Molecular Investigation of Some Virulence Factors of *Chlamydia abortus* Isolated from Aborted Ewes in Slemani Province

A Thesis Submitted to the College of Veterinary Medicine, University of Sulaimani, in Partial Fulfillment of the Requirements for the Degree of Doctor of Philosophy in Veterinary Medicine/ Bacteriology

By

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September, 2020

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بِسْمِ اللهِ الرَحْمنِ الرَحيمْ

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صَدَقَ اللهُ العَظيمْ

سورة طه :الآية *114*

DEDICATION

Dedicated to:

* **My great mother and late father**

* **My sisters and brothers**

* **All those who are the reason behind all the good things in my life.**

ABSTRACT

Enzootic abortion of ewes due to infection with the obligate intracellular bacteria *Chlamydia abortus* is a zoonosis leading to considerable economic losses worldwide. Abortion typically occurs in the last 2–3 weeks of pregnancy with the appearance of stillborn lambs and grossly inflamed placentas. This study was conducted to isolate and identify *C. abortus* in aborting ewes by cell culture and PCR. Also, to characterize the Mip (CAB080), Pmp18D (CAB776) and Momp (CAB048) proteins in *C. abortus*. During the lambing seasons from October 2017 to June 2018, 30 tissue samples of aborted fetuses and 50 vaginal swabs were collected from sheep herds that had a case history of abortion in Kalar, Said Sadiq and Chamchamal, which are districts of Slemani province. Microscopic examination of direct smears taken from the liver of aborted fetuses and vaginal swabs of aborted ewes using Giemsa stain showed that one out of the 30 tissue samples (3.33%) was positive for *Chlamydia* only from Kalar district. In contrast, the vaginal swabs had given a negative. The molecular detection showed that one out of the 30 tissue samples (3.33%) was positive for the *C. abortus* only from Kalar district, and the remaining 29 samples were positive for *Brucella abortus* (96.66%) and no *C. abortus* was detected from the vaginal swabs. The *ompA* gene sequence of *C. abortus* was deposited in the NCBI GenBank under the accession number MK643153 (*C. abortus* strain Sul/2017). *C. abortus* was isolated by using embryonated chicken egg inoculation and purification on Vero cell cultures. After isolation and purification, chlamydial elementary bodies are revealed by staining with Giemsa stain. Also, Eighteen BALB/c mice (pregnant and nonpregnant) were injected intraperitoneally with supernatant prepared from *C. abortus* culture in Vero cells to detect the expression of the genes *mip*, *pmp18D*, and *ompA* during different stages of

pregnancy by using qRT-PCR. The results showed apparent differences between the transcriptional levels of the genes in the samples taken during different time intervals of pregnancy. The results indicated that the different time intervals of pregnancy do not affect the expression of *mip*, *pmp18D,* and *ompA* in the pregnant mice. It is suggested to isolate and characterize *C. abortus* from placentae, fetuses, and vaginal discharges of aborting ewes and does in order to confirm the causes of abortion in small ruminants in the study area.

ACKNOWLEDGMENTS

First of all, I want to thank God the Almighty, the Most Gracious the Most Merciful, who enabled me to embark on postgraduate study and run through the program successfully without any significant difficulty or hindrance. Blessing and peace of Allah be upon Muhammad, the Messenger of Allah.

Special mention goes to my enthusiastic supervisor, Assist. Prof. Dr. Nahla Muhammad Saeed. A person with an amicable and positive disposition, she has always made herself available to clarify my doubts despite her busy schedules.

I extend my respect and express my sincere thanks and appreciation to the second supervisor, Prof. Dr. Shwan Kamal Rachid, for his scientific and practical guidance, patience, advice, and support from the initial to the final levels of my work which enabled me to develop an understanding of the subject.

Great thanks are due to Dean of our college Prof. Dr. Faraidoon Abdul Sattar , Head of Postgraduate Studies Unit, and Head Department of Microbiology for their support me through my postgraduate career.

I would like to express thanks to all the staff of the Cell Culture Unit / College of Medicine at the University of Babylon, especially Assist. Prof. Dr. Hamid Naji Obied Al-Kalabi, for all facilitations he offered me during the study.

My special thanks and appreciation go to Assist. Prof. Dr. Dyary Hiewa, for the advice in referencing and the technical issues of the thesis.

Many thanks go to the company Bio-Lab staff, especially Dr. Zana H. Mahmood, Dr. Hersh Abdul, and Dr. Karzan T. Shafiq for their help throughout this project.

I would like to mainly thanks Dr. Peshnyar Muhamad Atta, for his endless help and assistance in laboratory work; I wish to express my thanks to Assist. Prof. Dr. Nawazad Abdulrhman, Dr. Shakhawan Latif, Dr. Azher Ahemd, and Dr. Rana Al-Obaidi, for their help during this work. Finally, I wish to express my thanks to my great family for their encouragement and support me during this work.

DECLARATION FORM

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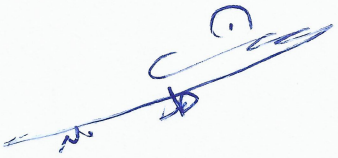
Signature: 

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LIST OF ABBREVIATIONS

Abbreviation	Meaning
BLAST	Basic Local Alignment Search Tool
bp	Base pair
cDNA	Complimentary Deoxyribonucleic acid
CFT	Complement fixation test
COMC	*Chlamydia* Outer Membrane Complex
Ct	Cycle threshold
DMEM	Dulbecco's Modified Eagle Medium
dNTPs	Deoxy nucleotides
EAE	Enzootic abortion of ewes
EB	Elementary body
ELISA	Enzyme-linked immunosorbent assay
FBS	Fetal Bovine Serum
GAPDH	Glyceraldehyde-3-phosphate dehydrogenase
Hsps	Heat shock proteins
IFA	Immunofluorescence test Assay
IM	Inner membrane
LPS	Lipopolysaccharides
Mip	Macrophage infectivity potentiator
MLST	Multi Locus Sequence Typing
MOMP	Major outer membrane protein
mRNA	Messenger Ribonucleic Acid
MW	Molecular weight
OEA	Ovine enzootic abortion
Omp	Outer membrane protein
PBS	Phosphate Buffered Saline
PCR	Polymerase chain reaction
Pmp	Polymorphic membrane protein
qRT-PCR	Quantitative reverse transcription PCR
RB	Reticulate body
RPM	Revolutions per minute
SPG	Sucrose Phosphate Glutamate
SPSS	Statistical Package for Social Science
T3SS	Type III secretion system
TBE	Tris/Borate/EDTA
WHO	World Health Organization
x g	Acceleration due to gravity

Chapter One

INTRODUCTION

Chlamydia abortus, formally called *Chlamydia psittaci* serotype 1, is a Gram-negative bacterium that belongs to the family *Chlamydiaceae*. *Chlamydiaceae* are obligate intracellular bacteria and are entirely dependent on host epithelial cells for their intracellular survival and growth (Selim, 2016). It is an economically important pathogen of small ruminants that has a specific predilection for the placental tissues (Elberbawy and Elkhabaz, 2014).

The genome of *C. abortus* is composed of a 1,144,377-bp circular chromosome with an overall GC content of 39. 87%. It possesses only single copies of the 23S, 16S, and 5S rRNA genes, in contrast to other *Chlamydia* species, which possess two copies (Thomson *et al.*, 2005).

C. abortus is a common cause of reproductive disorder in sheep and goats in almost every region of the world (Menzies, 2012). The infection is known as enzootic abortion of ewes (EAE), ovine enzootic abortion (OEA), or ovine chlamydial abortion (Spičic *et al.*, 2015). Due to the intrinsic nature of *C. abortus* infection, the organism cannot be detected either serologically or by direct detection of the pathogen until the time of abortion (Livingstone *et al.*, 2005).

As a result, the diagnosis of OEA depends on the isolation and identification of the causative agent from abortion materials (placenta, fetus, and vaginal discharge) in living cells. Embryonated chicken eggs and cell culture, combined with serology or detection of the nucleic acid of *C. abortus* using PCR

(polymerase chain reaction), are used to diagnose OEA (O.I.E, 2012). Amplification of *C. abortus* DNA by real-time PCR is considered to be the most sensitive and rapid diagnostic method available for OEA diagnosis (Sachse *et al.*, 2009).

The extent of *C. abortus* infections in Iraq has generally been documented serologically. At the same time, studies on pathogen isolation and detection by molecular methods such as PCR and real-time PCR are relatively rare in Slemani province, Iraq. Therefore, this study was conducted to achieve the following aims:

- Identification and molecular investigation of the *C. abortus* contributed to abortion in sheep.
- Molecular investigation of some virulence factors of *C. abortus* proteins that are assumed to be involved in pathogenicity and expressed during different stages of animal infection, such as: Gene encoding Mip (CAB080): Macrophage infectivity potentiator protein; Gene encoding Pmp18D (CAB776): Polymorphic outer membrane protein; and Gene encoding Momp (CAB048): Major outer membrane protein.

Chapter Two

LITERATURE REVIEW

2.1. Biology of *Chlamydiaceae*

2.1.1. History

Halberstaedter and von Prowazek first described chlamydial organisms in 1907 as intracytoplasmic inclusions containing large numbers of microorganisms in conjunctival epithelial cells of patients with trachoma **(**Figure 2.1). Considered to be protozoa, those microorganisms were named 'Chlamydozoa' after the Greek word 'Chlamys' for mantle. In 1930, Bedson and Western isolated small basophilic particles from blood and tissues of infected birds and humans during an outbreak of human psittacosis. That same year, the causative agent of Lymphogranuloma Venereum (LGV) was isolated from human tissue. Since these particles were not restrained by bacterial filters and could not be cultured on artificial media, they were classified as viruses in the psittacosis-LGV group (Hellerström and Wassén, 1930).

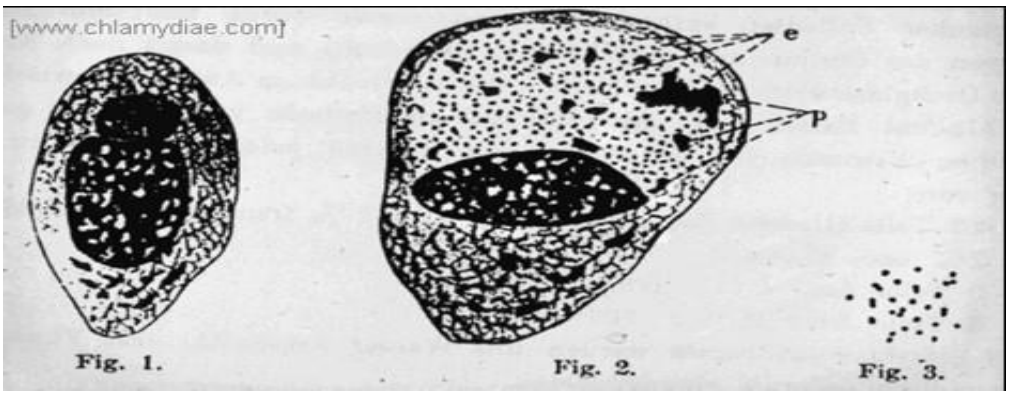

Figure 2.1. Original drawings of inclusion bodies by Halberstaedter and von Prowazek´s. It shows a normal conjunctival epithelial cell (left), an infected cell (middle), and free Chlamydial particles (right). The image was adopted from www.chlamydiae.com.

Two years later, the complicated developmental cycle was described for the first time when Bedson and Bland (1932) identified the reticulate body, additionally to the previously described elementary body. Still, these pathogens were considered to be viruses because of their obligate intracellular lifestyle. Because of their multiplication through binary fission, Bedson and Gostling (1954) considered these 'obligate intracellular parasites with bacterial affinities' more related to *Rickettsiae* than to bacteria. The development of electron microscopy and cell culture facilitated the clarification of the true nature of these pathogens. Because of their distinct developmental cycle, the presence of both DNA and RNA, the structure of the cell wall, and their sensitivity to antibiotics, the *Chlamydiaceae* were finally classified as Gram-negative bacteria (Moulder, 1966).

The first chlamydial infections in domestic mammals were described in 1936 after abortions in sheep in Scotland. However, the abortions were considered to be the result of environmental factors at that time (Greig, 1936). Only in 1950, it was demonstrated that these abortions were the result of an infectious disease caused by an organism of the psittacosis group (Stamp, 1951).

2.1.2. Taxonomy of Chlamydiales

The discovery of *Chlamydia*-like organisms (environmental Chlamydiae) in 1990 challenged the perception of chlamydial diversity, which was previously based on just the *Chlamydiaceae* family but now includes eight families (Horn, 2008). The family *Chlamydiaceae* is the only family in the order Chlamydiales, and all chlamydial species belong to this family. In the early years of the chlamydial taxonomy, the family *Chlamydiaceae* consisted of the single genus

Chlamydia that had two species, *C. trachomatis,* and *C. psittaci*. This classification was based on the differences in the morphology of intracytoplasmic inclusions, presence or absence of glycogen in the inclusions, susceptibility to sodium sulphadiazine and natural host (Forsey and Darougar, 1984).

In subsequent years, the development of DNA-based classification methods led to the designation of two *C. psittaci* strains into new species, *C. pneumoniae* (human respiratory pathogen) (Grayston *et al.*, 1989) and *C. pecorum*. The latter is responsible for encephalomyelitis, pneumonia, and polyarthritis in cattle and sheep (Fukushi and Hirai, 1992).

In 1999, Everett *et al*. proposed a revision of *Chlamydiaceae* classification, based mainly on phylogenetic analysis of the 16s and 23s rRNA genes. One additional genus and five new species were then created. Thus, the family *Chlamydiaceae* has now been divided into two genera, *Chlamydia* and *Chlamydophila* .The genus *Chlamydia* contains three species, *C. trachomatis* (human strain), *C. suis* (porcine strain), and *C. muridarum* (mouse and hamster strain). In comparison, the new genus *Chlamydophila* was assigned six species, namely *Cp. psittaci* (avian strain), *Cp. pneumoniae* (human strain), *Cp. pecorum* (ruminant and swine strain) *Cp. felis* (cat strain), *Cp. caviae* (guinea pig strain) and *Cp. abortus* (Figure 2.2), which is the classical serotype one responsible for OEA. Moreover, the order of the Chlamydiales was expanded with three additional families, which are *Parachlamydiaceae*, *Simkaniaceae*, and *Waddliaceae* (Everett *et al.*, 1999).

