences. 14: 3259-73).

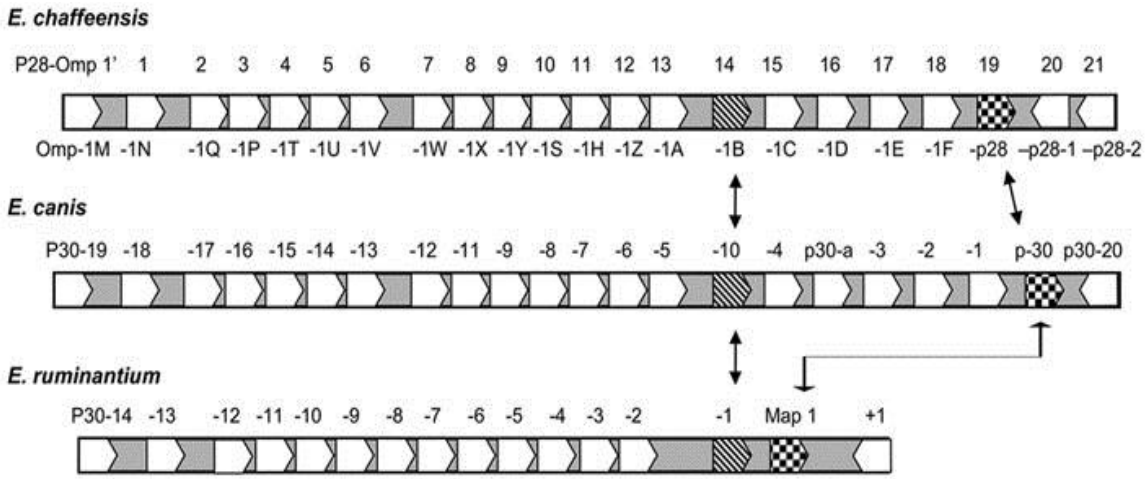


Figure 7 A cartoon representing the p28-Omp loci of *E. chaffeensis*, *E. canis* and *E. ruminantium* with identified expressed proteins from the p28-Omp genes in vertebrate macrophages (hatched boxes) and tick cells (checker board boxes) are presented (Reproduced with permission from *Frontiers In Biosciences*. 14: 3259-73).

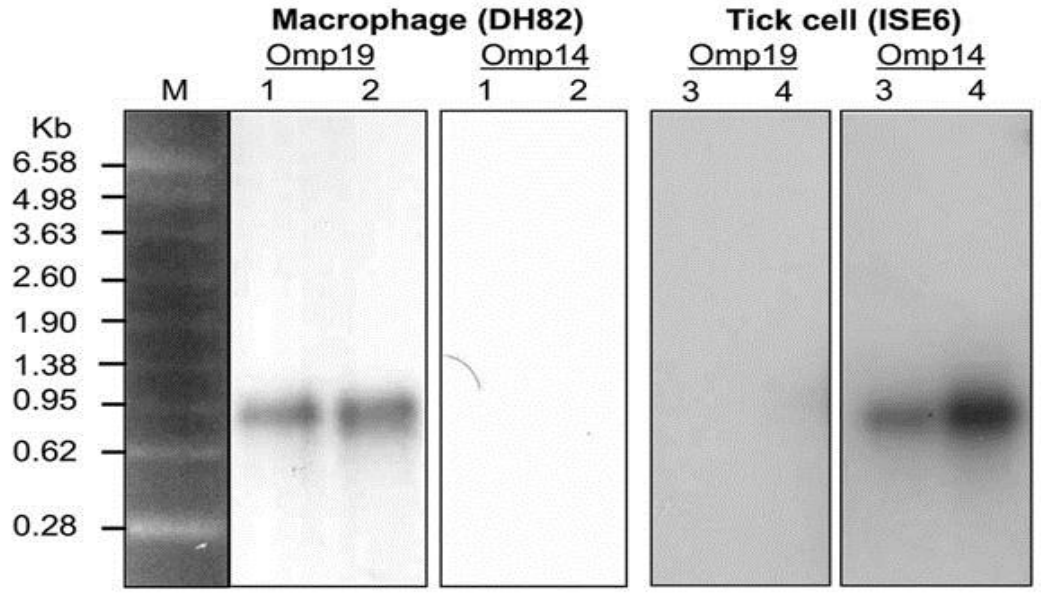


Figure 8 Northern blot analysis: DH82 (lanes 1 and 2) and tick cell line, ISE6 (lanes 3 and 4) assessed by Northern blot analysis using p28-Omp 14 or 19 gene-specific 32P-labeled probes. Gene 19 transcript of the size 0.9 kb is detected only in macrophage-derived RNA. Similarly, the tick cell-derived RNA contained 0.9 kb transcript for gene

CHAPTER II:-

STRUCURAL PREDICTION ANALYSIS OF *EHRLICHIA CHAFFEENSIS* OUTER MEMBRANE PROTEINS, P28 OMP-14 AND P28 OMP-19 ASSESSED BY CIRCULAR DICHROSIM AND PORIM ASSAY

Introduction

Ehrlichia chaffeensis, obligate intercellular bacterium, causes human monocytic ehrlichiosis (HME) [49, 61]. HME is considered as an emerging disease in people. *E. chaffeensis* resides in phaogosomes of monocytes or macrophages of vertebrate hosts [61]. This organism also infects several other vertebrates including dog, goat, coyote, and white tailed deer [20, 65-68]. *Amblyomma americanum* tick serves as the vector and white tailed deer serves as the reservoir host for this pathogen [62-64, 152].

E. chaffeensis may have evolved unique protein expression strategies in support of its growth in tick and vertebrate host environments. This organism persists in both vertebrate and tick hosts. Recent studies demonstrated changes in the protein expression of the organism replicating in macrophages and tick cells [143]. Host cell-specific differential protein expression may be essential for *E. chaffeensis* to adapt to vertebrate and tick hosts [112, 137, 138, 141]. Differentially expressed proteins of *E. chaffeensis* include several outer membrane proteins. The p28 Omp proteins are the most abundant outer membrane proteins expressed by *E. chaffeensis* [112, 141, 143].

In tick cells and macrophages, the p28-Omp proteins are differentially expressed. P28-Omp14 is the expressed protein in tick cells while p28-Omp19 is the major expressed protein in macrophages [128, 138]. The differential expression of these proteins is also confirmed for the pathogen in tick and vertebrate hosts [112, 137, 138, 141].

The p28 Omp proteins consist of three hyper variable regions are which contain hydrophilic domains and are recognized by the immune sera against *E. chaffeensis* [139, 140, 146]. Immunization with the p28-Omp recombinant proteins protects SCID mice against of *E. chaffeensis* infections [134, 142].

Typically, the outer membrane proteins serve as the permeability barriers to excrete noxious substances of bacteria and for the uptake of nutrients from the environment [153]. The precise functions of the p28-Omps in *E. chaffeensis* remain to be established.

In this study, we expressed the p28-Omp14 and p28-Omp 19 gene products of *E. chaffeensis* using *E. coli* expression system. The recombinant proteins were purified to near homogeneity and used to study the refolding in detergents to mimic structures in the outer membrane of the organism. The protein function of p28-Omp 14 and 19 was assessed by evaluating the porin activity after reconstituting the protein in a liposome.

Materials and method

In silco analysis:

We analyzed the protein sequences of p28-Omp 14 and 19 to predict the hydrophobicity using the ProtoScale program available at (http://expasy.org/tools/protscale.html). By calculating the average hydrophobicity and average amphilicity index, can structural predications can be made for a protein. The hydropathy plot is calculated with the help of index of Kyte and Doolittle and amphiphilicity indexes [154, 155]. The amphilphilicity of polar side chains is the

second parameter with a calculated index of amino acid charges. The transfer energy of the hydrocarbon part of a polar side chains are also used by the program to prepare the hydrophobicity plot [154].

Analysis of the two dimensional structures of the p28-Omp 14 and 19 was done with the help of the PRED-TMBB protein prediction program [155, 156] (http://biophysics.biol.uoa.gr/PRED-TMBB/). CPHmodels-3.0 Program is helpful in the predicting the 3D MODEL of the protein (http://www.cbs.dtu.dk/services/CPHmodels/) [157]. This program is useful in predicting a 3D structure of a protein. The 3D model predictions for p28 Omp-14 and 19 were performed by uploading the entire protein coding sequence except the first 25 amino acids into the program. To generate a 3D model, hydrophobic values and ampiphilic values obtained from ProtoScale program analysis were used as per the instruction provided at the program website.

Cloning and expression p28 Omp14 and p28 Omp19 gene products:

The protein coding sequences of the p28 Omp14 and p28 Omp19 were amplified from *E. chaffeensis* genomic DNA and cloned into pET 28 expression plasmid vector (Novgen, Gibbstown,NJ). Nco I and Xho I sites of were engineered into the PCR products to aid in the directional cloning into the pET 28 plasmid. The coding sequences excluded the first 25 amino acids, as they were considered as the signal sequences. Standard molecular cloning protocols were followed for preparing the recombinant plasmid constructs. Briefly, the PCR products and pET 28 plasmid were digested with Nco1 and Xho1 and the DNAs were purified by phenol/chloroform and ethanol purification precipitation method. The PCR products

and the linerzed plasmids were ligated (5 to 1 ratio) using T4 DNA ligase. The ligated products were then transformed into *E. coli* XL1blue and plated on agar plate containing kanamycin. Colonies were randomly picked and grown in LB media (Kanamycin resistance is conferred for the pET 28 plasmid). The presence of inserts in a recombinant plasmid was verified after preparing plasmid DNA from 3 ml liquid culture and the performing restriction enzyme digestion with Nco1 and Xho1. Plasmids containing ~0.9 kb inserts were identified following resolving the digested DNA on a 1% agarose gel. The integrity of sequence a recombinant plasmid was confirmed by performing DNA sequence analysis of the plasmid DNAs. The plasmids containing desired gene segments were retransformed into *E. coli* strain BL21 (DE3). Transformed BL21 (DE3) strain cultures were assessed for the presence of recombinant proteins. For this experiment, Culture was grown in 10 ml LB medium with kanamycin (10µg/ml final concentration) and when the culture reached to an optimum density of ~ 0.6 at 600, protein expression was induced with 1mM IPTG. The expression of the recombinant proteins was confirmed after analyzing cell lysates prepared from the cultures The 10 ml liquid culture were centrifuged for 5 min at 4000 rpm (Ependroff centrifugal 5810R). The supernatant was discarded and the bacterial pellet, I ml of lysis buffer (50 mM Tris-HCl, 5 Mm EDTA, 100 Mm NaCl, 0.5% Triton 100-X) containing lysozyme (2 µg/ml final concentrations) was added. The culture was resuspended by vortexing several times and subjected to sonication for 1 min using Sonic Dismembrator (Fisher scientific) at setting of 9. The cell lysates were separated to soluble and insoluble fractions by centrifugation at 4ºC for 5 min at 12,000 rpm (Ependroff centrifugal, 5810R). The presence of expressed proteins was evaluated in both the fractions, equal volumes of supernatant and 2x SDS gel loading buffer were mixed to prepare the protein

solution for analyzing on protein gel. To the insoluble fraction, 100µl of 1x SDS gel loading buffer was added and vortexed several times to solubilize the proteins. Twenty microliter each of the supernatant and insoluble fractions derived proteins were used to resolve on a 15 % polyacrylamide gel containing 10% SDS. The protocols to SDS for polyacrylamide gel electrophoresis were followed as described in book of Sambrook et al. The electrophoresed gel was stained with coomasie blue G250 staining solution and examined for the presence of proteins. The recombinant expressed proteins were identified in the insoluble fractions of expression constructs prepared for both p28-Omp14 and 19 genes. To verify the presence of specific products, lysates prepared from a non-recombinant plasmid transformed into *E. coli* BL21 (DE3) strain were used. Predicted 28 kDa proteins were observed in the insoluble fraction desired proteins of the p28-Omp 14 and 19 gene.