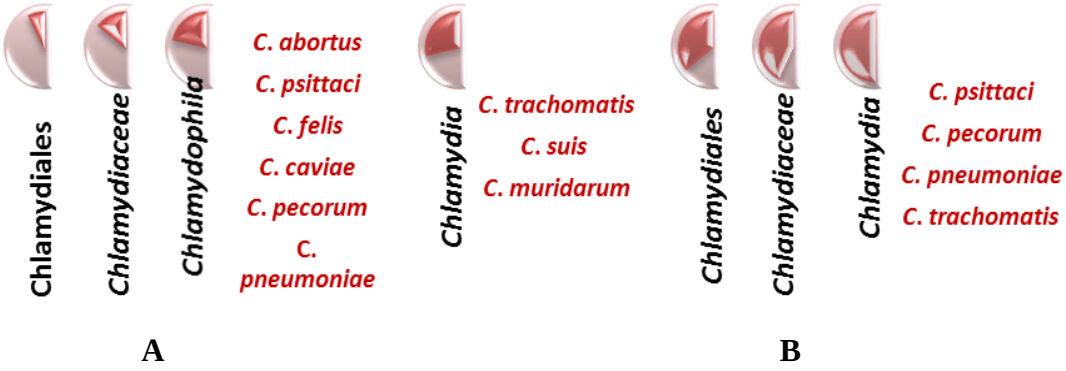

Figure 2.2. Genetic structure of the order Chlamydiales. A: The taxonomic revision of the Chlamydiales and it shows the family *Chlamydiaceae* consisted of the two genera, *Chlamydia* and *Chlamydophila*. B: The old taxonomy of the Chlamydiales and the family *Chlamydiaceae* consisted of the single genus, *Chlamydia* (Everett *et al.*, 1999).

This division was not widely accepted at the time. Although it was used extensively by veterinary chlamydiologists, it was perhaps most notably not adopted by groups working on the human pathogen *C. pneumoniae* (Stephens *et al.*, 2009).

In 2010, the taxonomy changed again, and all nine species now belong to the single genus of *Chlamydia*. These bacteria can cause a spectrum of diseases in a range of hosts. The primary sites of Chlamydial infection are mucosal surfaces, and in some cases, pathology is limited to these sites. However, some species and biovars within species can invade further to cause pathology at distal sites (Table 2.1).

Table 2.1. Members of the family *Chlamydiaceae*. Adapted from (Kerr *et al.*, 2005).

Species	**Host**	**Serovars**	**Clinical signs**
Chlamydia trachomatis	Humans (Nunes and Gomes, 2014)	D, E, F, G, H, I, J, and K	Sexually transmitted disease (STD), infection of the urogenital
		A, B, Ba, and C	Chronic conjunctivitis and blindness (trachoma)
		L1, L2, and L3	Lymphogranuloma venereum (LGV)
Chlamydia muridarum	Mice, hamsters		Respiratory tract infection, genital tract infection
Chlamydia suis b	Pigs		Diarrhea, pneumonia, conjunctivitis, reproductive disorders
Chlamydia pneumonia	Humans, koala		Pneumonia, bronchitis, encephalomyelitis, laryngitis, atherosclerosis, reactive arthritis
Chlamydia psittaci a	Birds		Respiratory tract infection
Chlamydia gallinaceae b	Chicken, guinea fowl, turkey		Respiratory tract infection
Chlamydia avium b	Pigeons, psittacine birds		Respiratory tract infection
Chlamydia abortus a	Ruminants, pigs		Reproductive disorders, abortion, and bad semen quality
Chlamydia pecorum	Ruminants, pigs, koala		Reproductive disorders, infertility, infection of the urine tract (koala) and abortion, enteritis, polyarthritis, encephalomyelitis, metritis, conjunctivitis and pneumonia (other animals)
Chlamydia felis b	Cats		Conjunctivitis and respiratory tract infection
Chlamydia caviae b	Guinea pigs		Ocular and urogenital tract infection

a = Zoonotic pathogen, b = Potential Zoonotic pathogen.

Recently, Sachse *et al*. (2014) proposed the addition of two new species to the current classification **(**Figure 2.3). These species are *C. avium sp. nov*., comprising strains from pigeons and psittacine birds, and *C. gallinaceae sp. nov*., comprising strains from poultry (Sachse *et al.*, 2014).

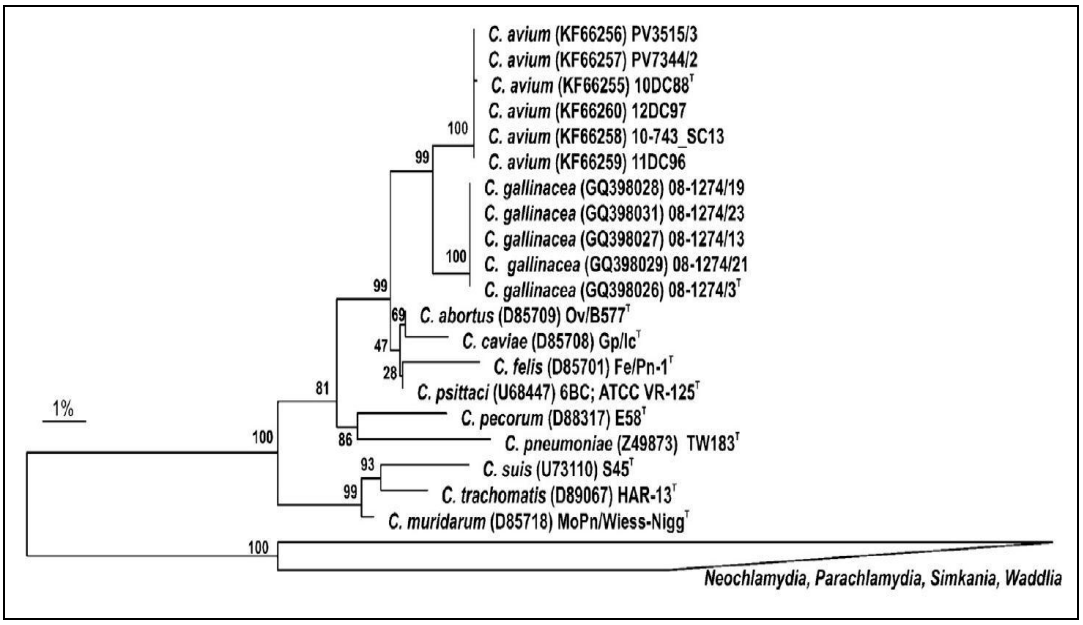

Figure 2.3. Phylogenetic reconstruction of the *Chlamydiaceae* classification, including the two new species *C. avium* and *C. gallinaceae* based on the alignment of almost complete 16S rRNA genes. The numbers on the nodes indicate the bootstrap support of each branch .Adapted from (Sachse *et al.*, 2014).

2.1.3. Morphology of *Chlamydia*

Members of the family *Chlamydiaceae* are obligate intracellular microorganisms that parasitize and replicate in a vast array of eukaryotic cells (Stephens, 1999). The developmental cycle of *Chlamydia* was first described by Bedson and Bland (1932). Chlamydial forms can be differentiated according to morphology, chronological appearance during multiplication, function, strength against mechanical agitation, cell wall permeability, and chemical composition of cell envelopes (Storz, 1971; Mårdh *et al.*, 1977). Infection can be confirmed later by electron microscopic studies (Soloff *et al.*, 1982; Matsumoto, 1988). Infection is biphasic, consisting of small extracellular infectious but metabolically inert elementary bodies (EBs) and larger intracellular reticulate bodies (RB). The

conversion of EBs to RBs and the reorganization of RBs back to EBs are unique stages in the chlamydial development cycle.

The EBs are dense, roughly nonmotile, and of most *Chlamydia*, coccoid. Some newly identified *Chlamydia*-like organisms, however, have been shown to possess elongated, rod-shaped, or even star-like shaped EBs, range from 0.3 to 0.6 μm in diameter (Štrus *et al.*, 2004; Thomas *et al.*, 2006; Corsaro *et al.*, 2007). They are extracellular infectious forms that possess highly condensed DNA (Baehr *et al.*, 1988; Barry *et al.*, 1992; Shen *et al.*, 2004; Sun *et al.*, 2008), and are believed to be metabolically inactive (RNA/DNA ratio of 1:1) (Ward, 1988; Shen *et al.*, 2004). The primary role of the spore-like infectious EB is to survive outside the host cell until the re-infecting new host cells. The EBs is well-adapted to the extracellular environment. They are osmotically stable and poorly permeable because of the highly cross-linked major outer membrane protein (MOMP) and other envelope proteins (Storz, 1971; Hatch *et al.*, 1984; Newhall, 1987). An electron-dense nucleoid is eccentrically located and is in contact with the plasma membrane. The remainder of the EB is less dense than the nucleoid and is composed of closely-packed ribosomes and moderately dense amorphous material (Mårdh *et al.*, 1977). Reorganization from infectious EBs to the noninfectious RBs proceeds through dispersing forms (Degraves *et al.*, 2004). They contain a granular matrix that is less dense that the nucleoid of EB but denser than RB.

The RBs are the intracellular, osmotically fragile, and surrounded by an inner and outer trilaminar membrane, ranging from 0.6 to 1.3 μm in diameter (Mårdh *et al.*, 1977). RBs represent the metabolically active stage and are highly transcriptionally active (RNA/DNA ratio of 3:1). The maximum expression of

genes involved in cell division, energy metabolism, protein folding, DNA replication, and DNA repair could be measured during this stage (Baehr *et al.*, 1988; Ward, 1988; Belland *et al.*, 2003; Nicholson *et al.*, 2003; Mäurer *et al.*, 2007). The internal structure of RB consists of numerous ribosomes and interspersed fine reticulated fibers. Smaller reticulate bodies are transformed, through condensing forms (intermediate bodies-IB), into the infectious elementary bodies.

In contrast to other gram-negative bacteria, *Chlamydia* lack peptidoglycan between the outer and inner membranes (Liao *et al.*, 1997). The outer membrane is composed of hexagonal subunits of the MOMP that connect to transmembrane channels (Giannati-Stefanou *et al.*, 1999). Ten to thirty hemispheric projections, which are hexagonally arranged at a center to center spacing of 50 nm, are present on the surface of EB (Wang *et al.*, 2001). Each projection emerges from a rosette-like pore that is approximately 30 nm in diameter and is anchored in the cytoplasmic membrane (Wilsmore *et al.*, 1990). These surface projections may be involved in the uptake of nutrients. However, they play no role in attachment of chlamydia to host cells (Grayston *et al.*, 1986). Genus-specific LPS and the MOMP are significant components of the Chlamydial cell wall (Cotter *et al.*, 1995). The MOMP is a transmembrane protein that is linked by disulfide bonds to other cysteine-rich membrane proteins to maintain structural rigidity. Exposure of the EB to reducing conditions in phagosomes breaks these disulfide bonds, making the cell wall less rigid and allows the structural changes that occur when the EB changes into an RB (Hatch *et al.*, 1982). Reduction also results in porin formation, whereby the outer membrane becomes permeable to nucleotides and amino acids required for RB metabolism (Hatch, 1996). Chlamydial LPS strongly

resembles the LPS of other gram-negative bacteria in its location in the outer membrane of the chlamydial envelope, chemical structure, and biological activity. The LPS contains at least three antigenic domains. Two of the domains are shared with the LPS of certain other Gram-negative organisms, including *Salmonella* rough mutants and *Acinetobacter calcoaceticus*, and the third domain is specific for *Chlamydia* (Williams *et al.*, 1989; Reichel *et al.*, 2018).

2.1.4. Developmental Cycle of *Chlamydia*

The developmental cycle of *Chlamydia* consists of five major phases (Figure 2.4). These phases start with the attachment and uptake of the EB, followed by the entry of the metabolically inert EB into the metabolically active RB. Then, the RBs start to grow and divide, and the maturation of noninfectious RB into infectious EB ensues. Finally, new EBs is released from the host cell.

The attachment of the EB to the eukaryotic cell surface forms the beginning of the acute infection. Binding preferentially occurs at surface microvilli. Since the base of the microvilli are areas of active transport of extracellular materials into the host cells, attachment to these sites might enable a rapid and efficient entry (Escalante-Ochoa *et al.*, 1998). Upon binding, the EBs is internalized in tight, endocytic vesicles called inclusions. There is currently no general convention on the mechanism of attachment and entry of EBs since several different mechanisms have been described (Wyrick *et al.*, 1989).

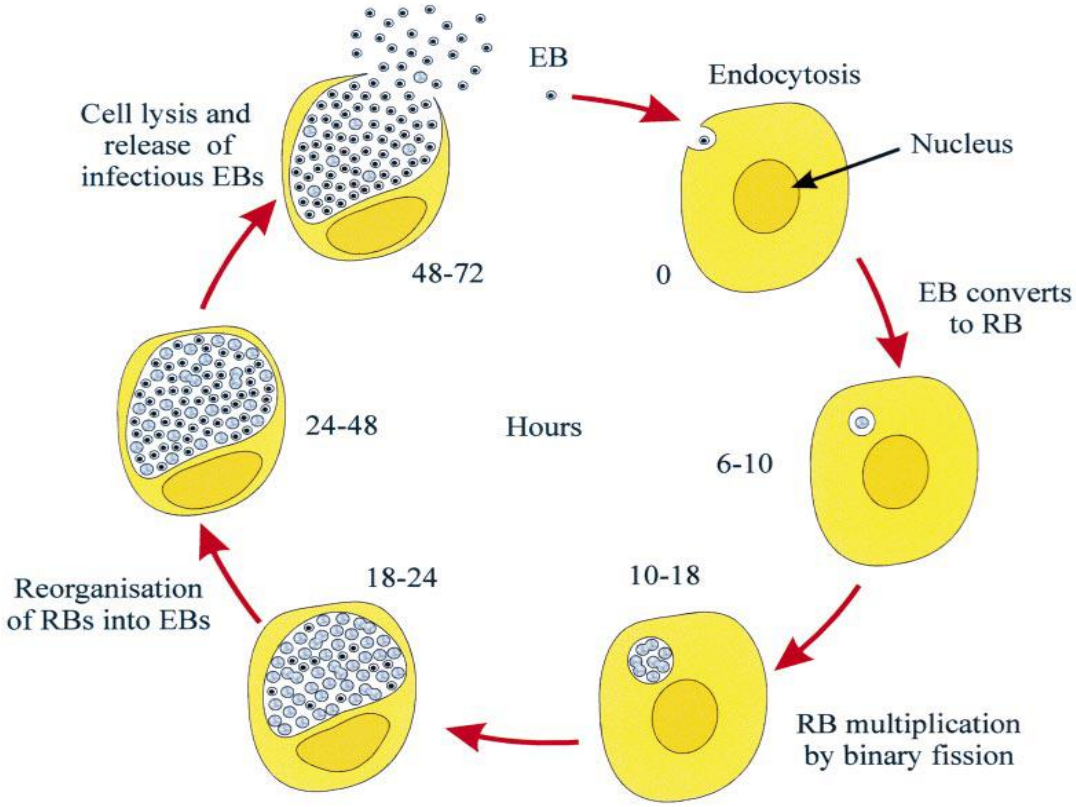

Figure 2.4. Chlamydial developmental cycle. The cycle was started with the attachment and uptake of the EB, followed by the entry of the metabolically inert EB into the metabolically active RB. Then, the RBs start to grow and divide, and the maturation of noninfectious RB into infectious EB ensues. Finally, new EBs is released from the host cell (Longbottom and Coulter, 2003).

These mechanisms include receptor-mediated endocytosis in clathrin-coated pits , pinocytosis in non-clathrin-coated pits, and microfilament-dependent phagocytosis (Prain and Pearce, 1989). The newly formed inclusions efficiently escape fusion with cellular lysosomes and EBs start to differentiate into RBs two hours post-infection. These RBs move towards the periphery of the inclusion and start replication from eight hours post-infection. As the RBs multiply, the inclusion rapidly fills and expands in size. After a period of 24–48 hours, the RB progeny recondenses back into metabolically inactive infectious EBs. The EB is stored in the lumen of the inclusion until they are released from the host cell at 48 to 72 hours post-infection through host cell rupture or exocytosis, and go on to

invade neighboring cells . The exact length of the developmental cycle is species-dependent (Moulder, 1991).

Both EBs and RBs have inner and outer membranes and a distinct but variable periplasmic space. Due to the lack of a cell wall, *Chlamydia* appears as Gram-negative on Gram's stain. EBs, when stained with basic dyes such as fuchsin in the Macchiavello or Gimenèz stains, they are visible as small red dots. However, their appearance is purple-blue in the Giemsa staining procedure (Figure 2.5). The development and replication of *Chlamydia* are dependent on the nutrient supply and metabolic status of the host. Delayed development due to nutrient deprivation can lead to enlarged, aberrant a degenerative chlamydial forms (Ahluwalia, 2010).

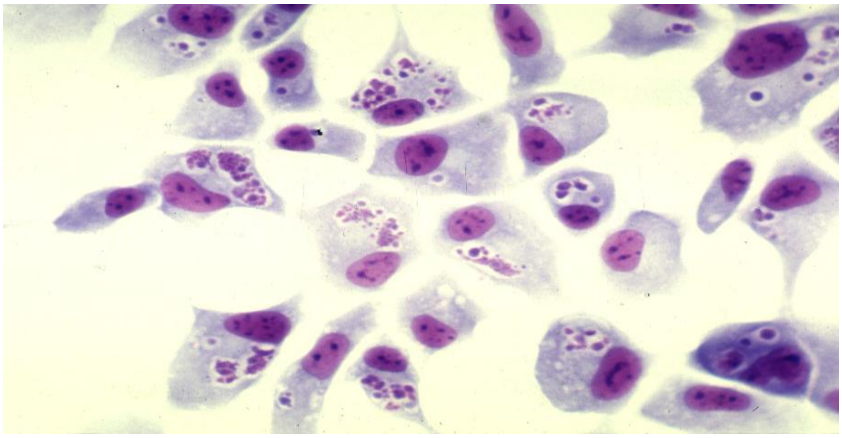

Figure 2.5. Mouse L 929 fibroblast monolayer cells infected with *Chlamydia psittaci* and stained by Giemsa's stain. Cells were cultivated in Minimum Essential Medium with Eagle's salts (Eagle's MEM). The intracellular inclusions contain few chlamydial developmental forms such as RB and EB, but also enlarged aberrant chlamydial organisms (Kaltenboeck and Wang, 2005).

The EB, in contrast to RB, is characterized by resistance to both physical and chemical factors in the extracellular environment as well as lack of metabolic

activity. This resistance is a consequence of the rigidity of the cell envelope, which is both osmotically stable and poorly permeable, and also of the significantly reduced surface area of the EB compared to that of RB. Thus, the EB is adapted for prolonged extracellular survival, which may mean many months outside the natural host (Longbottom and Coulter, 2003).

2.1.5. Chlamydial Effector Proteins

Members of the Chlamydial encode a Type III secretion system (T3SS) (Samudrala *et al.*, 2009). This efficient molecular syringe is essential for its entrance, growth, and survival in eukaryotic host cells through the transport of effector proteins to the host cell cytoplasm. The number of secreted anti-host proteins is estimated at 80 proteins for *C. trachomatis* (Betts *et al.*, 2009), although the exact function of many proteins remains to be uncovered. Within minutes upon attachment of EBs, the T3SS translocate the already produced effector proteins to the host cytoplasm, including Tarp (Translocated Actin-Recruiting Phosphoprotein). This protein is spatially and temporarily associated with the recruitment of actin at the site of internalization (Clifton *et al.*, 2004). The formation of an actin-rich pedestal underneath the attachment site eventually leads to the uptake of EBs into membrane-bound vesicles (Carabeo *et al.*, 2002; Coombes and Mahony, 2002; Subtil *et al.*, 2004; Beeckman *et al.*, 2007). The Tarp-mediated actin polymerization is not merely the result of a stable association between Tarp and actin but is more complex, involving multiple domains of Tarp (Jewett *et al.*, 2006).

2.1.6. Chlamydial Outer Membrane Complex (COMC)

Like all Gram-negative bacteria, *Chlamydiaceae* are surrounded by an outer membrane (OM) and a cytoplasmatic or inner membrane (IM), which is separated by a periplasmic space (Figure 2.6). The EB outer membrane contains phospholipids, lipids, lipopolysaccharides (LPS) and proteins. The proteins of the OM are essential for various purposes, including envelope architecture, virulence, transport, cell division, induction of inflammatory cytokine production, and immune evasion (Hatch, 1996; Stephens and Lammel, 2001).

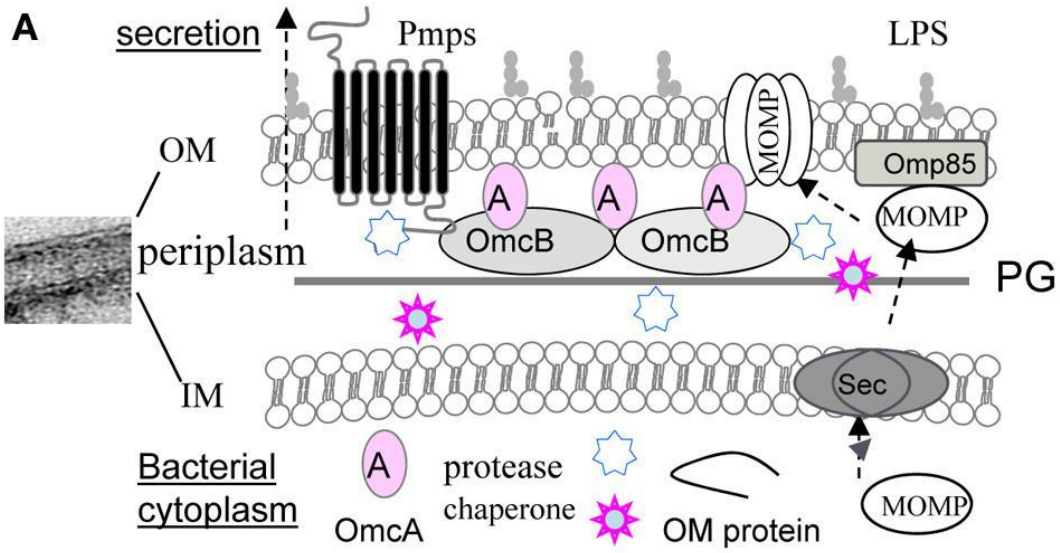

Figure 2.6. A schematic diagram of the envelope structure of *C. trachomatis*. Showing are there preventative OM associated components [Lipopolysaccharide (LPS), peptidoglycan (PG), MOMP, Pmps, OmcA, OmcB, and Omp85], and putative chaperone and protease in the periplasm in EBs. The potential process of MOMP or other OM protein transport across the inner membrane via the Sec pathway, and insertion and assembly into the OM is indicated (Caldwell *et al.*, 1981).

The Gram-negative cell wall strength is usually provided by peptidoglycan, covalently bound to lipoproteins. Typically, this part of the membrane is insoluble in sarkosyl, an anionic detergent. The *Chlamydiaceae* cell walls contain only a small amount of structural peptidoglycan but derive their

strength from crosslinks formed between the sulfur atoms of sulfur amino acid-rich proteins in the outer envelope (Moulder, 1993; Hatch, 1996). Still, part of their cell wall is also insoluble in sarkosyl, and this fraction is called the *Chlamydia* Outer Membrane Complex (COMC). This protein complex predominantly consists of MOMP, two cysteine-rich proteins (CRP), EnvA or OmcA and EnvB or OmcB, and the polymorphic membrane proteins (Pmps). Besides, the outer membrane also contains the proteins PorB, Omp85, OprB, heat shock proteins hsp60 and hsp70, CTL 0541, CTL0648, CTL0887, and Pal (Hatch *et al.*, 1984; Stephens and Lammel, 2001).

The Major Outer Membrane Protein (MOMP) is a cysteine-rich protein with a molecular weight of approximately 40 kDa. About 60% of the EB outer membrane and almost 100% of the RB is covered by MOMP (Hatch and McClarty, 1998). This protein is vital for the maintenance of the structural rigidity of the EB and functions as an adhesion, mediating nonspecific interactions with host cells (Su *et al.*, 1990). After the reduction of disulfide bonds, MOMP can also function as a porin for nutrient uptake. The *ompA* gene encodes the MOMP protein, which contains five conserved domains (CS1–CS5) with in-between four variable domains (VSI–VSIV). The domains are localized outside of the bacterial membrane (Figure 2.7) (Stephens *et al.*, 1987; Baehr *et al.*, 1988; Yuan *et al.*, 1989). MOMP is an immunodominant protein and contains genus-, species- and serovar-specific epitopes (Caldwell *et al.*, 1981). Moreover, mono- and polyclonal antibodies against MOMP can neutralize *Chlamydiaceae* infections both *in vitro* and *in vivo*. Therefore, MOMP is an interesting candidate to be selected as a family- and species-specific antigen (Caldwell and Perry, 1982; Zhang *et al.*, 1987).

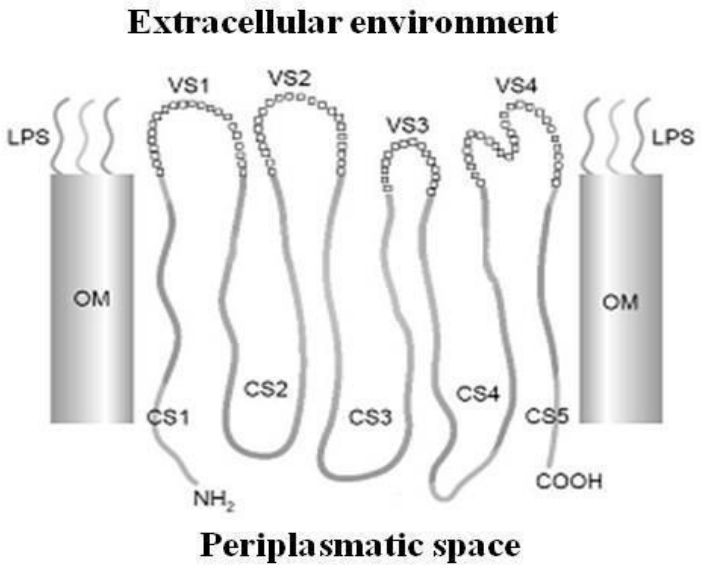

Figure 2.7. Model of the positioning of the MOMP protein in the outer membrane of the chlamydial cell wall. The conserved regions are represented as full lines and localized inside the outer membrane. The alternating lines represent the surface-exposed variable domains I to IV. Adapted from (Baehr *et al.*, 1988; Kim and DeMars, 2001).

The second most abundant proteins in the EB outer membrane complex, OmcA, and OmcB**,** are hardly present in RBs (Newhall, 1987; Everett and Hatch, 1995; Sanchez-Campillo *et al.*, 1999; Watts and Kennedy, 1999). Both proteins are highly immunogenic and contain many conserved cysteine residues. OmcA, also known as 'outer membrane protein 3' (Omp3) or EnvA, is a lipoprotein with a molecular weight ranging from 9 kDa in *C. trachomatis* to 12 kDa in *C. psittaci*. The OmcA protein is probably inserted into the outer membrane, considering the presence of the signal peptide (Everett and Hatch, 1991).

OmcB, also known as 'outer membrane protein 2' (Omp2) or EnvB, is a *Chlamydiaceae*-specific protein that can be used as a marker for chlamydial infections (Sanchez-Campillo *et al.*, 1999). This 60 kDa protein contains genus-specific epitopes, and its sequence is highly conserved, suggesting a crucial

function in the chlamydial life cycle (Watson *et al.*, 1989; De la Maza *et al.*, 1991). In contrast to *C. trachomatis* serovar E and *C. psittaci* 6BC, the OmcB protein of *C. trachomatis* LGV1 is surface exposed and functions as an adhesin (Watson *et al.*, 1994; Everett and Hatch, 1995; Stephens and Lammel, 2001; Fadel and Eley, 2007; Fadel and Eley, 2008).

Polymorphic Membrane Proteins or Pmps are unique to *Chlamydia* and were first discovered at the surface of *C. abortus* S26/3 (Longbottom *et al.*, 1996). The number of *pmp* genes varies from nine for *C. trachomatis* (Stephens *et al.*, 1998) to 21 for *C. pneumoniae* (Villegas *et al.*, 2010). Phylogenetic analysis indicated the existence of six subfamilies (A, B/C, D, H, E/F, and G/I) (Grimwood and Stephens, 1999). Table 2.2 provides an overview of the *pmp* genes and the associated nomenclature for each chlamydial species (Henderson and Lam, 2001).

Table 2.2. Overview of the number and associated nomenclature of the *pmp* genes present in the different chlamydial species. It was adapted from (Tan *et al.*, 2006).