Purification of recombinant proteins:

The *E. coli* strains containing pET 28 recombinant plasmids were grown at 37ºC in 10 ml of Laurie broth (LB) medium containing kanamycin (10µg/ml final concentration) for about 14 hours. These cultures were transferred to 1 liter of LB medium with kanamycin and incubated in a shaker incubator at 37ºC for expanding the cultures. When the cultures grown to 0.5 optical density units (measured at 600 nm), protein expression was induced by adding 1 mM isopropyl beta D-thiogalactopyranoside (IPTG). The cultures were continued to grow for six hours at 37ºC. The bacterial cultures were collected as fractions of 50 ml in Falcon tubes and centrifuged at 4000 rpm for 10 min (Ependroff Centrifuge, 5810 R). The culture pellets were pooled to four falcon tubes after resuspending in 25 ml of lysis buffer [500 mM Tris HCl, 100 mM NaCl, 5 mM EDTA, 0.1 mM

phenylmethanesulfonylfluoride (PMSF) containing of 25 µl lysozyme (20 mg/ml)] and then incubated at 37ºC for 30 min. The culture lysates were sonicated using Sonic Dismembrator (Fisher scientific) for 5 min at setting 9. The lysate was centrifuged at 4000 rpm for 10 min to separate the insoluble and soluble fractions. To the pellet containing the insoluble fraction were dissolved in 25 ml of lysis buffer and vortexed to mix the insoluble fraction. The mixture was then centrifuged from 10 min at 4000 rpm as described above and the supernatant was discarded. This step was repeated one more time to recover clean insoluble fractions that contained inclusion bodies with recombinant proteins. The final recovered inclusion bodies were solubilized in 20 ml of denature buffer [20 mM Tris-HCl buffer (pH 8.5) containing 8 M urea] and placed in a 65ºC water bath for 30 min to dissolve the pellet (votexing for every 10 min, If needed). After 30 min, the dissolved solution was centrifuged at 36,000 rpm for 90 min (Beckman ultracentrifuge- optima max high capacity, SW 50.1) to remove any non-soluble proteins or macromolecules. Clean supernatant containing solubilized proteins was transformed to a sterile 50 ml Falcon tube and stored at -20 ºC for further use. The presence of recombinant protein in this fraction was evaluated by subjecting 25 µl each of the fractions in a 15% SDS –polyacrylamide gel and stained in coomassie blue staining. After verifying the presence of recombinant proteins, the solutions were used to purify using ion exchange and size exclusion chromatography methods to prepare purified recombinant proteins.

Ion exchange and size exclusion chromatography:

Anion exchange chromatography was used for the initial steps of purification for p28-Omp14 and p28-Omp19 recombinant proteins. The size of the anion exchange column used for purification is 2.5 cm x 10 cm (Bio-Rad, Hercules, CA).

The column was packed up to 8 cm height of the column with anion-exchange beads (Q Sepharose Fast Flow, Amersham Pharmacia Biotech, Piscataway, NJ) and was equilibrated with 100 ml of 20 mM Tris HCl (pH 8.5) containing 8 M urea (denature buffer). Subsequently, the solution containing a recombinant protein was loaded on to the column and washed with another 100 ml of denature buffer. Bound proteins were eluted with a linear gradient of 0 to 1 M NaCl (200 ml volume) prepared in denature buffer. Total 100 fractions (2 ml each) were collected. The presence of protein in fractions was assessed at 280 nm by using U.V. spectrophotometer. Based on the values obtained from the U.V spectrophotometer, graph was plotted with fractions on X-axis and optical density values on Y-axis. Fractions containing the major protein peak(s) were further analyzed to identify the presence of recombinant proteins. Twenty-five microliter each of the fractions were resolved in a 15% SDS polyacrylamide gel and stained by following silver staining protocol. The fractions having the desired protein (~28 kDa) at the highest concentration were pooled (Figure: 2.10 for p28-Omp14 and Figure: 2.11 for p28-Omp19).

Size exclusion chromatography:

The ion exchange column purified pooled proteins representing ~ 28 kDa size proteins were concentrated to 1 ml by using Amicon ultra centrifugal filters (10,000 Da capacity, Millipore). Proteins solutions were transferred to the filtration unit and centrifuged at 4,500 rpm for 40 min in Ependroff Centrifuge, 5810 R. The concentrated p28 protein solutions were subjected to size exclusion chromatography. The size of the column used for this analysis is 1 cm x 170 cm (Bio-rad). It was packed with Superdex beads (Superdex ^{TM} Prep grade, Amersham Pharmacia Biotech, Piscataway, NJ) to fill the column bed up to 150 cm height

(approximately 200 ml of Superdex beads were used). The column was saturated with 20 mM Tris HCl (pH 8.5) containing 8 M urea (denature buffer) by washing with about 200 ml buffer. Subsequently, concentrated protein solution of p28-Omp 14 or 19 was loaded on to the column and eluted with 200 ml of denature buffer. Flow was adjusted to 1 ml per 5 min. Total 200 fractions (1 ml per fractions) were collected and analyzed using U.V spectrophotometer set at 280 nm to identify the presence of a protein. Graph was plotted with optical density on X- axis and fractions collected on Y-axis. Proteins fractions containing the highest absorption at 280 nm were collected and the presence of p28 proteins was identified by performing PAGE in presence of SDS (Outlined were mentioned above). The fractions containing 28 kDa proteins were pooled and concentrated to 1 ml solution by using Amicon ultra centrifugal filters as mentioned above. The protein concentration was estimated using the kit for RD-DC method as per the manufacturing instructions (Bio-Rad, Hercules, CA). The concentrated proteins were then stored at -20ºC until further use.

Refolding of recombinant protein by micelle formation:

The recombinant p28-Omp 14 or 19 proteins were concentrated further to 10 mg in 0.3 ml volume of Tris HCl buffer (pH8.5) containing 8 M urea. The proteins were diluted into 20 ml of detergent solution. Typically, 20 µl each of the protein solution was added to the detergent solution while vortexing for using a magnetic stirrer at 4ºC. The slow addition of protein solution was completed in about 2.5 hours. Three different detergents were used for the refolding of proteins. They are Dodecylphosphocholine (DPC), Dihexanoylphosphatidylcholine (DHPC) or β n-Octyl-β-D-Glucopyranoside (β-OG). The detergent solutions were prepared in 20 ml

of 20 mM sodium borate buffer (pH 10) containg150 mM NaCl and 1 mM EDTA for DPC (15 mM). The DHPC (24 mM) and β-OG (24 mM) detergent solutions were prepared using 20 mM sodium phosphate buffer (pH 10) containing 150 mM NaCl and 1 mM EDTA.

CD Spectrophotometer: The secondary structure of proteins was evaluated by performing CD Spectroscopy scans at wavelengths from 190–250 nm as described earlier [158]. CD spectroscopy analysis was performed for the detergent treated proteins in which proteins were imbedded to form micelles in a buffer. CD measurements were performed using a Jasco J-715 spectrometer (Jasco.co, Oklahoma City, OK). The spectra were measured in a 200 µl capacity 1 mm thickness cuvette with 1 cm path length using the protein solution imbedded in micelles (approximately 0.4 mg of protein/ml of solution). The CD measurements were expressed in terms of molar ellipticity. The CD data were assessed to identify the presence of α helices and β sheet folding structures. The protein structure was predicted, based on the negative minima observed in the wavelength scans. The minima occur at 222 nm and 210 nm is related to the presence of α helices structures. While bending is observed at the 218 nm is related to β sheet structure [158-161].

Liposome assay (for determining the porin activity):

Porin activity of the recombinant proteins was determined after reconstituting protein in detergents to form proteoliposomes as previously described with few modification [162, 163]. Typically, mixture of 2.4 picomoles of acetone-washed egg phosphatidylcholine (Sigma-Aldrich, St. Louis, MO) and 0.2 picomoles of diacetylphosphate (Sigma-Aldrich, St. Louis, MO) were dried under a stream of

nitrogen gas at the bottom of a test tube. Then the lipid film was resuspended in 0.2 ml of 20 mM Tris-HCl buffer containing 2 µg of purified protein solubilized using β-OG. The resuspension step was completed by vortexing for about a minute and then by a brief sonication (1 to 2 min) in a Branson bath-type 1510 sonicator (Qsonica, LLC, Newtown, CT) at setting of 2.5 for 5 min. The lipid-protein mixture was then dried by using a vacuum desiccator (Labconco, FreeZone Freeze Dry System, Labconco Corporation Kansas City, Missouri). To make proteoliposomes, the dried protein-lipid film was resuspended in 0.3 ml of 10 mM Tris-HCl buffer (pH 7.5) containing 15% dextran T-40 (Sigma- Aldrich, St Louis,MO). Resuspension of the lipid film in the solution was carried out by occasional gentle rotation of the test tube, followed by 30 min of occasional hand shaking of the tubes. Seventeen microliters of proteoliposome suspensions mixed in 600 µl a solution containing solutes was used for proteoliposome swelling assays. Proteoliposome swelling was measured by recording the change in the optical density at 400 nm at various time intervals (0-500 sec). Proteoliposome swelling, an indication of solute uptake, was monitored by the decrease in the optical density after proteoliposome and solutes were mixed. The assays were performed for the following solutions at 33 mM; glucose (MW=180), fructose (MW=180), sucrose (MW=342), stachyose (MW=666) and L-glutamine (MW=146). Assays were also repeated four different L-glutamine concentrations (10, 20, 33, 40 mM).

Results

In silco analysis:

Hydrophobicity of the p28-Omp protein sequences was analyzed using ProtoScale computer program. Output from this analysis suggested that the p28-Omp 14 and 19 proteins have 9 hydrophobic regions [Figure 9 (A) (B)]. We predicted that these 9 hydrophobic regions represent transmembrane domains of the proteins. The presence of hydrophobic domains is similar for both p28-Omp proteins. However, there are several differences in the peaks were observed. The hydrophobic domains observed for both proteins spanned between the hydrophilic segments and included at both N-terminal and C-terminal parts of the proteins. Similarities in the hydrophobicity of the p28-Omp 14 and 19 proteins may reflect structural similarities within these proteins. Secondary structure prediction analysis by PREDTMBB program supported the ProtoScale hydrophobicity data for both p28-Omp 14 and 19 proteins. Specifically, the predicted structure by PREDTMBB program also identified 9 hydrophobicity segments of both p28-Omp 14 and 19 proteins. This program further identified these segments to be part of the membrane bound structures [Figure 10 (A) (B)]. The hydrophilic segments were predicted as exposed to extracellular environment of the outer membrane. The membrane bound nine segments were also predicted by the program as β turn secondary structures.

Three dimensional structure analyses of the p28-Omp 14 and 19 protein sequences were performed with the help of CPH model 3.0 program. The output data from this analysis supported the observations of hydropathy plot analysis and secondary structure predicted analysis by the above mentioned programs. The 3D model analysis showed a porin like structure with β barrel formations which included

hydrophobic domains of both the proteins. The β barrel structure appeared very similar for p28-Omp14 and 19 protein sequences. Hydrophilic domains predicted as exposed to the extracellular environment by PREDTMBB program were also found to be present in the 3D predicted models by CPH 3.0 program [Figure 11 (A)(B)]. The program predicted only one major extracellular loop for the p28-Omp 14 sequence that is away from the β barrel structure, whereas two loops were predicted for the p28-Omp19 protein. These sequence *in silco* analysis suggested that the p28-Omp proteins may contain membrane imbedded structures with β- barrel structure formations. The predictions are in agreement with the previous reports demonstrating the presence of the proteins on the outer membrane of *E. chaffeensis.*

Recombinant protein expression and purification:

In support of performing more detailed investigation of p28-Omp 14 and 19 proteins to assess the secondary structures, the protein sequences were engineered into an *E. coli* protein expression construct pET 28. The p28-Omp 14 and 19 proteins were produced from the pET 28 recombinant plasmids in *E coli* strain Bl21 (DE3). The expression of proteins was induced with IPTG. The proteins were expressed as inclusion bodies (judged form the presence of recombinant proteins found in the insoluble fraction of *E. coli* lysates) (Figure 12).The insoluble proteins from *E. coli* lysates were recovered by repeated washing of the collected inclusion bodies with lysis buffer followed by solubilizing the proteins in the denature buffer. Subsequent purification to obtain highly purified proteins was accomplished by using ion exchange and size exclusion chromatography methods. Inclusion bodies solubilized in denature buffer were used to absorb on to the ion exchange column

and subjected to elution with 0 to 1 M NaCl gradient [Figure 13 (A)(B)]. Protein elution was monitored for the presence of proteins in the fractions collected from column by U.V spectrophotometer at 280 nm. A major peak of protein was identified in the eluted fractions at 0.25 to 0.4 M NaCl gradient (collected from 10 fractions of 2 ml each) for both p28-Omp 14 and 19 proteins. These fractions were further analyzed in a denaturing polyacrylamide gel [Figure 14 (A) (B)]. The fractions containing ~28 kDa proteins were concentrated from 20 ml to 1 ml and used for size exclusion chromatography. Size exclusion chromatography purification was performed to further purify the proteins. The beads of Superdex ^{TM} Prep grade having the resolution capacity for proteins with molecular weight ranging from 10,000 to 60,000 kDa were used for the experiment. The concentrated protein from ion exchange column was added on to the size exclusion column. After the void volume, fractions were collected and the protein fractions were analyzed by U.V spectrophotometer [Figure 15 (A) (B)]. The fractions containing the p28-Omp 14 or 19 proteins were observed in fractions 22 to 32 (each fractions represents 2 ml) after the void volume of 30 ml. The presence of the p28-Omp proteins were further confirmed by denaturing polyacrylamide gel analysis. The protein fractions with the purified proteins were pooled and concentrated to 7 to 8 mg/ ml using Amicon ultracentrifugal filter. Protein fractions were assessed from various purification steps (Figure 16).