Species	**Number of *pmp* genes**	**Nomenclature**
Chlamydia trachomatis	9	A to I
Chlamydia suis	9	A to I
Chlamydia psittaci	21	An, Bn, Dn, En, Hn, Gn
Chlamydia abortus	18	An, Bn, Dn, En, Hn, Gn
Chlamydia pneumonia	21	An, Bn, Dn, En, Hn, Gn
Chlamydia caviae	17	An, Bn, Dn, En, Hn, Gn
Chlamydia felis	12	An, Bn, Dn, En, Hn, Gn
Chlamydia pecorum	15	An, Bn, Dn, En, Hn, Gn
Chlamydia muridarum	9	A to I

n represents a number.

This classification is based on the subfamily to which the protein belongs. The existence of six *pmp* subfamilies proposes at least six different functions, most likely in chlamydial virulence (Henderson and Lam, 2001). Although

specific roles have been described for only some Pmps, they are generally responsible for avoidance of the immune response while maximizing virulence (Tanzer *et al.*, 2001; Tan *et al.*, 2006).

PorB or OmpB is a 37 kDa, cysteine-rich protein, expressed in meager amounts and is present in the outer membrane of EBs. The sequence is highly conserved among chlamydial strains. PorB probably functions as a substrate-specific porin, providing chlamydia with dicarboxylic acids to compensate for the incomplete tricarboxylic acid cycle (Iliffe-Lee and McClarty, 2000; Kubo and Stephens, 2001). PorB-specific antibodies have neutralizing activity and were first discovered in human sera (Sanchez-Campillo *et al.*, 1999).

Omp85 is a highly conserved outer membrane protein and widely present in a wide range of Gram-negative bacteria and mitochondria. Omp85 is generally considered to be essential for cell viability and is involved in the insertion of lipids and proteins into the bacterial outer membrane (Genevrois *et al.*, 2003; Voulhoux *et al.*, 2003). Omp85-specific antibodies neutralize Chlamydial infections *in vitro* (Stephens and Lammel, 2001).

The Chlamydial lipopolysaccharide **(**LPS) is present on both EBs and RBs and is a major antigenic component. This 10 kDa molecule has a lipid a part, containing two glucosamines (GlcN) bound to fatty acids, and contains a specific tri-saccharide of 3-deoxy-D-manno-oct-2-ulopyranosonic acids (Kdo). Two of these Kdo residues are linked through a 2 → 8 linkage, and this structure is unique for *Chlamydiaceae* (Brade *et al.*, 1987).

Heat shock proteins (Hsps) are present in the COMC of both EBs and RBs and are considered to play an essential role in chlamydial immunopathology (Zhong and Brunham, 1992). So far, the Hsp10, Hsp60, and Hsp70 proteins have been identified for *Chlamydia* (Kornak *et al.*, 1991). These proteins are structurally alike and highly conserved within chlamydial species. Moreover, Hsps are homologous to GroEL (Hsp60) and DnaK (Hsp70) of *Escherichia coli* and human mitochondria, with up to 50% protein sequence identity (Singh and Gupta, 2009).

2.1.7. Chlamydial Metabolism

The intimate relationship of *Chlamydia* with their eukaryotic host cells led to several adaptations of the bacterial metabolic processes to that of the host cell to effectively exploit the host organism. Within the relatively safe host compartment, intracellular bacteria have access to the universal energy-rich metabolic intermediates and, accordingly, the establishment of symbiosis has enabled the bacteria to reduce major metabolic pathways (Zientz *et al.*, 2004). *Chlamydiaceae* are therefore not able to synthesize purines, pyrimidines, NAD+, and several cofactors *de novo*. They are auxotrophic for most amino acids and lack critical enzymes of several biosynthetic pathways (Mcclarty, 1999). To compensate for missing metabolic intermediates and nucleotides, *Chlamydiae* encode a wide variety of transporter proteins (Mcclarty, 1999; Tjaden *et al.*, 1999; Winkler and Neuhaus, 1999). However, genome sequencing revealed that *Chlamydiae* are still able to synthesize essential precursor metabolites during glycolysis, the pentose phosphate pathway, and the tricarboxylic acid (TCA) cycle. Also, *Chlamydiae* produce energy in the form of ATP during oxidative

phosphorylation independently from the host. This surprising finding has changed the view of *Chlamydiae* as real "energy parasites" (Stephens *et al.*, 1998; Read *et al.*, 2000; Nicholson *et al.*, 2003; Villegas *et al.*, 2010). Recent proteome and transcriptome studies have shown that genes involved in energy metabolism are maximally expressed during the middle and later stages of the developmental cycle. While in its early stages, a dependence on host cell ATP exists, which is imported by constitutively expressed ADP/ATP translocases (Shaw *et al.*, 2002; Nicholson *et al.*, 2003).

2.1.8. Comparative *Chlamydiaceae* genomics

For many years, the whole-genome sequencing of different species has been the most vital tool to investigate chlamydial biology. The first sequenced genome was an isolate of *C. trachomatis* serovar D in 1998 (Stephens *et al.*, 1998), and genomes from ten chlamydial species were sequenced later (Hölzer *et al.*, 2016). The chlamydial genome is one of the smallest prokaryotic genomes. It is composed of one circular chromosome of only approximately 1 Mbp and is highly conserved in genes responsible for functions shared among the species, like metabolism genes (Clarke, 2011).

Comparative analyses of the *Chlamydia* genomes have demonstrated high levels of conservation across species and reduced genome size (Knittler *et al.*, 2014). The reduced genome size is likely due to the increased reliance on host-derived molecules that accompanies an obligate intracellular life cycle. Evidence of this in the *Chlamydiaceae* is demonstrated by the loss of several genes involved in metabolic pathways, as these molecules could be scavenged from the host (Thomson *et al.*, 2005). Many common genes with important functions have been

identified within all the *Chlamydiceae* genomes. Polymorphic membrane proteins (Pmps) and inclusion membrane proteins (incs) (Gupta and Griffiths, 2006) are thought to function in interactions with the host, including in adhesion and antigen-driven immune evasion (Nunes and Gomes, 2014). *Chlamydiaceae* also possess (T3SS), which is thought to be important for virulence as it allows the bacterium to insert effectors into the host cell. Translocated actin recruiting protein (TARP) is an example of a T3SS effector found in all sequenced *Chlamydiaceae* genomes, which is believed to be responsible for the remodeling of host cell actin to facilitate infection (Nunes and Gomes, 2014). The plasticity zone (PZ) has been identified as a hypervariable region present in all *Chlamydiceae* genomes. A number of genes have been identified within the PZ regions that are thought to contribute to chlamydial pathogenicity and niche tropism (Thomson *et al.*, 2005; Rockey, 2011). Comparative genomics has been used to compare *Chlamydiaceae* genomes and. The method revealed interspecies and intraspecies differences, which may explain host and tissue tropism (Bachmann *et al.*, 2014b).

2.1.8.1. *Chlamydia* Interspecies Gene Variation

There is a high level of conservation in both gene order and content despite the varied host range and tissue tropism of the various *Chlamydiaceae* species (Bachmann *et al.*, 2014a). Variability has been identified within the PZ region and *Pmp* genes between different species, which may be important for determining host adaptation (Bachmann *et al.*, 2014b). The PZ region of different species encodes several genes, including a tryptophan (*Trp*) operon, toxin genes,

and biotin synthesis genes, all of which have been implicated in interspecies variation.

The *Trp* operon encodes tryptophan synthase, which is responsible for the synthesis of tryptophan needed for bacterial survival. The genital serovars of *C. trachomatis* encode functional *Trp* operons, which allow the bacteria to synthesize tryptophan from indole found in the genital microflora (Coers *et al.*, 2009). The absence of the *Trp* operon means that the organisms are dependent on host cell tryptophan and are potentially more susceptible to IFN-γ mediated indolamine 2, 3-dioxygenase (IDO) activation, which mediates tryptophan depletion. *Chlamydia pneumoniae*, *C. muridarum*, and *C. abortus* all lack a functional *Trp* operon, which may explain why these bacteria colonize particular anatomical niches with greater access to host tryptophan or where they have protection from IFN-γ mediated degradation (Thomson *et al.*, 2005). IFN- γ avoidance genes may also have a role in determining host-tropism. *C. muridarum* has developed mechanisms to avoid IFN-γ inducible GTPases that are more important for immune defense against *Chlamydia* in mice than tryptophan depletion (Nelson *et al.*, 2005). IFN-γ is an essential cytokine in immune defense to chlamydial infections.

Genes encoding a cytotoxin, similar to the Enterohemorrhagic *E. coli* (EHEC) adherence factor, are present in the PZ region of *C. psittaci*, *C. felis*, *C. caviae*, *C. muridarum*, *C. pecorum*, and *C. pneumoniae* but absent in other chlamydial species (Voigt *et al*., 2012). *C. psittaci*, *C. trachomatis*, *C. pecorum*, *C. felis*, and koala *C. pneumoniae* isolates express membrane attack complex/perforin genes (*MACPF*) (Voigt *et al.*, 2012; Sait *et al.*, 2014). The

function of the cytotoxin and *MACPF* genes in chlamydial species is not yet known (Voigt *et al.*, 2012).

The *guaAB-add* gene cluster encodes accessory virulence genes thought to be involved in purine biosynthesis and is present in *C. pneumoniae, C. muridarum, C. felis,* and *C. psittaci* genomes but not in *C. trachomatis* or *C. abortus* (Read *et al.*, 2013).

Biotin synthesis genes have been found in *C. pneumoniae, C. abortus, C. psittaci, C. felis,* and *C. pecorum* genomes but were absent in *C. muridarum, C. caviae,* and *C. trachomatis* (Sait *et al.*, 2014). The presence of biotin synthesis genes within the *C. abortus* genome may be necessary for the bacteria to live in the placenta (Thomson *et al.*, 2005), as pregnancy has been shown to result in a biotin-deficiency in humans (Mock *et al.*, 2002).

The *pmp* genes were first identified on the outer membrane of *C. abortus* (Longbottom *et al.*, 1998) and have since been identified in all other *Chlamydia* species. The different species have varying numbers of *pmp* genes, from nine in *C. trachomatis* and *C. muridarum* to 21 in *C. pneumoniae* and *C. psittaci* (Knittler *et al.*, 2014). *Pmp* genes are immunogenic and vital to be involved in the adhesion of EBs to the host cell surface. The *pmp* genes also contribute to antigenic variation facilitating evasion of the host immune system (Longbottom *et al.*, 1998). They are characterized by a high rate of mutation between and within species, possibly indicative of adaptation to different hosts. The increased number of *pmp* genes in some species may be reflective of the ability to infect multiple hosts. Therefore, it is likely that the combination of several genetic differences,

and not a single gene, are responsible for determining host and niche tropism between species of *Chlamydia* (Knittler *et al.*, 2014).

2.1.8.2. *Chlamydia* Intraspecies Gene Variation

Chlamydial strains are isolates of the same species that are usually differentiated based on genetic variation, particularly in *ompA* that encodes the (MOMP). However, more recent typing studies have moved to a whole-genome approach. *C. trachomatis* is the only chlamydial species that contains serovars (Table 2.1), which are groups of strains separated based on MOMP differences (Nunes and Gomes, 2014). Different chlamydial serovars/strains can have varied host and tissue tropism. Studying the genetics of these strains will help identify the specific genes involved in host-pathogen interactions (Bachmann *et al.*, 2014b).

A study comparing *C. pecorum* strains from koala, sheep, and cattle found that the region with the most significant number of SNPs was the *pmp* gene region. A different region that includes genes encoding T3SS components and effector proteins also contained a large number of SNPs. Several pseudogenes, including a toxin gene, were identified in the koala strains that were found to be intact in the ruminant strains. The loss of gene function suggests that these genes were not necessary for infection of the koala host but potentially crucial for infection of the ruminant hosts. Three ruminant *C. pecorum* strains that caused different pathological conditions, such as asymptomatic enteric infection, polyarthritis, and metritis, were found to have highly conserved genomes (Bachmann *et al.*, 2014b). Cattle and sheep are often grazed together and are therefore likely to be exposed to similar pathogens, including *C. abortus*. This

species rarely causes abortion in cattle, which is interesting, considering it is an important cause of abortion in sheep. Whole genome sequencing of *C. abortus* strains isolated from cattle, sheep and goats revealed that the strains were highly conserved. Little variation was identified in the PZ region suggesting there was minimal intraspecies variation between *C. abortus* strains. The role of genetic variation between *C. abortus* strains in host tropism cannot be completely discounted (Miyairi *et al.*, 2011).

Genes encoding recombination elements, including *Chlamydia* phages and a conserved plasmid, can be found in many of the *Chlamydiaceae* genomes. Besides, there is evidence that gene exchange usually occurs between different strains of the same species (Nunes *et al.*, 2013; Nunes and Gomes, 2014). The analysis of multiple *C. trachomatis* strains has identified that recombination alters the sequence of important genes (Joseph *et al.*, 2012). Therefore, it is likely to be critical in promoting strain diversity. Isolates of *C. trachomatis* that do not possess the plasmid are rare, suggesting that it is vital for *C. trachomatis* biology. The plasmid has been shown to encode a gene that facilitates the accumulation of glycogen and normal inclusion morphology (Song *et al.*, 2013).

2.2. *Chlamydia abortus* Genome

The general features of *C. abortus* genome are shown in Figure 2.8 and summarized in Table 2.3. The genome is composed of a 1,144,377-bp circular chromosome with an overall GC content of 39. 87% (Thomson *et al.*, 2005). The origin of replication was assigned based on the GC deviation of the genome, as previously described (Parkhill *et al.*, 2001). Characteristically for the *Chlamydia*, *C. abortus* possesses only single copies of the 23S, 16S, and 5S rRNA genes, in

contrast to *Chlamydia* species, which possess two copies (Everett *et al.*, 1999). The annotation identified 961 predicted coding sequences (CDS), representing a coding density of 88%. Of the predicted CDS, 746 have been given functional assignments based on previous experimental evidence or database similarity and motif matches. For those with no functional assignment (215 CDS), 110 were only significantly similar to proteins from other members of the *Chlamydiaceae*, and 15 predicted CDS returned no significant database hits. In total, 38 tRNAs were identified, which corresponded to all the amino acids except selenocysteine. There is no evidence of recent horizontal gene transfer in *C. abortus*, including a complete lack of any phage genes that have been seen in other sequenced *Chlamydia* species (Read *et al.*, 2000; Read *et al.*, 2013). Significantly, *C. abortus* lacks any toxin genes, and also lacks genes involved in tryptophan metabolism and nucleotide salvaging, suggesting that the genetic basis of niche adaptation of this species is distinct from those previously proposed for other chlamydial species (Read *et al.*, 2013).