Refolding of recombinant proteins in detergents:

The purified p28-Omp 14 and 19 recombinant proteins in 8 M urea in 20 mM Tris-HCl were used to assess their folding in detergents; β-octyl glycoside (β-OG), dodecylphosphocholine (DPC) and dihexanoylphosphatidylcholine (DHPC). The

detergents were used to prepare micelles in which proteins were imbedded. Initial experiments were performed for p28-Omp 19 with varying concentrations of detergents and with a fixed concentration of 5 mg of the protein. Folding in β-OG was unsuccessful as the proteins were precipitated in it. Thus, the β-OG was rejected for subsequent analysis. To identify the concentration of detergents for the maximum refolding of proteins, we used varying concentrations of DPC (10, 12, 15, and 18 mM) and DHPC (22, 24, and 26 mM). The proteins folded in DPC and DHPC micelles were analyzed on a polyacrylamide gel in the presence of SDS. The analysis included proteins that were not treated with detergents (negative control). The p28-Omp 19 protein in all four concentrations of DPC had a protein band that migrated faster as compared to the control proteins (Figure 17). The relative abundance of the fast migrated protein band was the highest in 15 mM DPC. Similarly, DHPC treated protein also had the fast migrating protein band (Figure 18). This experiment was repeated for both p28-Omp 14 and 19 proteins using a fixed concentration of 15 mM DPC and 24 mM DHPC. The presence of folded proteins was also assessed by denaturing polyacrylamide gel. The p28 Omp 14 or 19 proteins in DPC and DHPC are considered folded if fast migrating protein band are detected. This analysis included controls where proteins were resolved in the absence of a detergent. In addition, detergent treated proteins after denatured by boiling were also assessed. The p28-Omp14 and 19 proteins in DPC and DHPC had the major fast migrating proteins bands (Figure 19 and 20). The fast migrating protein bands were not observed in the samples that lacked detergents or boiled samples after detergent treatment.

The secondary structures of the detergent treated p28-Omp proteins were evaluated by circular dichroism (CD) analysis. The CD scanning analysis was

performed at near ultra violet wavelength (190 to 280 nm) to differentiate proteins containing β sheet structures or α helices. β-sheet structures exhibit characteristic negative bend in the wavelength scan that peaks at 218 nm and this depression should be more pronounced for the proteins in the presence micelles compared to protein in buffer. If proteins contain α-helices structures, the wavelength scans show two negative peaks; one each at 208 nm and 220 nm. We performed CD spectra analysis for the recombinant p28-Omp 14 or 19 proteins [Figure 21 (A) (B)]. The analysis were performed for proteins in presence of 15 Mm DPC or 24 mM DHPC. Controls for this experiment included CD analysis performed for proteins in the absence of a detergent. Similarly, buffer scan also recorded to scan as non specific control. The CD analysis revealed the characteristic negative peaks at 218 nm for both p28-Omp14 and 19 proteins as expected for proteins having β-sheet structures. CD scans for buffer alone exhibited straight lines for the entire wavelength scans. The CD spectrum for the proteins in borate buffer alone also had the characteristic bend at 218 nm. The peaks however, were greater for the proteins in DPC and DHPC.

Liposome swelling assay:

A recent study based on the bioinformatics analysis, suggested that *E. chaffeensis* p28-Omp19 and Omp 1F (p28-Omp 18) (another protein made from the p28-Omp multigene locus) have overall folded structures similar to the porin proteins of Gram-negative bacteria [166]. The authors in this study performed porin assays for the proteins and demonstrated that possess the porin activity [151]. To do similar analysis, proteoliposome were prepared with p28-Omp 14 and 19 recombinant proteins using egg-phosphotidyacholine and dicetylephostphate [163]. The

proteoliposomes were then assessed for the porin activity by allowing the solutes of glucose (MW: 180 Da), fructose (MW: 180 Da), sucrose (MW: 342 Da), stachyose (MW: 666 Da) or L-glutamine (MW: 146.5 Da). Generally, proteoliposomes respond to a solute and swell if it passes through it. We have selected four different size sugars to make isosmotic solution and to assess if the proteoliposomes can permit the sugars to transfer. We also selected L-glutamine for proteoliposome swelling assay similar to studies reported by Kumagai and Rikihisa [151]. In the present study, L-glutamine selected because it is considered as important amino acid to be important from a host cell by *E. chaffeensis*.

Control for this experiment included swelling assays performed for proteoliposome with buffer alone. Similarly, swelling assays were performed with liposome prepared without the addition of recombinant proteins. In this control, we only used buffer alone on buffer containing 33 mM L-glutamine. No swelling was observed for the liposome as judged from the flat line on a time scale analysis (not shown). The swelling for the proteoliposome as assessed by rapid decrease in absorbance at 400 nm is the greatest for water. The swelling of proteoliposome was also observed for 33 mM glucose, fructose, sucrose, and stachyose. In this experiment, we also performed the porin activity as judged by the decreased absorbance for L -glutamine Three independent proteoliposome assays were performed with both p28-Omp14 or 19 recombinant proteins [Figure 22 and 23 (A, B, C)]. Although in all three experiments, we observed swelling in presence of sugars or L-glutamine. There appear to be experimental variation in the proteoliposome swelling. To estimate the impact of the size of the molecules, we performed size versus rate of swelling analysis from three independent experiments (Table 31). The analysis revealed no difference in the swelling of proteoliposome in presence of p28-

Omp 14 and 19 proteins. Secondly, the proteoliposome swelling is greater for smaller molecule and it decreased with increasing size of sugars used in the analysis.

The porin activity was also performed with four different concentration of L-glutamine with p28-Omp 19. The porin activity was the highest for 10 mM and it descends with the increase in the concentration of L-glutamine to 20, 33 mM and 40 mM.

Discussion

E. chaffeensis is a relatively recently discovered pathogen transmitted from infected ticks to vertebrate hosts, including humans [49, 61]. Much of the research on this pathogen has been focused on understanding how the organism is able to persist in ticks and vertebrate hosts. Recent studies suggest that the pathogen expresses numerous proteins in a host cell specific manner [137, 141, 143]. Tick cell derived *E. chaffeensis* persists longer time in the murine host compared to bacteria originating from vertebrate macrophages [128]. These results suggest that the difference in protein expression is an important contributor for *E. chaffeensis* adaptation to vertebrate host. However, little is known about the functional significance of differentially expressed proteins to the pathogen's growth.

In this study, we focused to characterize two differentially expressed proteins of *E. chaffeensis* to define their secondary structure and to map their biological activities. In particular, we studied two proteins that are differentially expressed on the outer membrane of *E. chaffeensis*; they are p28-Omp14 and p28-Omp 19. The p28-Omp 14 is primarily expressed by the organism in tick cells both under *in vitro* and *in vivo*, whereas p28-Omp 19 is the major expressed outer membrane protein of *E. chaffeensis* in infected cultured macrophages and infected vertebrate hosts [112,

141]. These proteins gained considerable importance because they are highly immunogenic and are recognized by the immune sera of infected vertebrates [133, 134, 142]. Moreover, these proteins are made from the multigene locus which includes 22 tandemly arranged paralogous genes [113, 139, 140, 146]. Their homologs are also found in other *Ehrlichia* species with differential expression reported [104, 105]. Despite their differential expression on the pathogens cell surface and their extensive shared homology in the primary protein structure of different paralogs, biological function of these proteins is not yet defined. Recent studies for two of these proteins p28-Omp 18 and p28-Omp 19, suggest that they are porin like proteins [151].

To define the function of the differentially expressed p28-Omp proteins, we first performed *in silco* analysis. The analysis supported earlier data that the p28-Omp 14 and 19 are membrane bound proteins. We utilized three different programs to predict the secondary and 3D structures of these proteins. The hydrophobicity analysis by ProtoScale program suggested the presence of 9 hydrophobic segments in both the proteins [Figure 9 (A) (B)]. The PREDTMBB program confirmed these predictions. The prediction analysis PREDTMBB program further suggested that the 9 hydrophobic domains are parts of the outer membrane protein embedded in the lipid bilayer [Figure 10 (A) (B)]. The hydrophilic domains identified by ProtoScale program are also predicted as the hydrophilic domains by PREDTMBB program and suggested that the hydrophilic segments are part of the protein exposed to extracellular environment. The prediction analyses data for p28-Omp 14 and 19 are very similar to the previous predictions made for p28-Omp 19 and 18 by kumugai and Rikihisa [151].

The 3D modeling analysis by CPH model 3.0 are also useful in predicting the structure of the membrane proteins [154, 157]. Analysis of p28-Omp 14 and 19 using this program also supported the predictions made by ProtoScale and PREDTMBB programs. The 3D structure prediction analysis suggested that the proteins fold very similar in a lipid bilayer with β- barrel like structures formed with several β helices. The β helices represent protein segments with higher hydrophobicity (Figure: 11 (A) (B). The 3D structure predictions did not reveal obvious differences between p28-Omp 14 and 19. The only notable difference is the presence of two extracellular loops; the loops appear to fold differently for these proteins.

In support of mapping the secondary structures and differences within, we prepared recombinant protein of p28-Omp 14 and 19 in *E. coli* and purified then to homogeneity by employing purification techniques including differential centrifugation, ion-exchange and size exclusion chromatography methods. The methods aided in the recovery of large quantities of highly pure recombinant proteins of p28-Omp 14 and 19. As these are membrane proteins, they remained insoluble in aqueous solutions and required 8 M urea for solubilization. The function of these proteins cannot be assessed in non-native insoluble form. Thus, it is important to reconstitute the proteins to mimic the native structure imbedded in the bacterial outer membrane. Solubilization of membrane proteins in detergents with molecular weights similar to those found in the lipid bilayers of the bacterial membrane is technique commonly utilized [160, 164, 167]. In this study, we selected three different detergents β-OG, DPC and DHPC for performing solubilization studies. Experiments were performed to fold the p28-Omp 14 and 19 in these detergents by following the protocols described earlier [160, 167]. The proteins in β-OG were insoluble at the concentration used (20 mM) and therefore this detergent was not used for

subsequent experiments. Following the solubilization in DPC or DHPC, the presence of folded proteins was assessed by SDS PAGE analysis. Folded proteins migrate faster in the gel compared to non-folded proteins and therefore this method is useful in identifying the presence and abundance of folded proteins. Folded proteins for p28-Omp14 and 19 proteins are clearly visible in the polyacrylamide gel as judged by rapid migration of protein bands compared to proteins in the absence of DPC and DHPC. The folding is high at 15 mM DPC and 24 mM DHPC. These concentrations of DPC and DHPC are considered critical concentration of detergents required for forming micelles [168]. The micelles folded proteins were assessed further by performing CD analysis to examine the nature of the secondary structures in the folded proteins. The CD analysis suggested a characteristic bend at 218 nm, which is an indication of the presence of rich β sheet structures that are typically present in membrane bond proteins of Gram-negative bacteria [159, 169]. Both DPC and DHPC treated proteins show a negative peak at 218 nm. These results indicating the presence of large amounts of β sheet structures. Hydrophobic domains of a membrane proteins form β sheet structures and get integrated into the lipid bilayers. The hydrophobic domains of the proteins are imbedded within the membrane surface of a lipid bilayer, thus, allowing membrane proteins to interact with their hydrophobic environments. Previous studies on two p28-Omp proteins of *E. chaffeensis* [p28-Omp 19 and p28-Omp 1F(Omp18)] suggest that the proteins form porin like structure and possibly serve as porins to allow metabolite exchange between the host and pathogen [151]. In this study, we assessed the proteoliposome as a measure for porin activity swelling for both p28-Omp 14 and 19 recombinant proteins after reconstituting the proteins to form in a proteoliposomes. The analysis suggested that the p28-Omp 14 and 19 possess the porin activity. Proteoliposome

assays of p28-Omp 14 and 19 proteins had shown the permeability for L-glutamine and for four sugars; glucose, fructose, sucrose, and stachyose. The swelling of proteoliposome appeared to be dependent on the size of a molecule and the concentration of solutes. Three independent proteoliposome assays suggested the highest permeability for L- glutamine fallowed by fructose, glucose, sucrose, and stachyose. The proteoliposome assay performed with p28-Omp 19 and L-glutamine suggested that the porin activity is concentration dependent and increasing concentration cause decrease in porin activity.

The availability of the recombinant proteins in pure form is valuable to further the critical structural differences in these highly homogenous proteins and to define the significance of differences in the primary structure to functional variations. The functional differences may be understood by more structural analysis performed by such as by performing X-ray crystallography and nuclear magnetic resonance studies.

Figures related to Chapter 2

Calculating Hydrophobicity

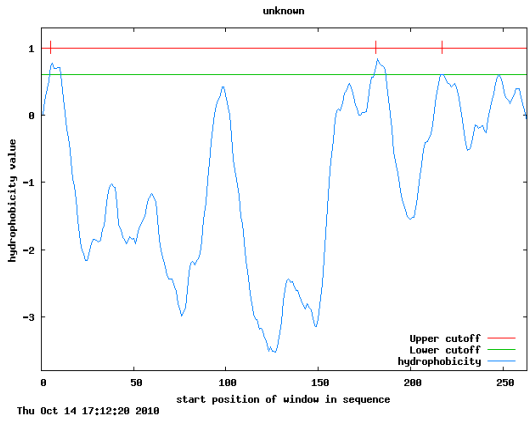


Figure: 9 (A)

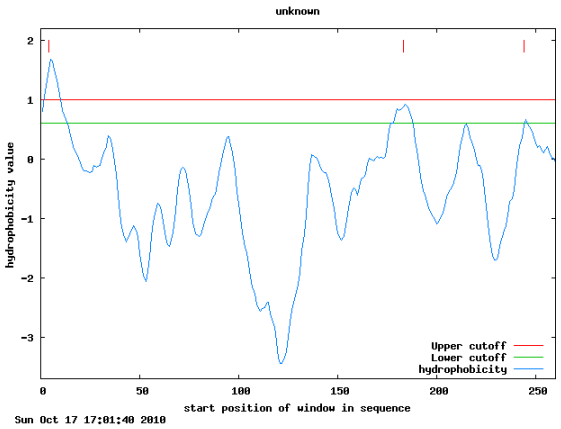


Figure: 9 (B)

Figure: 9 (A) (B) Calculating Hydrophobicity of p28-Omp14 and p28-Omp19 by PROTOSCALE program (Kyte and Doolittle)

2D structural prediction by PRED-TMBB Program

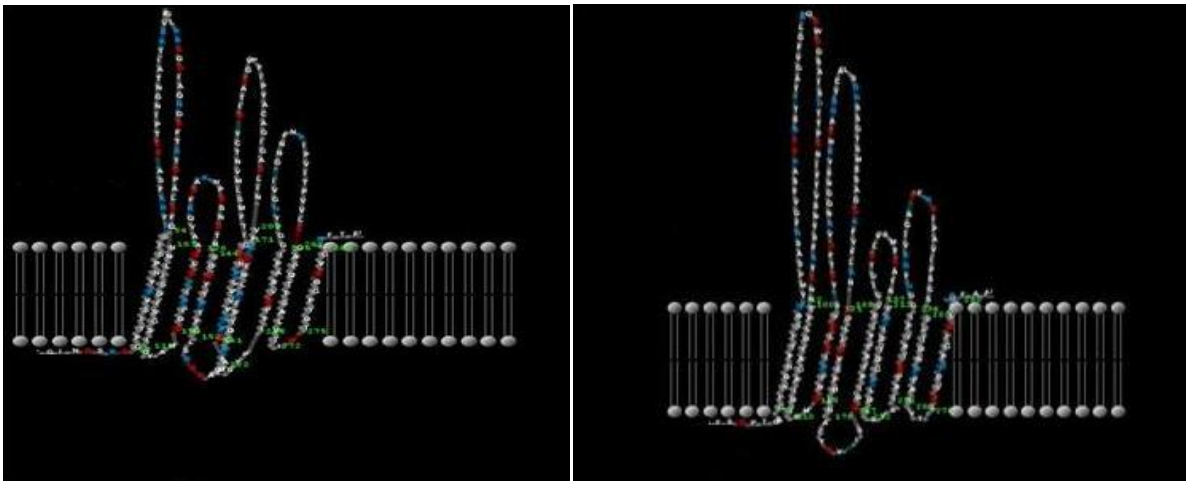


Figure: 10 (A) **Figure: 10 (B)**

Figure: 10 (A) (B): Predication of 2D model for p28-Omp14 and p28-Omp19 in transmembrane using PRED-TMBB program

3D model prediction by CPH MODEL 3.0

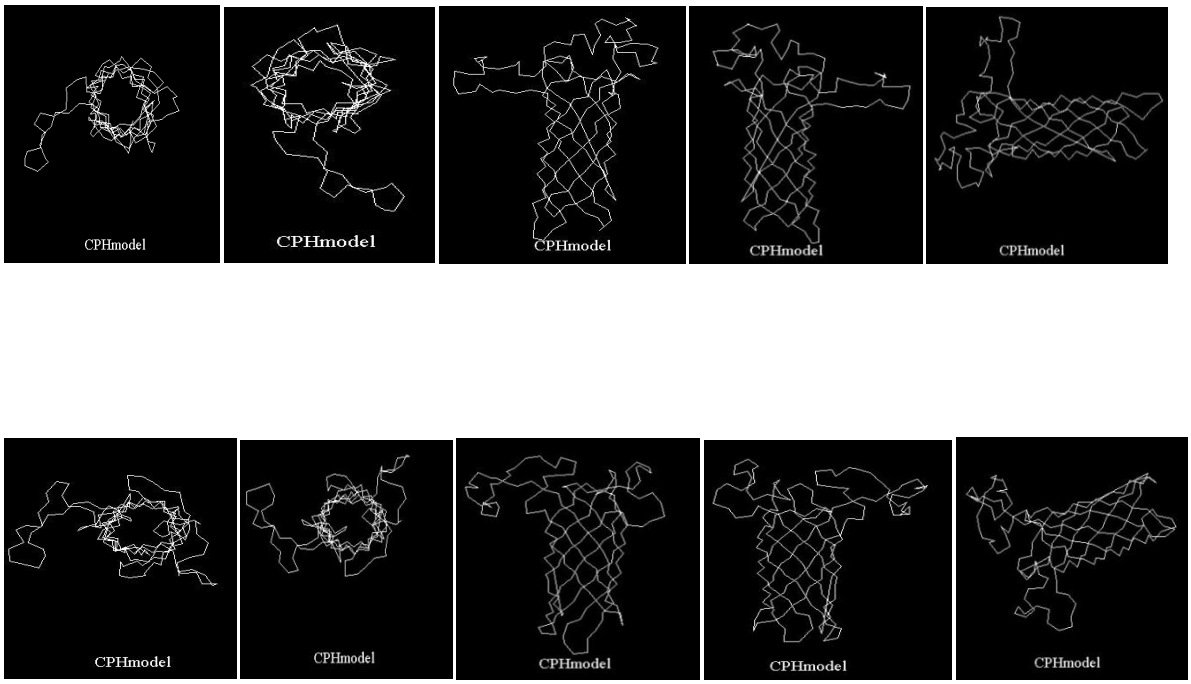


Figure 11 (B): Predicated 3D model of p28-Omp 14 and p28-Omp 19 by using CPH model 3.0.

SDS PAGE analysis for recombinant expressed protein

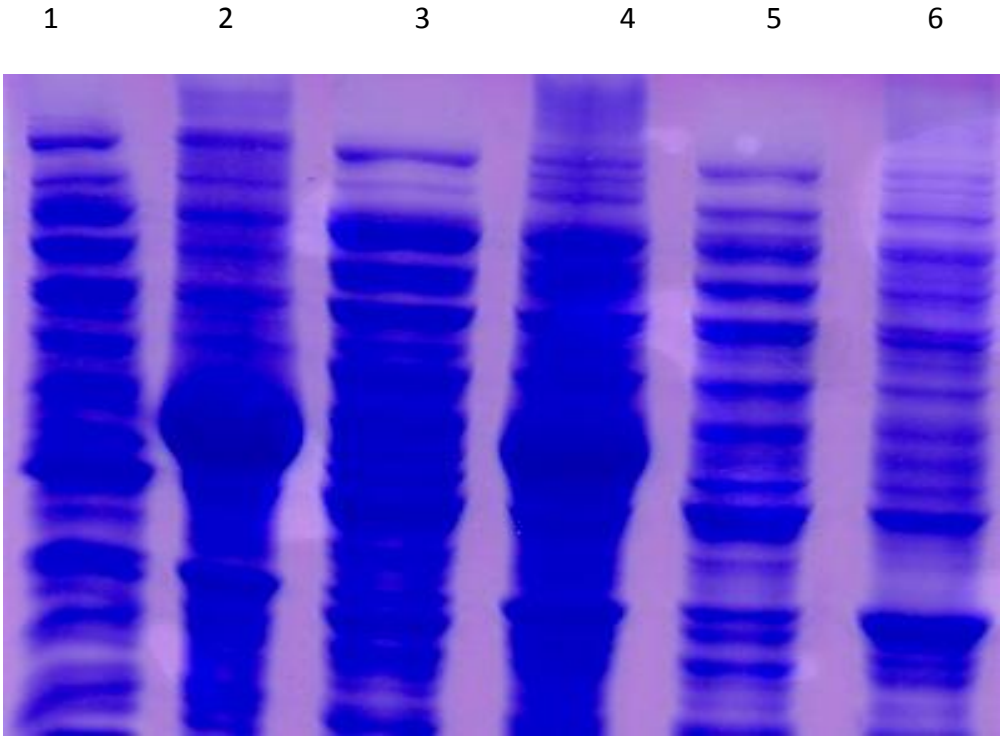


Figure 12: **SDS-PAGE analysis of p28-Omp 14 and 19:** The proteins were expressed in recombinant *E. coli* Bl21 bacterial strain. SDS- PAGE analyses were performed for verifying the presence of desired protein. 1: Supernatant of p28 Omp 14 after cell lysis, lane 2nd: Pellet of p28-Omp 14 cell lyste dissolved in 8 M urea, lane 3rd: Supernatant of p28 Omp 19 after cell lysis, lane 4th: Pellet of p28-Omp 19 cell lyste dissolved in 8 M urea Cell lysates. Whereas 5 and 6th lanes are bacterial culture without desired recombinant plasmid. Proteins were analyzed on a 15% acrylamide gel and visualized by Coomasie staining.

Ion exchange Chromatography analysis of recombinant gene 14 and 19 protein expressed elution.

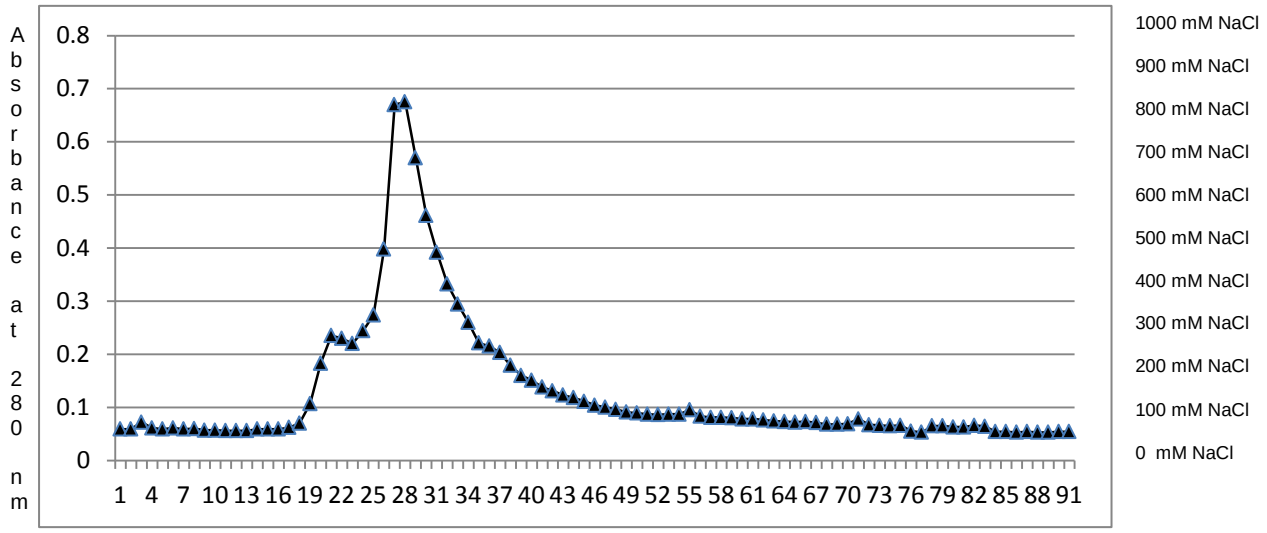


Figure 13 (A)

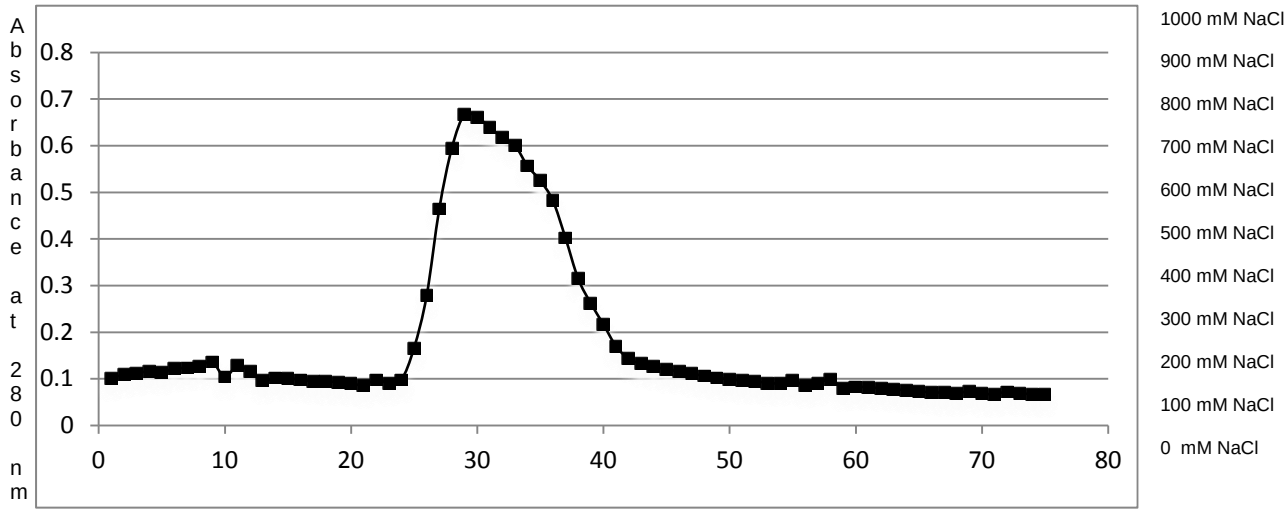


Figure 13 (B)

Figure 13 (A) (B): p28-Omp 14 and 19 protein fraction samples eluted from Ion exchange was analyzed by U.V spectrophometer.