Table 2.3. Summary of the *C. abortus* genome features. Adapted from (Thomson *et al.*, 2005).

Criteria	***C. abortus* (S26/3)**
Genome size (bp)	1,144,377
% GC of genome	39. 87
% GC of CDSa	40. 5
% coding	88. 2
No. of CDSa	961
Avg. aa % ID to *C. abortus* orthologs	—
No. of Pmp proteins	18
No. of Trna	38
No. of rRNA operons	1

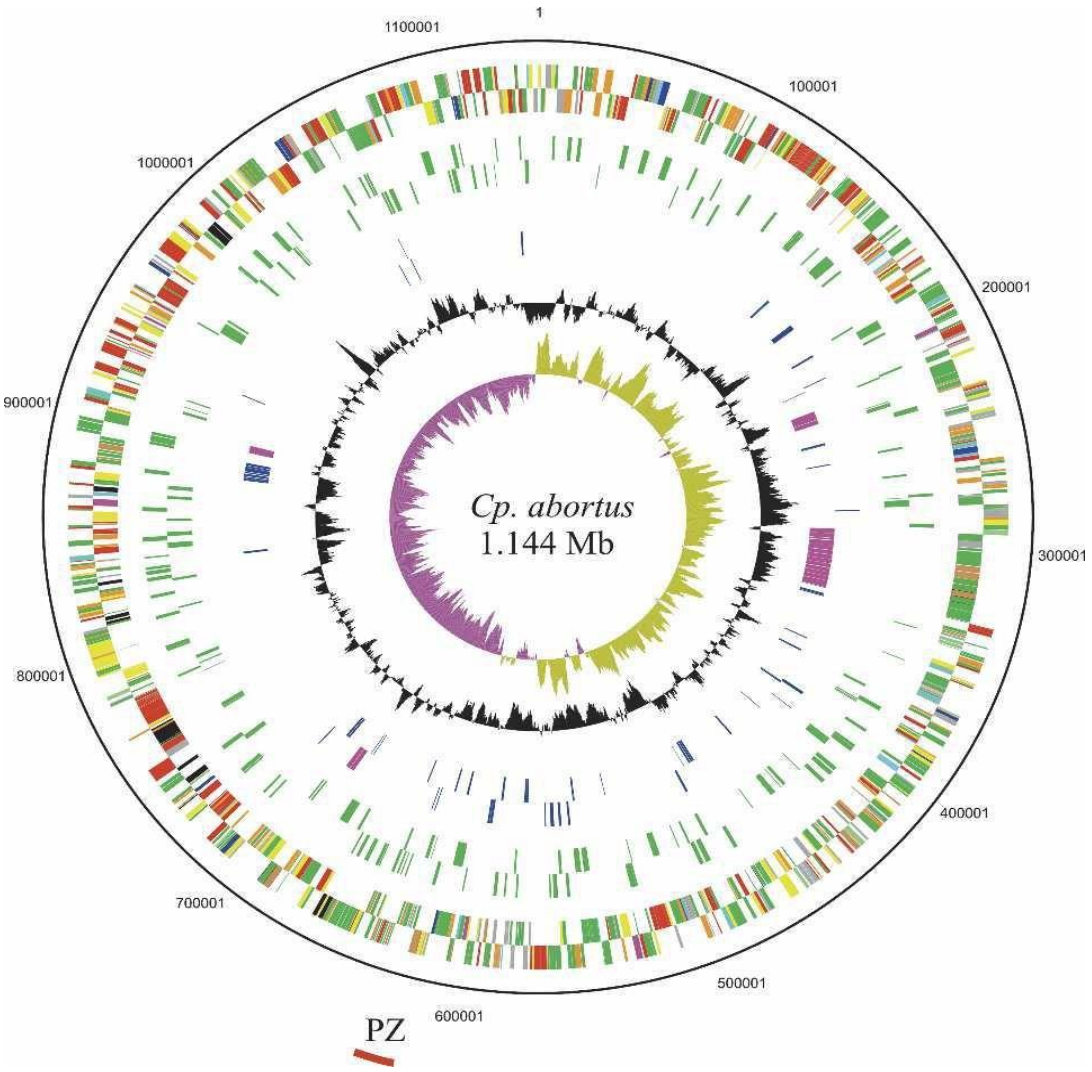

Figure 2.8. Circular representation of the *C. abortus* chromosome. The outer scale shows the size in base pairs.From the outside in, circles 1 and 2 show the position of genes transcribed in a clockwise and anticlockwise direction, respectively (for color codes, see below). Circles 3 and 4 CDS encoding all membrane proteins (green) minus the Pmp and TMH/Inc family proteins in the clockwise and anticlockwise directions, respectively. Circles 5 and 6 show members of the Pmp (purple) and TMH/Inc protein families (blue) in the clockwise and anticlockwise directions, respectively. Circle 7 shows a plot of G+C content (in a 10-kb window); circle 8 shows a plot of GC skew ([G_C]/[G+C]; in a 10-kb window). Genes in circles 1 and 2 are color-coded according to the function of their gene products. Dark green color reveals membrane or surface structures. Yellow color shows central or intermediary metabolism. Cyan indicates the degradation of macromolecules. Red is used to show information transfer/cell division. Purple shows small molecule degradation. Pale blue is used to show regulators. Dark blue shows pathogenicity or adaptation. Black is an indicator of energy metabolism. Orange means conserved hypothetical. Pale green means unknown. Brown is for pseudogenes. The position of the plasticity zone (PZ) is shown as a red arc outside of the scale ring (Thomson *et al.*, 2005).

2.3. Chlamydial Abortion

Ovine chlamydiosis, also known as enzootic abortion of ewes (EAE) or ovine enzootic abortion (OEA) is caused by the bacterium *C. abortus*. Chlamydial abortion in late pregnancy causes severe reproductive wastage in many sheep-rearing areas of the world, particularly where flocks are closely congregated during the parturient period (O.I.E, 2012). The first published description of the disease was made in sheep in Scotland by Greig (1936) , who considered that it was due to nutritional deficiency and named the condition "enzootic abortion." Greig, however, failed to experimentally confirm that dietary deficiency had any bearing on the disease (Greig, 1936; Longbottom and Coulter, 2003). Stamp *et al.* (1950) suggested that EAE was infectious , following an observation that immunity developed after abortion. They reported experimentally-induced abortion in ewes inoculated with filtrates and suspensions from organs of aborted fetuses obtained from farms with outbreaks of abortion. The group showed that the aborted fetal membranes possessed characteristic lesions that contained large numbers of small microorganisms. These microorganisms stained red with diluted Ziehl-Neelsen stain. Subsequently, Stamp (1951) reported that the organisms belong to the psittacosis lymphogranuloma venereum group of virus-like agents .The following year, Stamp *et al.* (1952) developed a complement fixation test for field diagnosis of the disease . Later, evidence was established that the organisms were prokaryotic bacteria that were fundamentally distinct from viruses, and were classified into the genus *Chlamydia* (Page, 1966).

2.3.1. Host Range for *C. abortus*

Chlamydial abortion is caused by *C. abortus*, which was formerly called *Chlamydia psittaci* serotype 1 (Everett *et al.*, 1999). All members of the family *Chlamydiaceae* are obligate intracellular bacteria and cause a wide range of disease conditions in man, other mammals, and birds. The diseases generally manifest in forms of abortion, infertility, enteritis, encephalomyelitis, conjunctivitis, polyarthritis, or pneumonia (Longbottom and Coulter, 2003). *C. abortus* is principally a pathogen of small ruminants that primarily affects the reproductive tract, causing late-term abortion (Wilson *et al.*, 2009). It is a recognized cause of abortion in cattle, but the incidences have been reported sporadically throughout the world (Wilson *et al.*, 2012). The organism can survive and remain infective for weeks at low environmental temperatures and for years at -70°C. However, its infectivity is destroyed at a temperature of 60°C, or by treatment with ether or formalin (Aitken and Longbottom, 2007). The pathogen has been isolated from abortion cases in horses, rabbits, and llamas and is a recognized cause of abortion in pigs (Wilson *et al.*, 2009). *C. abortus* has been associated with ovarian hydrobursitis syndrome in camels (Ali *et al.*, 2012). Mice are used as laboratory models in the study of the pathogenesis of abortion caused by *C. abortus* infection (Rodolakis *et al.*, 1998). Few data are available on the prevalence and relevance of *C. abortus* infection in wildlife hosts (Salinas *et al.*, 2009). The organism was isolated from an abortion case in the springbok antelope in the Paris Zoo (Berri *et al.*, 2004). Serological evidence of infection with the organism was detected in many wild ruminants such as red deer, fallow deer, roe deer, alpine ibex, Iberian ibex and mouflon in Spain and Italy (Salinas *et al.*, 2009), as well as yaks in China (Chen *et al.*, 2014).

Human infection with *C. abortus* is relatively uncommon. However, the biggest threat is to pregnant women because of the ability of the organism to colonize the human placenta. The infection, in most cases, is directly associated with exposure to infected sheep or goats and their aborted fetuses and offspring. The outcome of such infection is an acute influenza-like illness for some days, followed by spontaneous abortion in the first trimester of pregnancy. In contrast, later infection causes stillbirth or preterm labor. Untreated infection in pregnant women may progress to septicemia, hepatic, and renal dysfunctions, pneumonia, and disseminated intravascular coagulation, which may result in death (Longbottom and Coulter, 2003).

2.3.2. Transmission of *C. abortus*

A major route of transmission of *C. abortus* into a naïve sheep flock is through the introduction of infected replacement ewes (Milne *et al.*, 2009). Infected ewes can shed large numbers of bacteria in vaginal discharge and aborted material, such as placenta and coats of lambs (Essig and Longbottom, 2015). Large amounts of infectious *chlamydiae* are excreted in these materials, which lead to contamination of lambing/kidding environment, feed, and pasture. Susceptible ewes and does become infected through ingestion or inhalation of *C. abortus*-infected materials (Livingstone *et al.*, 2005; Gerber *et al.*, 2007). The bacteria can then remain viable within the environment for several days, which may be prolonged with colder weather facilitating further transmission (Longbottom and Coulter, 2003). Following oral-nasal ingestion, the bacteria can then persist, at a site as yet unknown, asymptomatically within the nonpregnant ewe. It has been suggested that following contact with infectious *Chlamydiae*, the

EBs become localized in the pharyngeal lymphoid tissues and tonsils, from where they disseminate systemically via blood or lymphatic circulation to reach the uterus and other organs (Papp *et al.*, 1994; Longbottom *et al.*, 2013).

In the subsequent pregnancy, *C. abortus* infects the placenta and initiates pathology at around day 90 of gestation (Kerr *et al*., 2005), which causes abortion usually within the last 2–3 weeks of gestation (Rocchi *et al.*, 2009). Often the first sign of the disease is the discovery of an infected dead lamb, which makes disease control difficult (Essig and Longbottom, 2015). If a ewe is infected after around 110–120 days of gestation, they will generally deliver normally but may still abort during their next pregnancy. Interestingly, not all ewes that are infected with *C. abortus* will abort. Once an ewe has aborted due to *C. abortus*, it does not abort again with re-infection. However, the immunity that develops within these ewes is not necessarily sterile, as they have been shown to shed at estrus and with subsequent lambing, facilitating further transmission (Livingstone *et al.*, 2009).

The *C. abortus* transmission cycle shows that after the introduction of a small number of infected ewes into a flock, there are usually few abortions within the first year. However, an abortion storm, which can affect around 30% of the flock, occurs in the second year as more ewes are exposed to infected material (Milne *et al.*, 2009). Papp and Shewen (1996), suggested that venereal transmission or mechanical transfer of the organisms by rams during mating could play an essential role in the spread and perpetuation of ovine enzootic abortion within a flock.

2.3.3. Pathogenesis of *C. abortus* infection

2.3.3.1. Latent Infection

Ovine enzootic abortion typically presents itself in late gestation as a result of latent infection with *C. abortus* acquired before pregnancy (Entrican *et al.*, 2012). These latently infected animals are difficult to identify since they do not typically exhibit clinical signs before abortion. The term latency could be defined as a static phase of the pathogen that is asymptomatic for the host (Rocchi *et al.*, 2009). The main recognized feature of *C. abortus* infection in nonpregnant sheep/goat is the establishment of latency and subsequent recrudescence (emergence) to invade the placenta at a given time during pregnancy (Longbottom *et al.*, 2013). Study of the early phase of the pathogenesis of OEA showed that chlamydial antigens could be detected in epithelial cells and lymphocytes in some organs and lymph nodes, but attempt to isolate the organism from these tissues proved abortive (Amin and Wilsmore, 1995). Systemic dissemination of the organism to other organs was reported to be suppressed by the host immune response as early as 30 days post-infection, thus forcing it into latency (Navarro *et al.*, 2004). However, the exact location where latency is established remains unknown (Navarro *et al.*, 2004; Entrican *et al.*, 2012).

The underlying mechanisms that control the series of events which lead to the recrudescence of *C. abortus* from the site of latency and subsequent invasion of the placenta at a particular stage of gestation are poorly understood (Rocchi *et al.*, 2009; Longbottom *et al.*, 2013). The establishment of latency depends on the dose of chlamydial EBs taken by the susceptible host. Lower uptake results in insufficient immunological stimulation to induce protective immunity, thereby

permitting a latent intracellular infection to persist and recrudescence in the next pregnancy. In contrast, higher uptake appeared to invoke a robust immune response that prevents the establishment of latency, thereby resulting in much lower abortion rates (Longbottom *et al.*, 2013).

Recrudescence of latent infection is thought to occur as a result of immune modulation that allows chlamydial multiplication and intermittent low-level chlamydemia (Entrican *et al.*, 2001; Entrican *et al.*, 2012). Then, the bacteria initiate infection of the placenta, which is particularly vulnerable to *C. abortus* after 90 days gestation (Buxton *et al.*, 1990; Longbottom *et al.*, 2013). Susceptible animals that acquire the infection while pregnant will abort during the same pregnancy without developing latency (Navarro *et al.*, 2004).

2.3.3.2. Placental Infection

Following disruption of the state of latency and subsequent dissemination of the organisms to the gravid uterus, the chorionic epithelial cells (trophoblasts) are the first to be invaded by *C. abortus* (Entrican *et al.*, 2001). Although reports indicated that the placental infection starts in the maternal epithelial cells of the placentomes, its dissemination on the maternal side is sufficiently controlled by the maternal immune system (Navarro *et al.*, 2004). It was suggested that unique immunologic suppressor mechanisms operate at the materno-fetal interface of the placentomes to prevent the attack of the maternal immune system against the fetal placenta and permit the acceptance of the semi-allogeneic fetus (Hansen and Liu, 1996). This special environment favors the development of pathogens, such as *C. abortus* and makes the fetus vulnerable to attack by such organisms (Entrican *et al.*, 2001). *C. abortus* has a particular affinity to fetal trophoblast cells, and these

cells serve as the primary targets for multiplication and dissemination of the organisms (Navarro *et al.*, 2004).

The average gestation period of sheep and goats is 147 days (Entrican and Wheelhouse, 2006). *C. abortus* could be detected in these species from 60 days of gestation within the maternal (hematomata) in the hilus of the placentomes (Aitken and Longbottom, 2007). It is presumed that infection gains access to the maternal hematomata in latently infected animals via the blood circulation. Moreover, the direct contact of the fetal trophoblasts with the maternal blood in this region enables the transfer of the organisms from the mother to the fetus (Entrican *et al.*, 2001). However, the placenta continues to be relatively resistant, and no pathological changes appear until 90 days of gestation (Rodolakis *et al.*, 1998). Therefore, complex mechanisms are thought to operate at this stage to permit the release of *C. abortus* from the site of suppression to enable colonization of fetal trophoblast cells (Entrican *et al.*, 2001). Following the establishment of the organisms in the fetal trophoblasts in the hilus of most of the placentomes, rapid exponential replication leads to local necrosis and contiguous spread of the infection into surrounding intercotyledonary membranes and apposing endometrium. These events ultimately lead to abortion, which occurs around 125 to 135 days of gestation (Buxton *et al.*, 1990).

2.3.4. Mechanism of Abortion in *C. abortus* Infection

The specific mechanism that is responsible for abortion as a result of *C. abortus* infection is unclear (Longbottom and Coulter, 2003). However, current knowledge points to a combination of different factors, which include damage to the placentomes, destruction of chorionic epithelium, chorioallantoic arteritis,

vascular thrombosis (Entrican and Wheelhouse, 2006; Sammin *et al.*, 2009), and hormonal imbalance due to loss of endocrine function of the placenta (Leaver *et al.*, 1989). The inflammatory process and subsequent destruction of chorionic epithelial cells at the feto-maternal interface result in reduced efficiency of the exchange of oxygen, nutrients, and waste products between the dam and fetus (Sammin *et al.*, 2009). Likewise, vascular thrombosis and chorioallantoic arteritis impinge on blood flow and functional capacity of the placenta. These effects are detrimental to the viability of the fetus (Sammin *et al.*, 2009). Vascular thrombosis is a consequence of placental cellular infiltration and excessive expression of the pro-inflammatory cytokine tumor necrosis factor-alpha (TNF-α) which is incompatible with successful pregnancy (Buxton *et al.*, 2002; Entrican and Wheelhouse, 2006). The expression of TNF-α is most likely induced by chlamydial lipopolysaccharide (LPS) since LPS has been shown to experimentally induce the production of TNF-α by inflammatory mononuclear cells (Aitken and Longbottom, 2007). TNF-α mRNA expression is also readily expressed in the mononuclear cells within the infected arterioles and arteries, as well as in the inflammatory exudates associated with the chorionic epithelium (Longbottom and Coulter, 2003; Aitken and Longbottom, 2007). The high concentration of TNF-α at the materno-fetal interface is incompatible with pregnancy. Therefore, its production, which is not found in uninfected ovine placentas, has been suggested to cause damage to the placenta, thereby contributing to abortion or premature birth (Longbottom and Coulter, 2003). Progesterone is necessary for the maintenance of pregnancy by inhibiting myometrial contraction throughout pregnancy (Noakes *et al.*, 2001). This hormone is produced by chorionic epithelial cells in sufficient concentration to maintain pregnancy from 55 days gestation in

sheep (Allen *et al.*, 2002) and interacts with estradiol and prostaglandin to control the onset of parturition (Entrican *et al.*, 2001). Rapid destruction of the chorionic epithelium as a result of chlamydial infection can lead to disruption of secretion of progesterone and subsequent loss of balance of pregnancy maintaining hormones, which may trigger premature labor and abortion (Entrican *et al.*, 2001; Entrican *et al.*, 2002; Rocchi *et al.*, 2009). Chlamydial infection in sheep has been shown to induce premature decline in plasma progesterone level and premature rise in estradiol 17β and prostaglandin E2 concentrations, which is thought to contribute to the initiation of premature labor and subsequent fetal expulsion (Leaver *et al.*, 1989).

2.3.5. Clinical Signs of *C. abortus* infection

Following infection, *C. abortus* usually establishes an asymptomatic infection within ewes. Occasionally, behavioral changes and vulval discharge can be detected for up to 48 hours before the abortion. However, often the ovine enzootic abortion is clinically characterized by abortion, which mostly occurs in the last three weeks of gestation (Aitken, 1986; Essig and Longbottom, 2015). In addition to abortion, stillbirth and birth of weak or moribund low birth-weight lambs/kids that fail to survive beyond 48 hours are also encountered (Papp *et al.*, 1994; Rodolakis and Mohamad, 2010). Some infected ewes may give birth to healthy lambs and it is not uncommon for an infected ewe to deliver one dead and one weak or healthy lamb (Longbottom and Coulter, 2003). This death of premature live-born lambs and kids contribute to the sum of reproductive wastage caused by chlamydial infection (Aitken and Longbottom, 2007). After abortion, the affected animals recover rapidly or continue to shed dirty brown infectious

exudates from the vagina for further seven to ten days (Appleyard *et al.*, 1983). Occasionally, the placenta is retained and associated metritis develops, leading to loss of condition and death as a result of secondary bacterial infection (Aitken and Longbottom, 2007). The placenta shows extensive necrosis and thickening of intercotyledonary areas. The thickened areas are covered with dirty brownish exudates over the surface, which is a characteristic feature of *C. abortus*-infected placenta (Smith, 2001). After the first abortion, ewes and does develop protective immunity and breed successfully in subsequent pregnancies with no evidence of *Chlamydiae* in the vaginal discharges, placental or neonatal samples (Rodolakis *et al.*, 1998; Smith, 2001). The disease can take a cyclic nature in a flock/herd where after five years of 5–10% prevalence of abortion, a repeat episode of abortion storm occurs, where all the yearlings in a flock/herd will abort (Rodolakis *et al.*, 1998; Rodolakis and Mohamad, 2010).

2.3.6. Diagnostic Techniques *C. abortus* Infection

2.3.6.1. Identification of the Causative Agent

Diagnosis of OEA depends on the isolation and identification of the causative agent from abortion materials such as the placenta, fetus, and vaginal discharge. Isolation can be combined with serology or detection of the nucleic acid of the agent using PCR. Where the clinical history of the flock and the character of lesions in aborted placentae suggest enzootic abortion, a diagnosis can be attempted by microscopic examination of smears made from affected chorionic villi or adjacent chorion (Essig and Longbottom, 2015). Several staining procedures are satisfactory, such as modified Machiavello, Giemsa, *Brucella* differential, or modified Ziehl–Neelsen stains (ZN). In positive cases stained by

ZN method and examined under a high-power microscope, large numbers of small (0.3 μm) coccoid elementary bodies are seen singly or in clumps stained red against the blue background of cellular debris. Under dark-ground illumination, the elementary bodies are pale green. If placental material is not available, smears may be made from vaginal swabs of females that have aborted within the previous 24 hours. Swabs may also be taken from the moist fleece of a freshly aborted or stillborn lamb that has not been cleaned by its mother, or from the abomasal content of the aborted or stillborn lamb. In general, such preparations contain fewer organisms than placental smears. *C. abortus* resembles the *Rickettsia Coxiella burnetii,* which, in some circumstances, may provoke abortion and which, in humans, causes Q fever. Antigenic differences between *C. abortus* and *Coxiella burnetii* can be detected serologically. Fluorescent antibody tests (FATs) using a specific antiserum or monoclonal antibody may be used for the identification of *C. abortus* in smears (O.I.E, 2012).

2.3.6.2. Isolation of *C. abortus*

C. abortus can only be isolated in living cells such as embryonated chicken eggs and cell culture (O.I.E, 2012). The success of this procedure depends on the acquisition of good quality, well-preserved diagnostic tissue samples, and the availability of specialist culturing facilities and expertise (Longbottom *et al.*, 2002). Isolation in cell culture can be carried out in a variety of cell types such as McCoy cells, Buffalo green monkey (BGM) cells, and baby hamster kidney (BHK) cells (O.I.E, 2012). Besides, Schiller *et al.* (2004) demonstrated that Caco-2 cells and human colonic adenocarcinoma perform markedly better for the isolation of *C. suis*, compared to African green monkey kidney (Vero) cells. Due

to the zoonotic nature of *C. abortus*, isolation and identification procedures must be carried out under biosafety level 2 conditions (O.I.E, 2012). The preferred cell line appeared to be strain-dependent, and thus adaption of the cell line used for isolation to the nature of the sample might be recommended. Moreover, other than the requirement for advanced facilities and expertise, the need for rapid and cooled transport of samples for efficient recovery of chlamydial pathogens, reduces the suitability of culture for chlamydial screening. Still, culture remains essential to demonstrate the viability of a field strain, to generate and characterize new isolates and to study pathogenesis (Sachse *et al.*, 2009).

2.3.6.3. Immunological tests for *C. abortus* infection

Serology depends on the demonstration of raised or rising levels of *C. abortus*-specific immunoglobulin G (IgG) antibodies in the animal sera between the acute and convalescent phases of infection (Griffiths *et al.*, 1996). The serological techniques employed in the diagnosis of OEA include complement fixation test (CFT), immunofluorescence test, and enzyme-linked immunosorbent assay (ELISA) (Longbottom *et al.*, 2002). CFT is the most commonly used assay for diagnosis and screening of sheep and goats for ovine enzootic abortion (O.I.E, 2012). However, the test lacks specificity because it detects genus-specific LPS antigen, which cross-reacts with other members of the genus *Chlamydia*, such as *C. pecorum* (Vretou *et al.*, 2007; Wilson *et al.*, 2009). Several serological assays, such as indirect immunofluorescence test and ELISA using purified LPS, have been developed in an attempt to produce more specific techniques that could distinguish between *C. pecorum* and *C. abortus* infections. However, most of these assays were not sufficiently sensitive and specific (Longbottom *et al.*, 2002;

Wilson *et al.*, 2009; Rodolakis and Mohamad, 2010). More specific indirect ELISAs based on recombinant antigen preparations such as MOMP and POMP (polymorphic outer membrane protein) that express a part of a protein at 80–90 kDa have been developed. These assays have shown to be more sensitive and specific than CFT in differentiating animals infected with *C. abortus* from those infected with *C. pecorum* (Longbottom *et al.*, 2002; Vretou *et al.*, 2007; Rodolakis and Mohamad, 2010; O.I.E, 2012). However, most of these tests are mainly used as in-house laboratory research tools and few of them have been commercialized (Vretou *et al.*, 2007; O.I.E, 2012).

There are several commercialized serological tests available for the detection of *C. abortus*. However, a weakness of the current serological tests is that they cannot differentiate between naturally infected and vaccinated animals (Sachse *et al.*, 2009).

Immunohistochemical staining of histological sections with monoclonal antibodies directed against chlamydial surface antigens, such as LPS or MOMP, are commonly used to detect chlamydial species for diagnostic purposes. These methods are also used for epidemiological and pathogenesis studies (Juvonen *et al.*, 1997; Tsakos *et al.*, 2001; Hotzel *et al.*, 2004; Navarro *et al.*, 2004; Borel *et al.*, 2006). The used of monoclonal antibodies are directly conjugated with the enzyme horseradish peroxidase or detected using a fluorescein-conjugated secondary antibody. Labeling can be enhanced using the streptavidin-biotin method. Immunohistochemical staining procedures can be automated and are routinely applied in many laboratories. However, poor tissue penetration of the primary antibody may impair the sensitivity. Besides, immunohistochemical

assays often show poor specificity due to nonspecific binding of the antibodies with proteins, such as collagen, or the presence of endogenous peroxidases or biotin. Moreover, quantification of the amount of antigen present is challenging and the reproducibility is often low (Szeredi *et al.*, 1996; Buxton *et al.*, 2002).

2.3.6.4. Molecular Diagnosis of *C. abortus*

The introduction of molecular methods has considerably improved the sensitivity and specificity of the detection of *Chlamydiae*. However, as a large number of PCR tests are currently in use, it is not always clear whether these tests have been adequately validated (Berri *et al.*, 2009; Sachse *et al.*, 2009). Amplification of *C. abortus* DNA by real-time PCR is considered to be the most sensitive and rapid diagnostic method available for diagnosis of OEA and has been made available in commercial kit form for veterinary application (Sachse *et al.*, 2009; O.I.E, 2012).

Everett *et al.* (1999) designed assays for amplification and sequencing of the 16S and 23S rRNA signature sequences. These assays are widely used and adapted to identify the involved chlamydial species (Lutz-Wohlgroth *et al.*, 2006; Englund *et al.*, 2012; Di francesco *et al.*, 2013). Besides, sequencing of *omp2* and *ompA* genes was applied for chlamydial species identification (Schiller *et al.*, 1997; Hoelzle *et al.*, 2000; Kauffold *et al.*, 2006). However, sequencing is time-consuming compared to other nucleic acid-based tests, such as real-time PCR. Moreover, the sensitivity of the used assays is variable and sequencing is less attractive for large-scale studies.

Pantchev *et al.* (2010) developed six separate real-time PCR assays for the detection of all chlamydial species of veterinary interest (*C. psittaci*, *C. abortus*, *C. pecorum*, *C. caviae*, *C. felis*, and *C. suis*) . The *ompA* gene appeared suitable for discrimination of all species, except for *C. suis*. Instead, the 23S rRNA gene seemed to be a more convenient target. However, this PCR assay was developed for the examination of veterinary samples and cannot distinguish *C. suis* from the genetically closely related human pathogen *C. trachomatis*. Schautteet *et al.* (2013) used real-time PCR assays based on the *ompA* and 16S rRNA genes for the detection of *C. abortus* (Livingstone *et al.*, 2009), *C. psittaci* (Geens, 2005), and *C. pecorum* (Wan *et al.*, 2011) in pig samples.