Fractions from p28-Omp 14 ion exchange chromatography

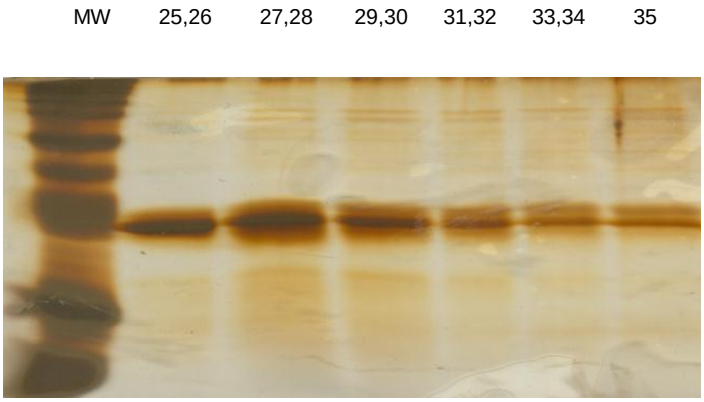


Figure 14 (A)

Fractions from p28-Omp 19 ion exchange chromatography

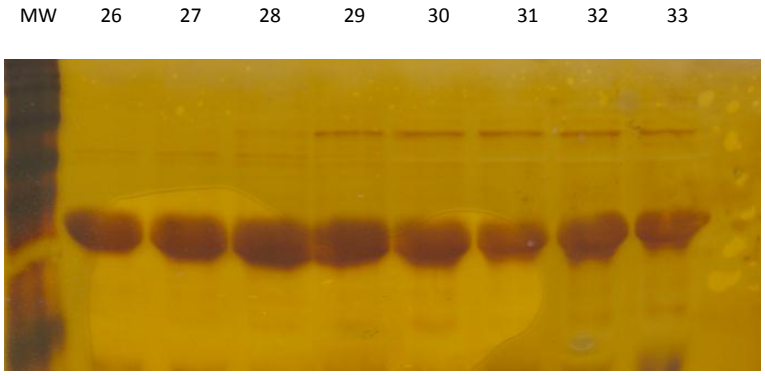


Figure 14 (B) : **Ion exchanged Purified p28-Omp 14 and 19 analysis on SDS-Polyacrylamide gel**: Maximum homogeneity protein fractions were collected (range from 25 to 35 fractions of p28-Omp 14 and 26 to 33 fractions for p28-Omp 19, around 0.4 to 0.5 M NaCL gradients) were analyzed on SDS PAGE. Proteins were separated on a 15% acrylamide gel and visualized by Silver staining method.

Size exclusion chromatography

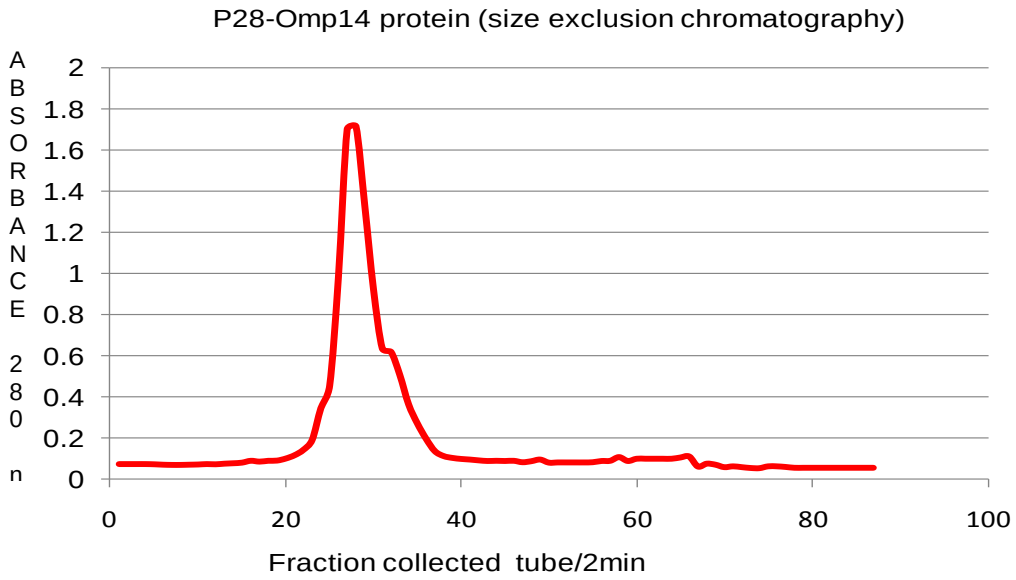


Figure 15 (A): **Purification p28-Omp 14 by size exclusion chrmotography**

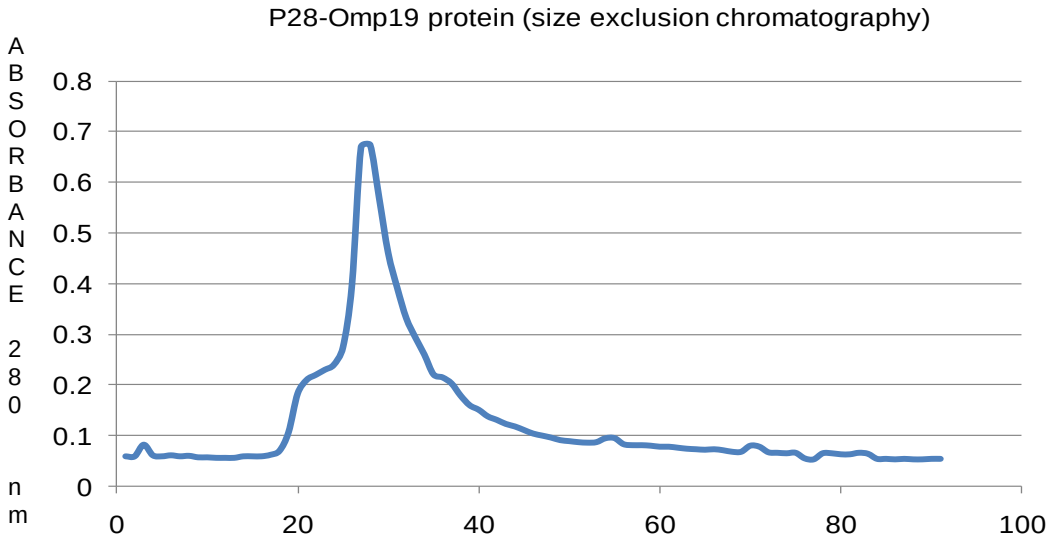


Figure 15 (B): **Purification p28-Omp 19 by size exclusion chromotography**

Figure 15 (A) (B): Elution profile obtained from the size-exclusion chromatography step using the 1*175 cm column containing Superdex beads75 (26/60) column. Flow rate at 1 mL/min with 20 mM Tris HCl buffer (pH 7.5) and 100 mM NaCl. Eluted fractions were collected after void volume of 20 ml (Ranges from 25 to 35) for p28-Omp 14 and p28-Omp 19.

Protein fraction assessed from various purification steps

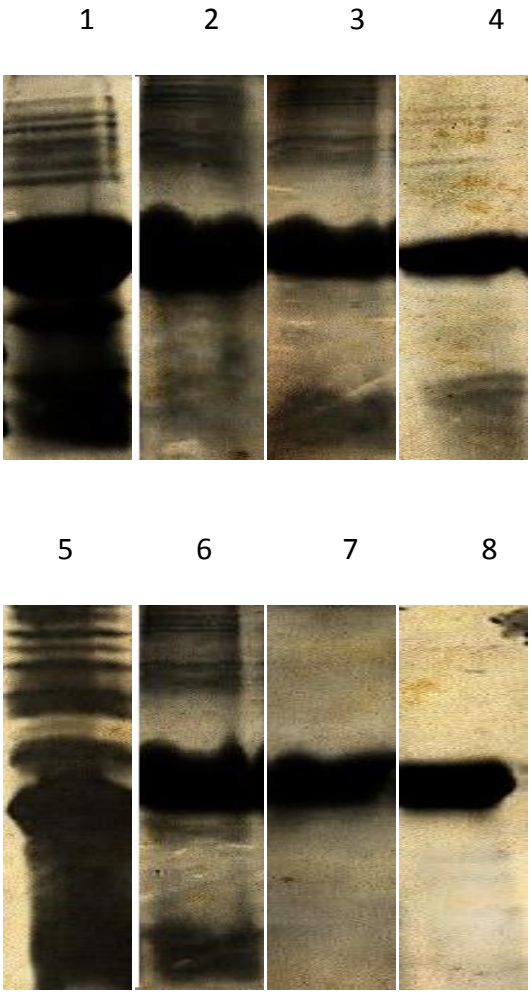


Figure 16: Protein sample from each Purification step was anyalsed by SDS-Polyacrylamide gel.

p28-Omp 14: lane1: cell lystes, lane 2: Centrifugation separated, lane 3rd: Ion exchange purified and 4th lane: Size exclusion chromatography purified

p28 – Omp 19: Lane 5th: cell lystes, lane 6th: Centrifugation separated sample, lane 7th:Ion exchange purified sample , lane 8th : Size exclusion chromatography purified sample

Detergent treatment

Various concentration of DPC micelle treatment with p28-Omp19

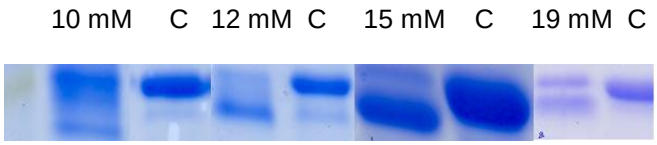


Figure 17: p28 Omp 19 was folded in 10 mM, 12mM, 15 mM and 19 mM. all solution were buffered with 20 mM sodium phosphate buffer, pH 8.0 at 4ºC for overnight. Protein was visualized by staining with coomasie blue. C is control; protein without detergent treatment.

Various concentration of DHPC micelle was used p28-Omp19 refolding.

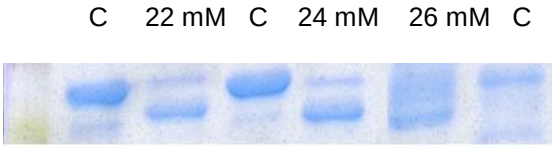


Figure 18: p28-Omp 19 was treated with in 22 mM, 24 mM, and 26 mM of DHPC. All solution were buffered with 20 mM sodium phosphate buffer, pH 8.0 at 4ºC for overnight. Protein was visualized by staining with coomasie blue. C is a control: protein without detergent treatment. 15% SDS PAGE were used for analyzing.

p28-Omp 14 and 19 treated with DPC detergent and analyzed by 15% SDS Page gel

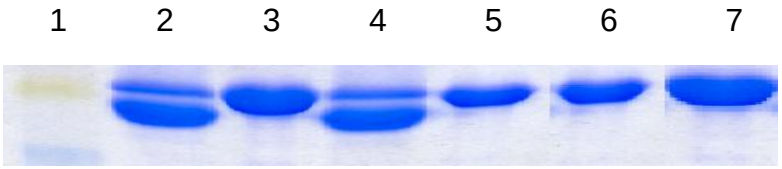


Figure 19: Both p28-Omp 14 and 19 were treated with 15 mM DPC at 4ºC for overnight. Lane 1: Marker lane 2nd: p28-Omp 14 treated with detergent, lane 3 :(Control) untreated p28-Omp 14. lane 4th: p28-Omp 19 treated with detergent, lanes 5th : untreated p28-Omp 19, lane 6th and 7th were heat modifiability of DPC detergent treated p28 Omp14 and 19 All solution were buffered with 20 mM sodium phosphate buffer, pH 8.0 . Proteins were visualized by coomasie staining.

p28-Omp 14 and 19 treated with DHPC detergent and analyzed by 15% SDS Page gel

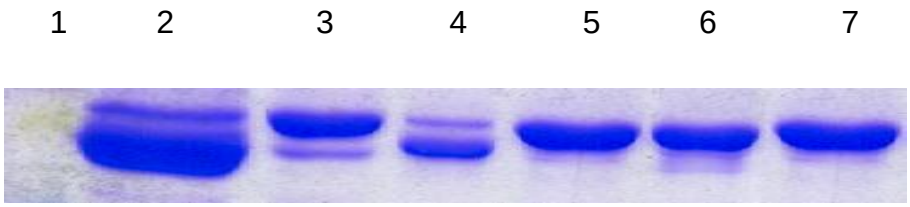


Figure 20: **Analysis of DHPC detergent folded protein:** Both p28-Omp 14 and 19 were treated with 24 mM DHPC at 4ºC for overnight. Lane 1: Marker lane 2nd: p28-Omp 14 treated with detergent, lane 3rd: Heat modifiability of DHPC detergent treated p28-Omp 14, lane 4th: p28-Omp 19 treated with detergent, lanes 5th : Heat modifiability of DHPC detergent treated p28-Omp 19, lane 6th and 7th were untreated p28 Omp14 and 19 proteins. All solution were buffered with 20 mM sodium borate buffer, pH 8.0 . Proteins were visualized by coomasie staining.