2.3.7. Control and Preventive Measures *C. abortus* infection

Following chlamydial abortion, the bacteria are shed into the environment. Therefore, measures should be taken to limit contamination by isolating the ewe. The area should then be decontaminated and contaminated material safely destroyed (Essig and Longbottom, 2015). The importance of good biosecurity was emphasized in a recent survey in which the majority of *C. abortus* was detected in flocks where other abortifacients, such as *Toxoplasma* and *Campylobacter*, were present (Longbottom *et al.*, 2013). The introduction of infected replacement animals is the primary route of transmission between farms. Maintaining closed flocks or only buying replacements from EAE accredited flocks is the best way of preventing the introduction of disease (Longbottom *et al.*, 2013).

Since OEA has bacterial etiology, treatment with antibiotics is possible. Antibiotic therapy should be considered an option when the diagnosis is made or when the active chlamydial infection is thought to be present in a flock/herd of

pregnant animals. Antibiotics such as oxytetracycline can be used effectively to limit disease severity (Entrican *et al.*, 2001) and reduce further losses during abortion storms. Intramuscular injection of long-acting oxytetracycline at a dose of 20 mg/kg body weight to pregnant sheep reduces the overall incidence of abortion and increases the number of viable lambs (Aitken and Longbottom, 2007; Mearns, 2007). To obtain the best result, treatment should be given as soon as possible after 95 days of gestation when the placental infection may have commenced, and repeated at 10 to 14 days intervals until lambing (Smith, 2001). However, antibiotics are not guaranteed to prevent abortion or shedding of the bacteria. Besides, due to the absence of clinical signs with *C. abortus*, they are not an effective control strategy (Entrican *et al.*, 2012) Thus, some abortions and stillbirths will occur despite treatment (Aitken and Longbottom, 2007). Due to this limitation, antibiotic treatment is not considered a sustainable strategy for the control and prevention of OEA. The better approach is to use a combination of flock/herd management and vaccination (Entrican *et al.*, 2001; Longbottom and Coulter, 2003; Entrican *et al.*, 2012).

Flock/herd management is aimed to create a flock/herd free of enzootic abortion. This goal is achieved by keeping a flock/herd "closed" through breeding own replacement animals or buying replacement stock from farms known to be free of EAE (Stuen and Longbottom, 2011).

The best approach to the control of *C. abortus* is through protection by vaccination. Nonpregnant healthy animals can be vaccinated at any time until four weeks before breeding (Entrican *et al.*, 2001). All breeding females should be vaccinated in the first year that the disease is first diagnosed in a flock/herd and

this should be repeated after three years or sooner if the flock/herd is heavily infected. All new entrants into the breeding flock/herd for the first time should also be vaccinated (Entrican *et al.*, 2001). There are currently three vaccines that are commercially available to prevent OEA: Enzovax®, CEVAC *Chlamydia*®, and Mydiavac®. The first two vaccines are based on the live-attenuated 1B strain of *C. abortus,* which is a temperature-sensitive mutant that can grow at 35°C but not 39.5°C (the body temperature of sheep) (Longbottom and Livingstone, 2006; Entrican *et al.*, 2012). Mydiavac® is composed of inactivated whole organism with a mineral oil adjuvant and has only recently been reintroduced by Benchmark Animal Health (Essig and Longbottom, 2015). Enzovax® and CEVAC *Chlamydia*® should not be administered to pregnant sheep or handled by pregnant women (Stuen and Longbottom, 2011). An advantage of Mydiavac® is that it can be used in pregnant ewes (Longbottom and Livingstone, 2006). Both the live attenuated and inactivated vaccines have been successfully used to prevent OEA (Longbottom and Coulter, 2003; Essig and Longbottom, 2015). Both vaccines offer proper protection against OEA and significantly reduce the shedding of infective organisms, a factor that is important in limiting the spread of infection to other animals (Longbottom and Livingstone, 2006).

Chapter Three

MATERIALS AND METHODS

3.1. Equipment and Apparatus

The equipment and apparatus used in the study are listed in Table 3.1.

Table 3.1. The equipment and apparatus used in the study.

Equipment	**Company**	**Origin**
Autoclave	Olching	Germany
Centrifuge	Centurion	U.K.
Chick incubators and hatchery	Aflo	U.A.E.
CO_2 incubator	Heraeus	Germany
Freezer (-20°C)	Sharp	Japan
Deep freezer (-80°C)	MAAN	Sweden
Digital camera	Am scope	China
Gel electrophoresis system	Cleaver	U.K.
Hemocytometer (slide chamber)	Neubauer	Germany
Hood	Labtech	Korea
Inverted microscope	Motic	China
Laminar airflow cabinet (class 2)	Heraeus	Germany
Light microscope	Leica	U.S.A.
Oven	Binder	Germany
Microcentrifuge (cold)	MAAN	Sweden
Multicolor real-time PCR detection system	Bio-Rad (IQ5)	U.S.A.
pH .meter	WTW	Germany
Portable phlegm suction	Lab Tech	Korea
Refrigerator (4°C)	Arcelik	Turkey
Sensitive balance	MettlerToledo	Switzerland
Thermal cycler	Prime	U.K.
NanoDrop 8000 Spectrophotometer	Thermo Scientific	U.S.A.
Thermo shaker	Memmert	Germany
U.V. Transilluminator	Syngene	U.K.
Vortex mixer	J. P. Selecta	Spain

3.2. Chemicals

The chemicals which were used in the study are illustrated in Table 3.2.

Table 3.2. The chemicals used in the study.

Chemicals	**Company**	**Origin**
Ethanol 99%	Tosel	Turkey
Dimethyl sulfoxide (DMSO)	Carl Roth	Germany
Methanol	Himedia	India
Phosphate-buffered saline (PBS) liquid and sterile	Chemical Point	Germany
Phosphate-buffered saline (PBS) powder	Sigma	U.S.A.
TBE Buffer (10X)	Ge Net Bio	Korea
Trypsin – EDTA liquid and sterile	Carl Roth	Germany
Water, DEPC treated (ddH_2O) Cat. No. B-2013	GeNet Bio	Korea
Acetone	J.T. Baker	Germany

3.3. Other Materials

The other materials used in the study listed in Table 3.3.

Table 3.3. The other materials used in the study.

Materials	**Company**	**Origin**
Agarose powder	Gene Direx®	U.S.A.
Amphotericin B (100 mg vial)	Myers	U.S.A.
Antibiotics (streptomycin, gentamicin, kanamycin)	Fluka	Germany
Fetal bovine serum- Heat inactivated sterile	Biowest	South America
Gentamicin (80 mg / 2 mL) vial	Arab pharm.	Jordan
Giemsa stain	Syrbio	Syria
Prime Safe dye (10 mL)	GeNet Bio	Korea
Streptomycin (1 g . vial)	Troge Medical GMBH	Germany
Trypan blue	Sigma	U.S.A.
DMEM high glucose (liquid and sterile)	Gibco	U.K.
DMEM culture medium (powder)	Sigma	U.S.A.
Nutrient broth	Oxoid	U.K.
Ketamine (50 mg/mL)	Holden Media	India
Xylazine (20 mg/mL)	Ceva Sante animale	France
Hydrochloric acid 36%	Labachemie	India
Sodium hydroxide	Labachemie	India
Eggs		Iraq
SPG medium with supplement		Iraq
Vero cells (vial)		

3.4. Animal Samples

Samples were collected from different flocks in three districts of Slemani province, during the lambing seasons from October 2017 to June 2018 (Table 3.4). A total of 80 samples, 30 from aborted fetuses, and 50 vaginal swabs were collected from sheep herds that have the case history of abortion. The samples were transported in a polyethylene coolbox with ice packs to the Research Center at the College of Veterinary Medicine/University of Sulaimani for isolation and identification of *C. abortus*.

Table 3.4. Details of specimens collected from aborted sheep in three different districts of the Slemani province.

Districts	**Type of specimen**	
	Vaginal swabs	**Fetus (liver, lungs, and spleen)**
Kalar	35	15
Said Sadiq	5	10
Chamchamal	10	5
Total	50	30

3.4.1. Aborted Fetuses

Thirty aborted fetuses were collected and submitted to the laboratory under sterile conditions for direct microscopic examination. After the samples were transferred to the laboratory for anatomical processing, they were cleaned and disinfected with ethanol 70%. The carcasses were then opened under sterile conditions. The direct impression smear was prepared from the liver by cutting the surface of hepatic tissue and then blotting with a filter paper to remove excess blood. Later, the liver piece was placed and pressed gently onto a microscopic slide for an impression smear and stained with Giemsa stain for direct

examination. The slides were examined microscopically for the demonstration of Chlamydia organisms (Selim, 2016).

3.4.2. Vaginal Swabs

A total of 50 sterile vaginal swabs were collected directly from the vaginas of aborted ewes within a maximum time of 72 hours .After cleaning the external vaginal orifice with ethanol 70 %, one vaginal swab per animal was then taken by rotating cotton tipped swabs around the wall of the vagina, approximately 2 cm distal to the cervix. The swabs were brought to the laboratory in 2 mL Sucrose Phosphate Glutamate (SPG) transport medium. All swabs were stored at -20 °C prior to DNA extraction (Livingstone *et al.*, 2009; Elberbawy and Elkhabaz, 2014).

3.5. Staining Method

Giemsa stain was performed according to Dagnall and Wilsmore (1990) , and Elberbawy and Elkhabaz (2014) as follows:

a. All smears from the aborted fetal liver and vaginal swabs were allowed to air-dry.

b. The samples were fixed with absolute methanol alcohol for 3–5 minutes.

c. The dry fixed slides were immersed in the Giemsa solution inside a Coplin jar for 10 minutes.

d. After that, the slides were taken out of the jar, washed with distilled water, and dried.

e. The stained slides were examined microscopically under oil immersion (1000X) for the detection of EBs of *Chlamydia*, which appeared as purple-colored bodies.

3.6. Molecular Identification of *Chlamydia abortus*

3.6.1. Materials used for PCR technique

3.6.1.1. Prime Prep Genomic DNA isolation from tissue

The Prime Prep Genomic DNA isolation Kit (Tissue, K-3000), was used for DNA extraction from tissue and vaginal swabs (Table 3.5)**.** Small pieces of tissue from each collected organ were cut, pooled, and then minced with a micro pestle until completely homogenized (Alem *et al.*, 2017) .Vaginal swabs were put in 2 mL SPG and vortexed vigorously for 20 seconds. One milliliter was taken and put into a clean tube and centrifuged at 14,000 RPM for 10 minutes in a microcentrifuge. Genomic DNA was extracted from the resulting pellet (Livingstone *et al.*, 2009).

Table 3.5. Composition of the Prime Prep Genomic DNA extraction kit from tissue.

Buffer	**Volume (mL)**	**Origin / Cat. No.**
Buffer TL (Tissue lysis)	20	GeNet Bio, Korea, (K-3000)
Buffer GB (Genomic Binding)	12	
Buffer GW1 (Genomic Washing 1)	20	
Buffer GW2 (Genomic Washing 2)	10	
Buffer GE (Genomic Elution)	10	
Proteinase K solution	1.2	

3.6.1.1.1. Protocol of genomic DNA Extraction

The genomic DNA was extracted and prepared following the manufacturer's recommendations (GeNet Bio/ Korea). About 20 mg of the homogenized tissue was transferred into a microcentrifuge tube, and 200 µL of tissue lysis (TL) buffer with 20 µL of proteinase K solution were added to the tissue and vaginal swab samples and mixed by vortexing. The mixture was then incubated at 56°C (Thermo shaker) for one hour until the tissue was completely lysed. Later, the tube was briefly spanned down to remove any drops from the inside of the lid.

After that, 200 µL of genomic binding (GB) Buffer was added to the samples and mixed well by pulse-vortexing for 15 seconds. Then, the samples were incubated at 56°C (Thermo shaker) for 30 minutes, after which 200 µL of absolute ethanol was added to each sample and then mixed well by pulse-vortexing for 15 seconds. The tubes were spun down briefly to get the drops clinging under the lid. The lysates were transferred carefully into the upper reservoirs of the spin column (fitted in a 2-mL tube) without wetting the rims, which was then centrifuged at 10,000 RPM for one minute. The spin columns were transferred to new 2-mL collection tubes for filtration.

Then, 500 µL of genomic washing 1 (GW1) buffer was added to each spin column and centrifuged at 10,000 RPM for one minute. After centrifugation, the flow-through was discarded, and each spin column was transferred to a new 2-mL collection tube. The volume of 500 µL of genomic washing 2 (GW2) buffer was added to each spin column and centrifuged at 10,000 RPM for one minute. After centrifugation, the flow-through was discarded, and re- the spin columns were

assembled on collection tubes. The tubes were centrifuged again at 12,000 RPM for two minutes to remove the washing buffer and traces of ethanol completely.

The spin columns were transferred to new 1.5-mL tubes for elution. About 200 µL of genomic elution (GE) buffer was added to each spin column and incubated for 5 minutes at room temperature. Finally, the chromosomal DNA was eluted by centrifugation of the column at 10,000 RPM for one minute.

3.6.1.2. Primers

These forward and reverse primers as shown in Table 3.6 were in lyophilized forms and were supplied by (Macrogen, Korea). To prepare 100 pmol, according to the manufacture's instruction, primers were suspended in a volume of sterile nuclease-free distilled water. The preparation was used as the stock and stored at -20°C until use. The working solution was prepared by ten-fold dilution of the primer stock solutions to prepare 10 pmol /µL of the primers for use in PCR reactions.

Table 3.6. The oligonucleotide sequences used for the detection of *Chlamydia abortus* (*ompA* gene) and *Brucella abortus (bcsp31*gene) in aborted ewes.

Gene / **Primer**		**Oligonucleotide sequences (5'-3')**	**Product (bp)**	**Ref.**
ompA	omp-F	ATGAAAAAACTCTTGAAATCGG	1058	(Arshi *et al.*, 2011)
	omp-R	CAAGATTTTCTAGACTTCATTTTGTT		
bcsp31	B4-F	TGGCTCGGTTGCCAATATCAA	223	(Baily *et al.*, 1992)
	B5-R	CGCGCTTGCCTTTCAGGTCTG		

3.6.1.3. PCR Mixture kit

Table 3.7. Composition of Prime taq premix Master Mix kit.