Circular dichroism analysis p28-Omp 14 treated with DPC and DHPC

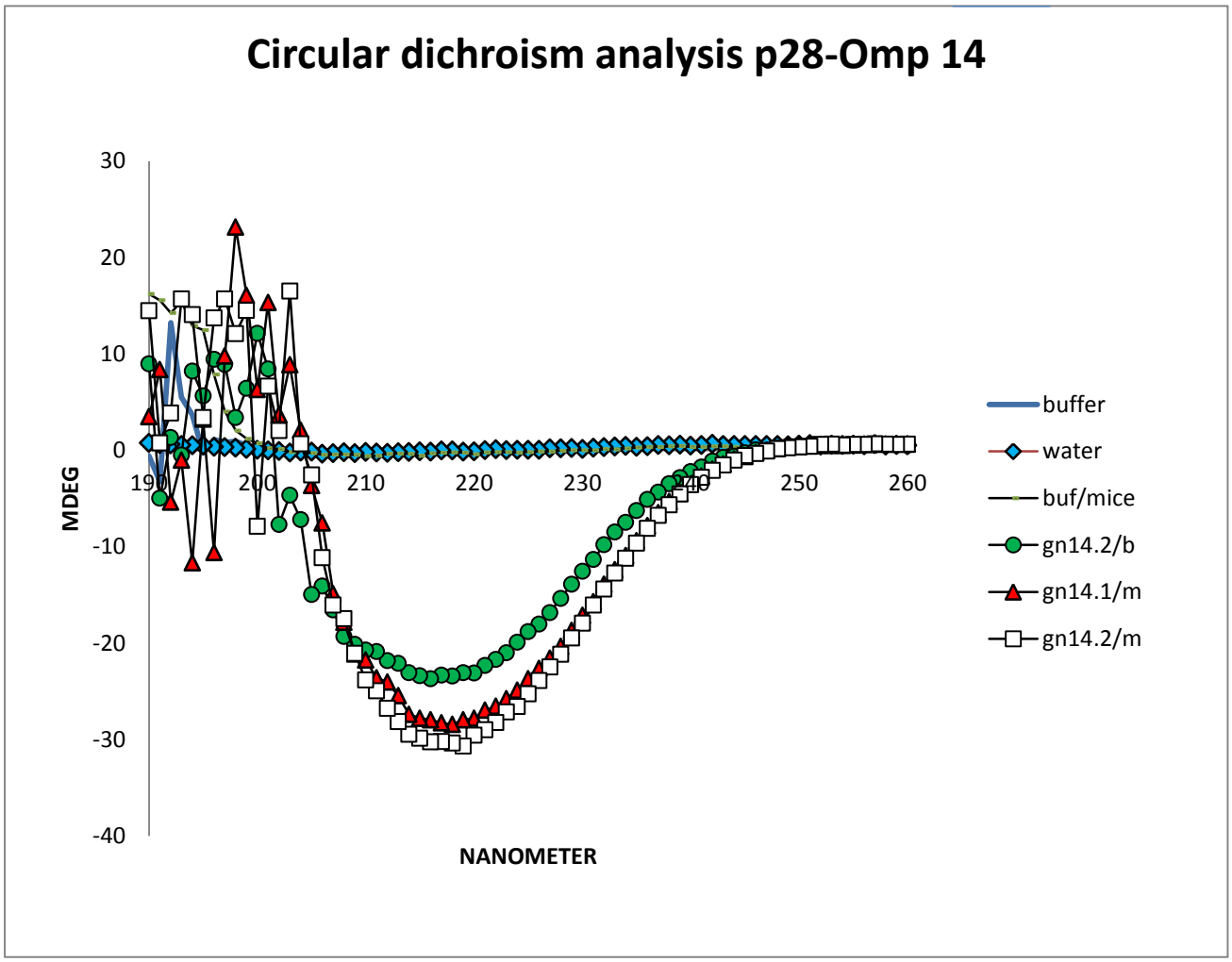


Figure 21 (A): Comparison between water soluble and detergent folded of p28-Omp 14. DPC detergent in Sodium borate buffer and DHPC detergent in Sodium phosphate buffer at pH8.0 used for refolding.

CD SPECTRA of (-) Buffer, (◆) Water, (--) Buffer/Detergent, (▲) p28-Omp 14/DPC detergent, (□) p28-Omp 14/DHPC detergent,(O) p28-Omp 14W/o micelles.

Circular Dichroism analysis of p28-Omp 19 treated with DPC and DHPC detergent

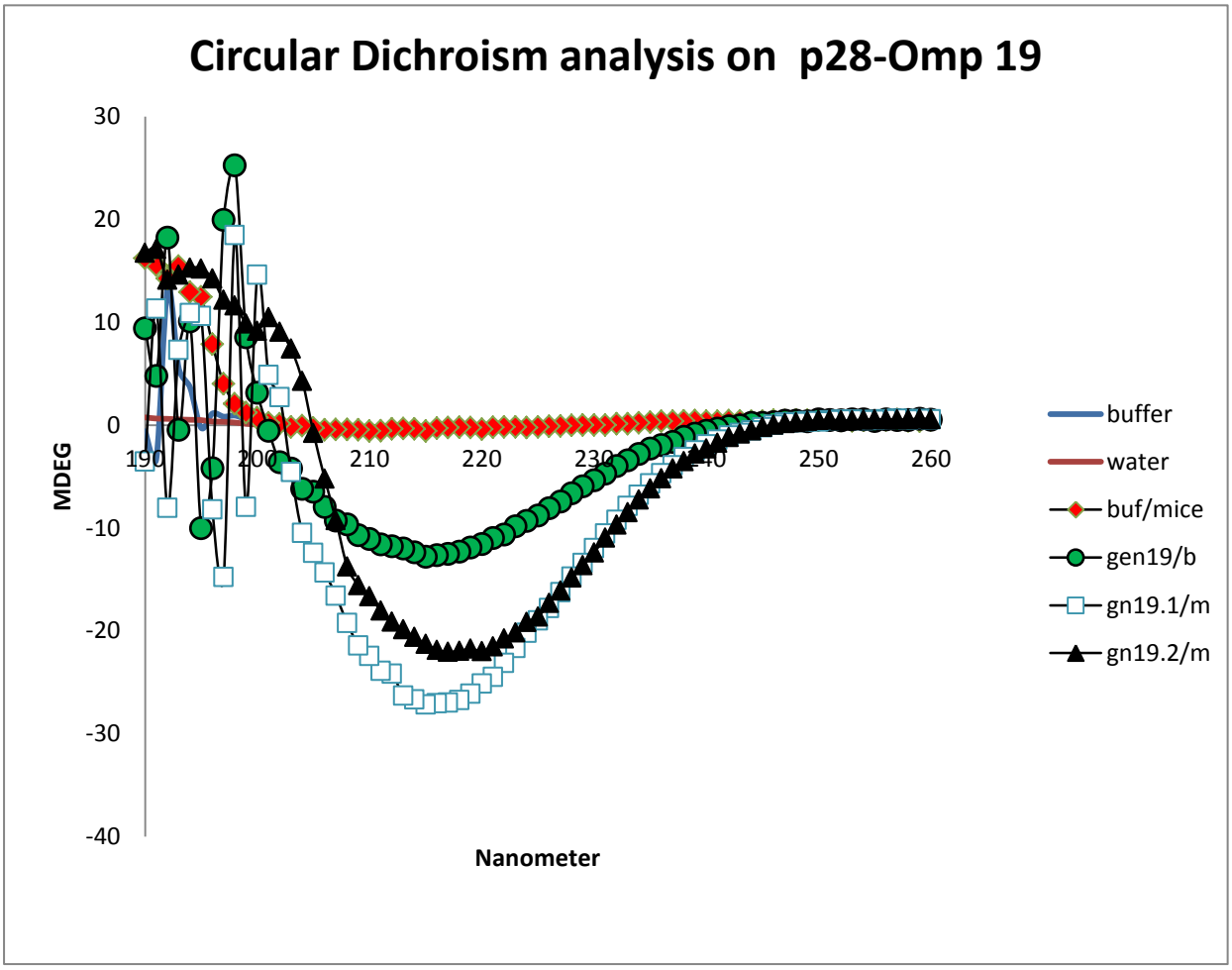


Figure 21 (B): Comparison between water soluble and detergent folded of p28-Omp 19. DPC detergent in Sodium borate buffer and DHPC detergent in Sodium phosphate buffer at pH8.0 used for refolding.

CD SPECTRA of (◆) buffer, (-) water, (--) Buffer/Detergent, (□) p28-Omp 19/DPC micelles, (▲) p28-Omp 19/DHPC micelles, (O) p28-Omp 19W/o micelles

Figure: 22 (A) (B) (C) Proteoliposome assay p28-Omp 14

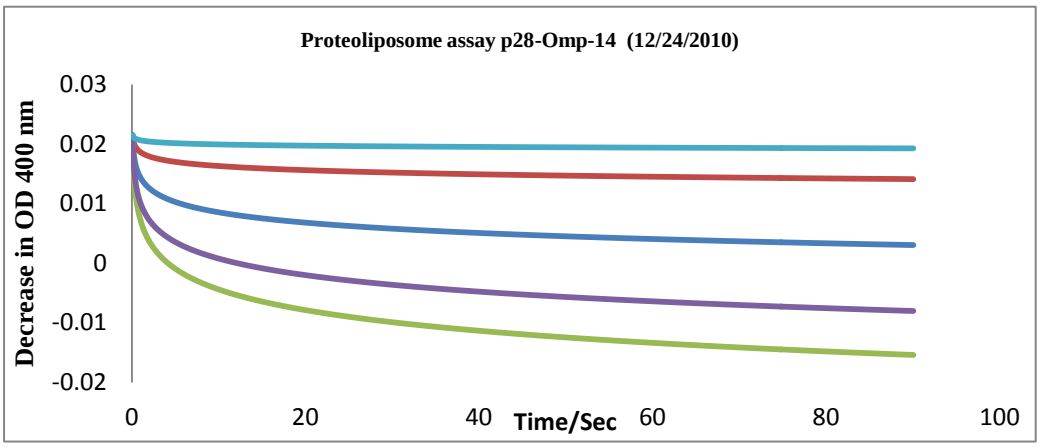


Figure 22(A)

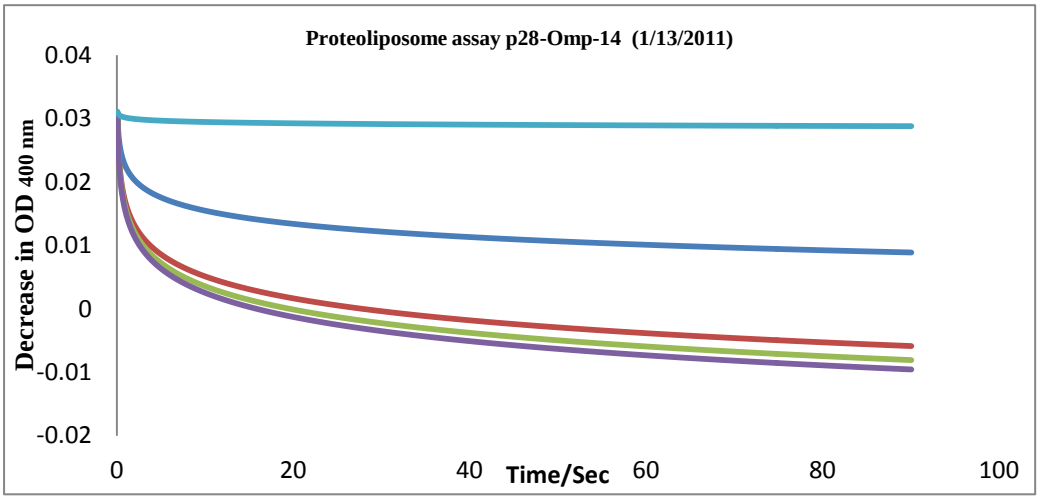


Figure 22(B)

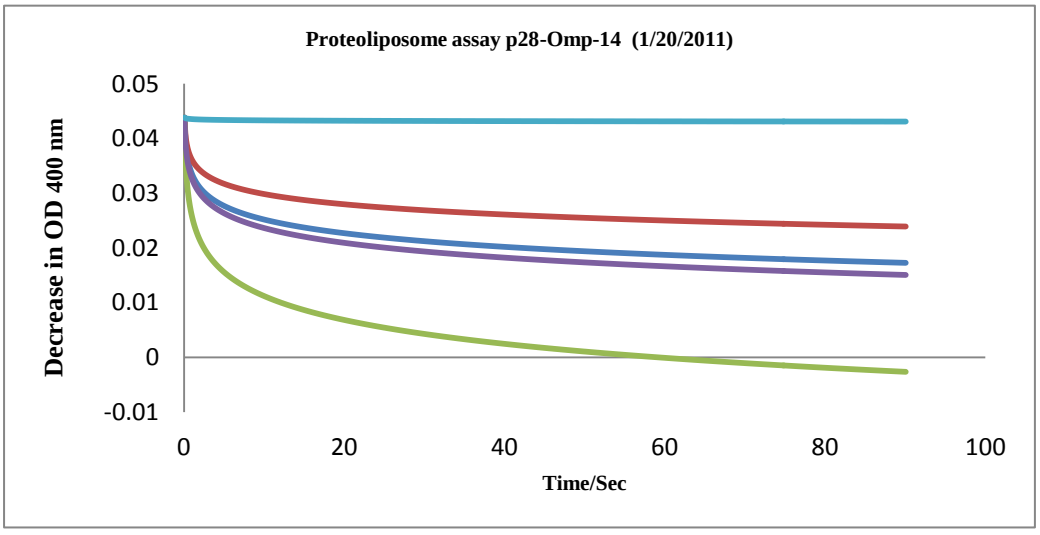


Figure 22(C)

Figure 22 (A) (B) (C): Porin activity of the *E. chaffeensis* outer membrane p28-Omp 14(recombinant). The diffusion rates of solutes into proteoliposome reconstituted with *E. chaffeensis* outer membrane proteins were monitored as the decrease in OD400. When proteoliposome were diluted in isosmotic solution of 33 mM Glucose (-) (MW=180), 33 mM sucrose (-) (MW=342), 33 mM fructose (-) (MW=180), 33 mM, L- glutamine (-) (MW=156) and 33Mm stachyose (-) (MW=666). (Three independent experiments are shown performed on (A) 12/24/2010, (B) 1/13/2011, (C) 1/20/2011).

Figure: 23 (A) (B) (C) Proteoliposome assay p28-Omp 19

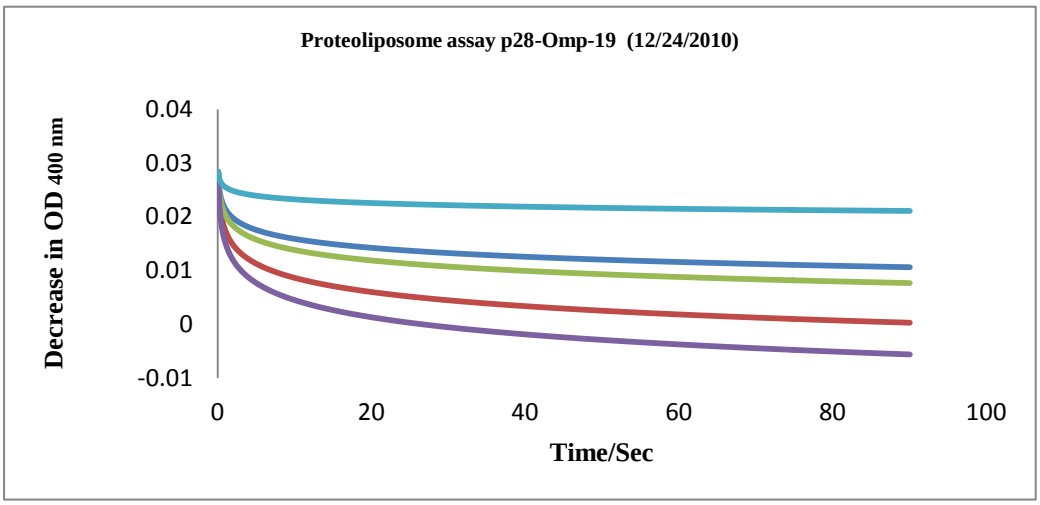


Figure 23(A)

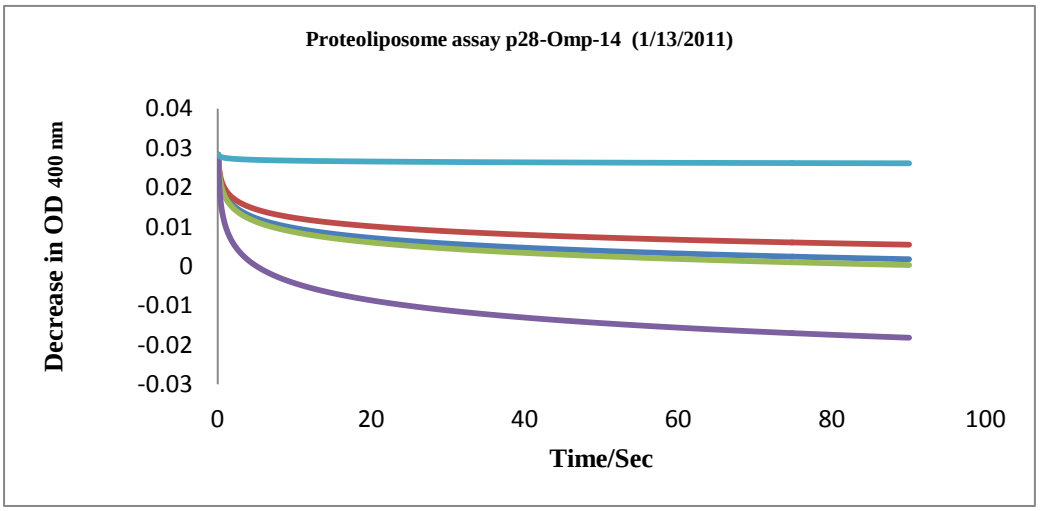


Figure 23 (B)

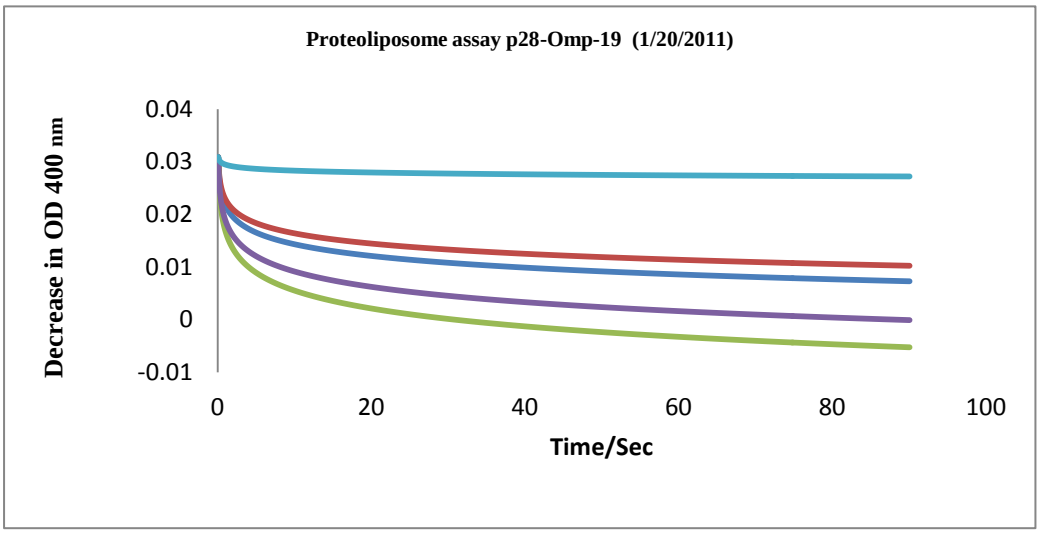


Figure 23(C)

Figure 23 (A) (B) (C): Porin activity of the *E. chaffeensis* outer membrane p28-Omp 19(recombinant). The diffusion rates of solutes into proteoliposome reconstituted with *E. chaffeensis* outer membrane proteins were monitored as the decrease in OD 400. When proteoliposome were diluted in isosmotic solution of 33 mM Glucose (-) (MW=180), 33 mM sucrose (-) (MW=342), 33 mM fructose (-) (MW=180), 33 mM, L-glutamine (-) (MW=156) and 33Mm stachyose (-) (MW=666). (Three independent experiments are shown performed on (A) 12/24/2010, (B) 1/13/2011, (C) 1/20/2011).

Rate of proteoliposome swelling assay (Average of independent experiment)

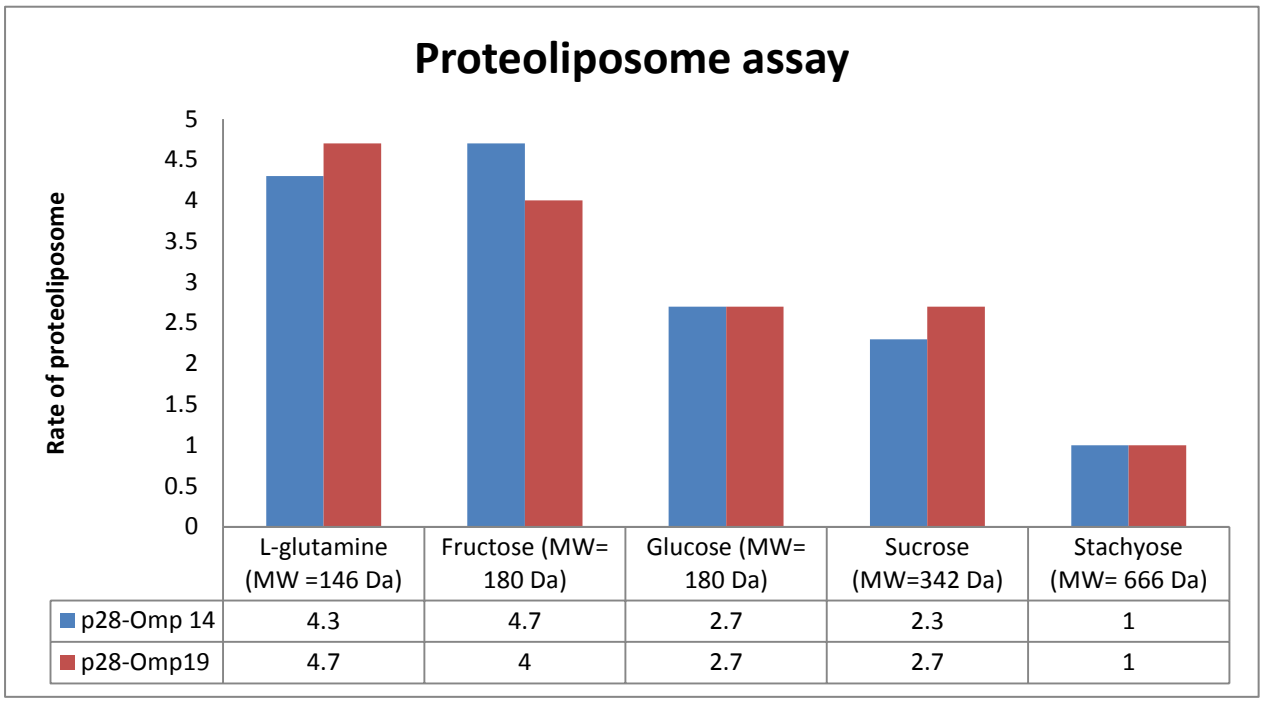


Table: 2: **Proteoliposome assay:** Based on decrease in OD position, number is allotted and graph is represented. (Average of three independent experiment was taken)

Proteoliposome assay (p28-Omp 19) with four different concentration of L-glutamine

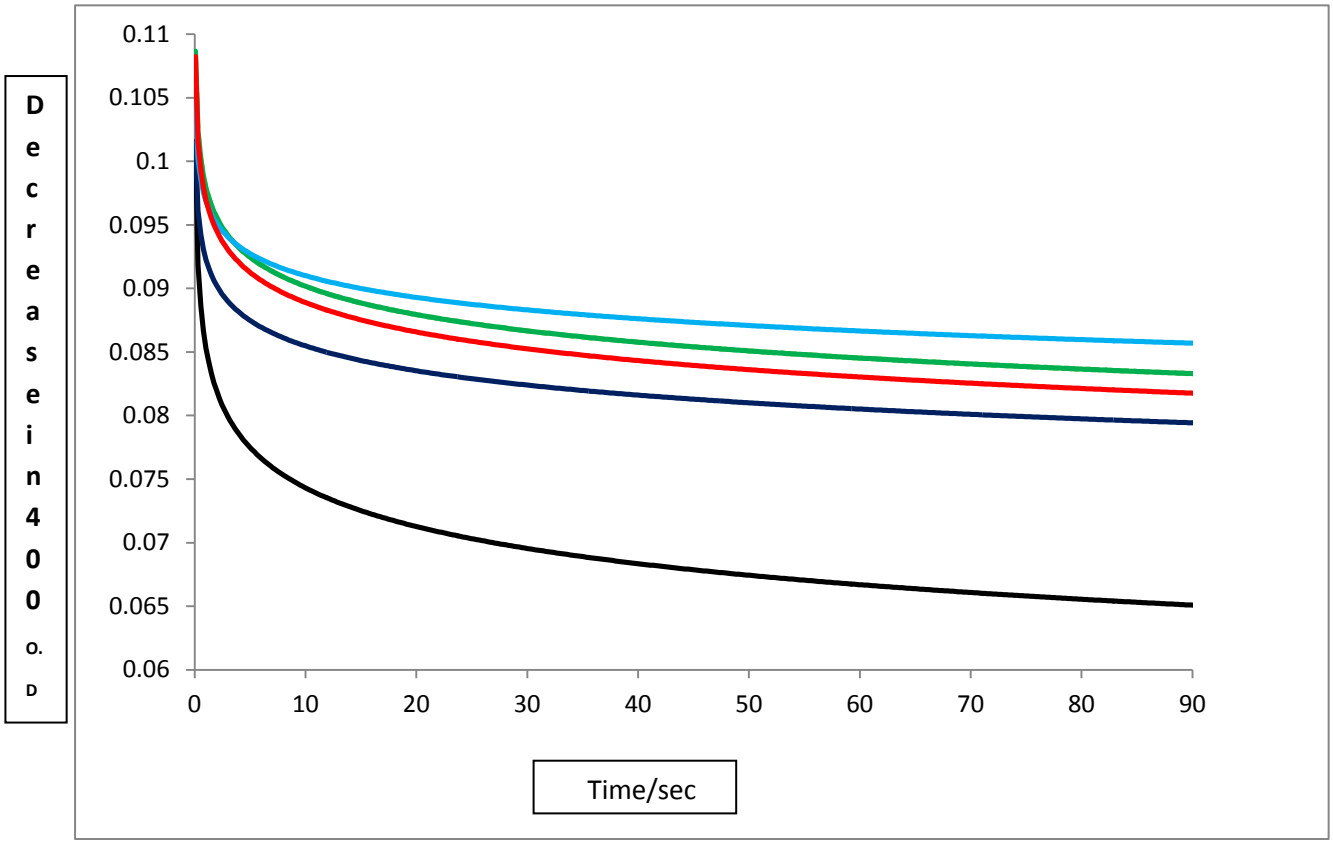


Figure 24: Porin activity of the *E. chaffeensis* outer membrane p28-Omp 19(recombinant) with L- glutamine.

The diffusion rates of solutes into proteoliposome reconstituted with *E. chaffeensis* outer membrane proteins were monitored as the decrease in OD400. When p28-Omp 19 proteoliposome were diluted in isosmotic solution of 10 mM L-glutamine (-), 20 mM L-glutamine (-), 33 mM L-glutamine (-), 45 mM L-glutamine (-). For control, water with proteoliposome (-) and water with liposome (-) were taken. The representative results of more than three independent experiments are shown.

Protocols for molecular biology techniques

Polymerase chain reaction (PCR): All PCR‘s were performed in final reaction volume of 25 µl. Each reaction contains a final concentration of 1X PCR reaction buffer, 50 nmoles of MgCl2, 10 nmoles of dNTP's, 10 pico moles each of forward and reverse primers, about 1ng of template DNA, and 1 unit of taq DNA polymerase (Invitrogen technologies, Carlsbad, CA). For PCR reactions that require proof reading, the PCR conditions are; 1x Pfx amplification buffer, 1 U of platinum Pfx DNA polymerase (Invitrogen Technologies, Carlsbad, CA), 5 nmoles of dNTP's, and rest of the reagents concentrations are maintained same as in above described reaction. The PCR temperature cycles include an initial heating to 95°C for 2 min, followed by 40 cycles of 95°C denaturation for 30 sec, primer annealing for 30 sec and carried out at appropriate temperatures calculated for each primer set, 72 °C extension for 30 sec. The extension temperature for platinum Pfx DNA polymerase is 68 °C. The extension temperatures were increased to 1min per each kb of expected amplicons length. Each reaction set included a negative control, which lacked a template but contained all the other reaction components. After reaction is complete the products were resolved on agarose gels containing ethidium bromide and visualized under UV light.

Restriction enzyme digestions: Typically restriction enzyme digestion reaction is performed in a 20 µl volume. The reaction included 1x restriction enzyme reaction buffer, 1 µg of DNA, 1-5 units of enzyme, 0.5 µl of BSA (10 µg/ µl), and water to a final volume of 20 µl. Typically the restriction enzyme digestion is carried out at 37°C for 2 h unless an enzyme requires a specific temperature. For all the reactions that utilized two restriction enzymes, a buffer optimal for both the enzymes is utilized.

Phenol purification of DNA: DNA fragments from PCR, restriction enzyme digestion, and filling-in reactions are purified phenol purification method. Typically, 3 M sodium acetate is added to final concentration of 0.3 M into a microcentrifuge tube containing DNA and final volume was adjusted to 200 µl with TE buffer. Two hundred micro liters of phenol (pH, 8.0) is added, vertexed to mix and centrifuged at 15,000 g for 15 min at 4°C. The top aqueous layer is transferred into a clean microcentrifuge tube and added a 200 µl of phenol:chloroform:isoamylalcohol (25:24:1) mixture. The contents are mixed by vortexing and centrifuged at 4°C at 15,000 g for 15 min. The top layer is transferred into another clean microcentrifuge tube. Thease steps were repeated with pheol:chloroform:isoamylalacohol and then with chloroform:isoamylalacohol. To the final removed aqueous layer, 0.5 ml absolute cold ethanol is added, incubated at -20°C for 15 min followed by centrifugation at 15,000g for 15 min. The DNA pellet is washed with 0.5 ml of 70% ethanol. Final pellet was air dried, resuspended in 20 µl TE buffer and stored at -20 °C until use.

Ligation reactions: A typical ligation reaction included approximately 25 ng of linearized purified plasmid vector DNA, 5 to10 molar excess of insert DNA, 1x ligation buffer, 5 units of T4 DNA ligase (Promega Corporation, Madison, WI) in a 20 µl reaction volume. The ligation reaction is carried out by incubating the contents at 15°C for 16 h. Following the ligation, 1 µl of ligation mix is used for transformation by chemical method. Alternatively, for use in transformation by electroporation method, the DNA is purified by phenol:choloform:isoamyl alcohol method described above.

Preparation of *E. coli* cells for use in chemical transformation methods: The *E. coli* strains utilized to prepare competent cells included Top 10 cells (Invitrogen

Technologies, Carlsbad, CA), DH5α (Stratagene, La Jolla, CA), and BL21 (DE3) (Novagen Inc., Madison, WI). Top 10 stain of *E. coli* is always grown in the presence of streptomycin (35µg/ml). DH5α and BL21 (DE3) stains are grown in a plain LB medium. To prepare chemical competent cells, an *E. coli* colony of a desired strain is cultured in 3 ml of LB medium overnight in a 37°C incubator, shaking at 250 rpm. Subsequently, *E. coli* culture is re-inoculated into 100ml LB medium and grown in a 37°C incubator. After the cells are grown to 0.4 OD (measured at 600nm), the cultures are harvested by centrifuging at 2,500 g for 5 min at 4°C. The cell pellet is resuspended in 10 ml of freshly prepared 10 mM Tris-HCl (pH, 7.5) and 50 mM CaCl2, and incubated on ice for 30 min. The cultures was centrifuged again at 2,500 g for 5 min at 4°C and the pellet is suspended in 2 ml of 10 mM Tris-HCl (pH, 7.5) and 50 mM CaCl2 and stored at 4°C. The competent cells made by this procedure are utilized within 24 h after their preparation.

Transformation: Transformation of ligated products into *E. coli* cells is achieved either by a chemical method or through an electroporation procedure. To transform by chemical method, 200 µl of chemical competent *E. coli* cells are mixed with 50 of 100mM CaCl2 and 49 µl of sterile water. One µl of ligation products are added to this suspension and mixed by gentle tapping of the tube. The contents are then incubated in ice for 15 min, followed by a heat shock at 42°C for 2 min. The cells are incubated at room temperature for 10 min, added 1 ml of LB medium, and incubated at 37 °C in a shaker incubator set at 200 rpm for 1h.

Preparation of Luria-Bertani (LB) media: The LB liquid medium and LB agar plates were utilized to grow *E. coli* cultures. To prepare 1 lit of LB liquid medium, 15

g tryptone, 10 g of yeast extract and 10 g of sodium chloride were dissolved in 1 lit of double distilled water and pH of the solution was adjusted to 7.0 with the help of 10N NaOH. The LB medium was autoclaved at a liquid cycle. LB agar plates preparation included similar preparation as described above but 15 g of cell culture grade agar powder was added to the medium prior to autoclaving. After autoclaving, the LB agar medium was allowed to cool to nearly 60°C and a desired concentration of appropriate antibiotic was added to the medium. Approximately 15 ml of medium was poured into sterile agar plates. After solidification of the agar medium, the plates were wrapped and stored at 4°C until use.

Selection of recombinant clones: Two hundred micro liters of transformed bacterial cultures are transferred onto LB plates containing appropriate antibiotic specific to a recombinant plasmid. The culture is uniformly dispersed onto the agar plate using a bacterial culture spreader. To grow the transformed *E. coli* the plates were incubated overnight in a 37°C incubator. The presence of transformants is assessed by comparing plates containing appropriate controls (ligation controls, no transformation controls). Subsequently, several colonies are selected, inoculated in a culture tube containing LB medium with appropriate antibiotic and are grown overnight at 37 °C in a shaker incubator to isolate the plasmid DNA.

Isolation of Plasmid DNA: From overnight grown *E. coli* cultures plasmid DNA is isolated by following boiling preparation method (336). To isolate plasmid DNA, 1.5 ml of overnight grown bacterial cultures are transferred into a micro centrifuge tube and centrifuged 12,000 g for 5min. The supernatant was aspirated carefully with the

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

Isolation of genomic DNA: Genomic DNA of *E. chaffeensis* grown in tick or macrophage cultures is isolated by sodium dodecyl sulfate-proteinase K-phenol, chloroform-isoamyl-alcohol method (336). Briefly, 1.5 ml of *E. chaffeensis* cultures are harvested by centrifugation at 12,000 g for 15 min and the cell pellet is resuspended in 0.5 ml of DNA extraction buffer (10 Mm Tris-Cl pH 8.0, 0.1 M EDTA, and 0.5% SDS) containing 0.5 mg/ml proteinase K (Sigma Chemical Co., St. Louis, MO). The contents are mixed by vertexing and incubated for 2 h at 60°C.

Phenol:chloroform:isoamyl alcohol extraction method is used to remove proteinecious material and ethanol is added to concentrate DNA as described previously. DNA pellet is air dried and resuspended in 100 of TE buffer. To remove the contaminating RNA, the DNA is treated with 1 µl RNase A (10 mg/ml) at room temperature for 10 min. The DNA samples were stored at -20°C until use.

Agarose gel electrophoresis: Plasmid DNA, restriction digestion products or PCR products are analyzed by resolving them on a 0.9% agarose gels by subjecting to electrophoresis. The agarose gels are prepared after dissolving agarose powder in 1x TAE buffer (40 mM Tris-acetate, 1 mM EDTA; final pH 8.0) containing 0.1 µg/ml of ethidium bromide. The contents are poured on a gel holding device and are allowed to solidify at room temperature. The gel is placed in an electrophoresis chamber containing 1X TAE buffer with 0.1 µg/ml of ethidium bromide dissolved in it. About 5 µl of DNA is loaded into the wells. Molecular weights markers are also loaded in a separate well and resolved to help in determining the approximate molecular weight of the DNA. The DNA is subjected to electrophoresis in the agarose gel at 70 V for 60-90 minutes and is visualized under UV illumination. The images are captured using Kodak gel imaging system.

Automated sequencing: Recombinant DNA clones were sequenced to verify the accuracy and orientation of the insert DNA using CEQ Genetic Analysis System and by following the manufacturer's recommendation (Beckman & Coulter, Fullerton, CA). Prior to performing a sequencing reaction, the recombinant plasmid DNA is purified by phenol:chloroform:isoamyl alcohol (25:24:1) method as described above. Following the purification, the concentration of the DNA was estimated by Nanodrop

method. The purified plasmid DNA is sequenced using a forward or reverse primer (plasmid derived sequence primers) specific to a sequence upstream or downstream to insert DNA, respectively. Sequencing reaction is performed utilizing DTCS sequencing kit by following the manufacturer's recommendations (Beckman and Coulter, Fullerton, CA). Sequence analysis is performed using Genetics Computer Group (GCG) (87) or Vector.

Silver stain preparation: Dilute Silver Stain 10 folds (e.g. Dilute 5 ml of the stain in 45 ml de-ionized water), then add 65:l of Sensitizer-I per 50ml diluted Silver Stain. Soak the gel in diluted and Sensitizer-I added Silver Stain for 20-30 min with gentle rocking of the gel, depending upon the thickness of the gel.

Silver staining Developer preparation:

While the gel is staining, prepare the developer. Add one heaping spoonful (3-5gm) of Developer to 100ml of de-ionized water. After the developer is dissolved, add 65 :l of Sensitizer-I and 65:l of Sensitizer-II. Rinse the gel 10-20 seconds with de-ionized water. Soak the gel in Developer-Sensitizer-I & II. Gently rock the gel until bands are visible. Band intensity will develop quickly.As soon as band intensity reaches an acceptable level, stop development with 2% acetic acid. Transfer the gel into theacetic acid solution and incubate for 10 min. Gel may be stored in 2% acetic acid or water.

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