Solution	Composition	Origin / Cat. No.
Prime premix 2x conc. x10 µL in 0.2 mL	1 unit Prime Taq DNA polymerase- 2.0 mM dNTP mixtur(dATP, dCTP, dGTP, dTTP) 4mM $MgCl_2$ Enzyme Stabilizer Loading dye (pH 9. 0) PCR enhancer Trise-HCL	GeNet Bio, Korea (G-3000)

3.6.1.3.1. Protocol of PCR technique

The PCR constituents were mixed in a 200-µL eppendorf tube. The DNA templates, primers, Prime Taq Premix (2X) master mix were mixed in specified volumes. The volume was then completed to 20 µL by adding diethylpyrocarbonate (DEPC)-treated water, as shown in Table 3.8.

Table 3.8. Components required for Prime taq premix Master Mix.

Components	Volume (µL)
Prime Taq Premix	10
Primers (forward and reverse)	1+1
DNA template from sample	5
DEPC water	3
Total	20

3.6.1.3.2. Optimization of PCR Reactions

The PCR reaction involves several steps, which required different annealing temperature and incubation time as shown in Table 3.9.

Table 3.9. Optimized PCR cycling program for *Chlamydia abortus* and *Brucella abortus*.

Step	***Chlamydia abortus***		***Brucella abortus***	
	Time	**Temperature (°C)**	**Time**	**Temperature (°C)**
Initial denaturation	5 min	95	2 min	94
3-step cycles (repeated 40 times)			3-step cycles (repeated 35 times)	
Denaturation	30 Sec	95	30 Sec	94
Annealing	30 Sec	58	30 Sec	57
Extension	45 Sec	72	80 Sec	72
Final extension	5 min	72	5 min	72

3.6.2. Agarose Gel Electrophoresis

3.6.2.1. Protocol for agarose gel electrophoresis

For the prepared 1X TBE buffer , the 10X TBE buffer was diluted ten-fold by adding 100 mL of 10X TBE with 900 mL of ddH_2O in a suitable container. DNA samples were tracked using an electrophoresis of the horizontal agarose gel. The agarose gel was prepared 1% according to Wang *et al.* (2011) .

The gel electrophoresis was started with the preparation of the agarose gel by mixing 1 g of agarose with 100 mL 1X TBE buffer in a 250-mL capacity conical flask. The flask was heated until the gel solubilized. After that, it was left to cool to about 50°C. The comb was fixed at one end of the gel electrophoresis tray for making the wells used for loading the DNA sample, and the agarose was poured gently into the tray and allowed to solidify at room temperature.

After gel solidification, the comb was gently removed from the tray, and both of the rubber ends of the trays were removed. The tray with gel was fixed on the electrophoresis tank, which was filled with 1X TBE buffer so that the buffer covered the entire surface of the gel. The volume of 3 µL of blue-DNA dye

(Bromphenol) was added to 7 μL of PCR product samples and mixed by pipetting. The mixture was then carefully loaded into individual wells.

The electrophoresis power supply was set at the 120 volts for 50 minutes. The amplified DNA bands were visualized using dark blue plates. The size of PCR products was estimated according to the pattern of migration of a 100 bp DNA ladder. The running DNA bands were then documented by photographing.

3.6.3. Direct sequencing

The PCR products of *C. abortus ompA* gene were sequenced in the Macrogen by using sanger sequencing method. The sequence was verified by comparison of the fastA data with the chromatograms of the sanger sequencing. The gene sequence was submitted to the GenBank.

3.6.3.1. Phylogenic analysis

Phylogenic trees were generated based on *ompA* genes obtained from 75 different published isolates of *Chlamydia* species. The sequences were obtained from the GenBank and the MLST website of Chlamydiales (http://pubmLst.org/chlamydiales/). Multiple alignments of these sequences were performed using the ClustalW method (Thompson *et al.*, 1994). The MEGA 7 online bioinformatics tool was used to perform phylogenetic analysis with Neighbor-Joining. The bootstrap values were determined from 1000 replicates of the original data.

3.7. Isolation and purification of *Chlamydia abortus*

3.7.1. Inoculation of Embryonated Chicken Eggs

Inoculation of embryonated chicken eggs was used for the isolation of *C. abortus* from fetus tissue samples like (liver, lung, and spleen) as follow:

a. One gram of tissue was homogenized by grinding using a sterile pestle and mortar in 9 mL of SPG medium to make a 10% suspension. The suspension was centrifuged at 2000 RPM for 5–10 minutes, and then the supernatants were collected and aliquoted in small volumes. Part of the supernatant was used immediately for the isolation of *C. abortus* in embryonated chicken eggs and cell culture or for the extraction of DNA, and the remainder was stored at -70°C (Soomro *et al.*, 2012).

b. For isolation of *C. abortus* in the yolk sac membranes of embryonated hen's eggs, 6 to 8-day-old embryonated hen's eggs were inoculated with the tissue suspension of the aborted animals. The embryonated chickens' eggs were 0-day-old at arrival. The inoculated eggs were incubated at 37°C in a humid atmosphere (50–70% humidity) for six days (Barhoom, 2015).

c. At the beginning of the sixth day, the vitality of the egg to be inoculated was checked with a candling lamp, Candling (holding an intense light below the egg to observe the embryo in a darkened room) was used to determine and mark the location of the embryo and egg's air sac (Soomro *et al.*, 2012).

d. The shell was cleaned with 70% ethanol, and the air sac was marked with a pencil, followed by drilling a small hole into the shell over the top of the air

sac. About 200 µL inoculum (10% suspension of tissue with 200 µg/mL streptomycin) was injected into the yolk sac. The hole was sealed with nail varnish or wax, under sterile conditions, and incubated at 37°C (Kalender *et al.*, 2013).

e. The vitality of the embryo was tested daily. Eggs that showed embryonic death during 4–10 days after inoculation were harvested. Those eggs in which the embryo died within 24 hours after infection were discarded (Li *et al.*, 2015).

f. The eggs containing dead embryos were kept at 4°C overnight, and the yolk sac membranes were aseptically harvested in a safety cabinet after washing the eggshells with 70% ethanol. The yolk sac was washed twice with PBS (pH 7.2) and then cut into small pieces to be ground with a pestle and mortar to prepare a cell suspension as described earlier (Soomro *et al.*, 2012).

g. The presence of Chlamydial progeny in yolk sacs was detected by *C. abortus*-specific PCR. Positive cultures were inoculated in cell culture monolayer to identify the inclusion bodies of *Chlamydia*, followed by staining with Giemsa (Li *et al.*, 2015).

3.7.2. Purification by Cell Culture

3.7.2.1. Preparation of Reagents and Solutions

3.7.2.1.1. Phosphate buffered saline (PBS)

The content of one vial of PBS was dissolved in one liter of deionized distilled water (ddH2O) and mixed with continuous stirring. Sterilization was done by autoclaving at 120°C for 20 minutes and kept sterile in a closed bottle until use (Kenkel, 2013).

3.7.2.1.2. Antibiotic stock solutions

The content of 1 g streptomycin vial dissolved in 5 mL of ddH_2O, to yield a stock solution of 200 mg/mL. This stock solution was then kept at -20°C until use. A vial of Gentamicin vial of 80 mg/2 mL solution was considered as a stock solution stored at 4°C for uses (Kenkel, 2013).

3.7.2.1.3. Chemical solutions

One normal NaOH was prepared by the addition of 40 grams of NaOH to approximately 900 mL deionized water with cooling and stirring until it dissolved completely, then it was completed to one liter. Sterilization was done by filtration through a 0.2 μm Nalgene filter (Kenkel, 2013).

One normal HCL was prepared by the addition of 58.8 mL of the 36% concentration HCl drop by drop to 900 mL of deionized water in a volumetric flask with continuous stirring. Then it was completed by addition of deionized water to 1000 mL. Sterilization was done by filtration using a 0.22 μm Millipore filter unit and kept sterile until need (Kenkel, 2013).

Trypsin-EDTA solution (0.05% w/v trypsin + 0.53 mM EDTA) was prepared and pH of the solution was adjusted to 7.2, and the final volume was completed to 1.0 L. Subsequently, the solution was sterilized by filtration using 0.22 μm Millipore Nalgene filters and stored at -20°C until use (Kenkel, 2013).

Stock solution of 4 mg/mL trypan blue dye was prepared by adding PBS buffer to 0.4 g of trypan blue inside a graduated cylinder with mixing until

dissolved. The volume was completed by addition of PBS to 100 mL and stored at room temperature (Kenkel, 2013).

3.7.2.2. Preparation of culture medium

3.7.2.2.1. Tissue culture media

Liquid tissue culture medium was prepared from powdered medium according to the US biological product manual as follows:

From the DMEM culture media powder, with HEPES buffer, L-glutamine and phenol red, 16.65g was dissolved in approximately 900 mL of deionized water in a volumetric flask. The other components included 2 g sodium bicarbonate powder, 2.5 mg amphotericin B dry powder, 50 mg gentamicin solution, and 50 mg streptomycin solution. The chemicals were added with continuous stirring. The volume was finished to about one liter, and the pH of the medium was adjusted to 7.4 by using 1 N NaOH and a pH meter. The volume was completed to one liter by adding ddH_2O. Sterilization was done by 0.4 and 0.2 μm Nalgin filter subsequently. After the end of the procedure, five mL of the medium was incubated in a sterile flask for four days at 37°C with daily examination for signs of bacterial and fungal contamination. It was considered sterile only in case of no signs of contamination during four days of incubation. Then the media was stored at 4ºC until use (Spector *et al.*, 1998).

3.7.2.2.2. Maintenance medium

Its preparation was similar to those of tissue culture media 3.7.2.2.1 except it was free of fetal bovine serum (Freshney, 1994).

3.7.2.2.3. Freezing medium

Ten mL of stock solution was prepared from 6 mL serum-free medium, 3 mL fetal bovine serum, and 1 mL dimethyl sulfoxide (DMSO), which was added drop by drop with mixing. The stock was stored at -20°C in between uses (Ausubel *et al.*, 1990).

3.7.2.3. Preparation and Processing of Vero cell line

Vero cell line in a frozen vial was supplied thankfully from Assist. Prof. Dr. Hamid Naji Obied Al-Kalabi from the Cell Culture Unit of Medicine College/Babylon University in Iraq. The medium was transported with dry ice in a cool box. Vero cells were grown in a 25-mL capacity tissue culture flask with a growth medium containing 10% FBS and antibiotics. The cells were incubated at 37°C.

3.7.2.3.1. Freezing of Vero cell line

A frozen stock of Vero cells was prepared for future use. Freezing was done by harvesting the monolayer of cells near the exponential growth phase and washing twice with 5 mL of PBS. After that, 3 mL of warm trypsin was added to the culture flask. Half of the trypsin volume was decanted after one minute. The flask was incubated at 37°C until the cell layer detached, and the cells were aided to disaggregate into single cells by gentle rocking on the flask sides. The flask content was transferred into a 15 mL sterile plastic centrifuge tube. Centrifugation was done at 800 RPM for 10 minutes. The supernatant was decanted and the cell pellet was resuspended with 1 mL of the freezing medium and transferred into 1.5

mL sterile freezing vial. The vial was kept for 10 minutes at room temperature and transferred to -80°C deep freezer for 24 hours. The cryovial was then stored for a long time in the liquid nitrogen tank at -196°C (Spector *et al.*, 1998).

3.7.2.3.2. Thawing of Vero cell line

The cell line frozen vial was removed from the liquid nitrogen container with caution and directly placed into a beaker containing pre-warmed sterile ddH_2O in a water bath at 37°C. The vial was removed from the water before the ice floccule dissolved completely and wiped with 70% alcohol. Without delay, the cell suspension content of the vial was pipetted under a laminar flow hood into a 15-mL sterile plastic centrifuge tube containing 10 mL of pre-warmed growth medium. Centrifugation was done at 800 RPM for 10 min, and the supernatant was aspirated and decanted. The cell pellet was re-suspended into 5 mL of warm fresh growth medium with 10% FBS and transferred into a 25-mL size cell culture flask. The flask was incubated at 37°C, and the growth medium was replaced on the next day (Freshney, 1994).

3.7.2.3.3. Harvesting of cells

Harvesting is a technique that uses the proteolytic enzyme trypsin to detach and disaggregate the adherent monolayer cells from the bottom of the culture vessel. This procedure was performed whenever the cells needed to be harvested for passage and cell count and it included different steps, which are illustrated in the next paragraph.

When the cultured cell growth reached a monolayer, and before the exponential phase, the medium was aspirated and discarded, and the cells were washed with few milliliters of warm PBS solution. Next, about 3 mL of warm trypsin EDTA solution was added to cover the monolayer with the gentle rocking of the flask dish 4–5 times to flood the monolayer. The flask dish was incubated at 37°C until the monolayer was detached, after which the flask was removed from the incubator, and complete separation of the cells was done by rocking the flask from side to side. The cells were gently pipetted up and down to disrupt cell clumps into single cells. Cells were counted and resuspended into the desired number in a growth medium with 5–10% FBS, which was used to inactivate trypsin. The cells were subcultured into two flasks or cultured on a culture plate (Freshney, 1994).

3.7.2.3.4. Subculture of adherent Vero cell lines

After the cells became confluent as a monolayer, the subculture was done according to the protocol mentioned by Ng and Schantz (2010).

Briefly, cultures were viewed using an inverted microscope to assess the degree of confluence and confirm the absence of bacterial and fungal contaminants. The medium was removed and the cell monolayer was washed with PBS without Ca^{2+}, Mg^{2+} using a volume equivalent to half the volume of culture medium. This wash step was repeated if the cells were known to adhere firmly. Trypsin- EDTA was pipetted into the washed cell monolayer using 1 mL per 25 cm^2 of surface area. Flask was rotated to cover the monolayer with trypsin and the excess trypsin was decanted. After that, the flask was returned to the incubator and left for 2–10 minutes. The cells were examined using an inverted microscope

to ensure that all the cells are detached and floating. The side of the flask was gently tapped to release any remaining attached cells. The cells were resuspended in a small volume of fresh serum-containing medium to inactivate the trypsin. Next, about 200 µL of the cell suspension was transferred into a 1.5 mL microcentrifuge tube, followed by the addition of 300 µL of PBS and 500 µL of 0.4% trypan blue solution and trypan blue viable cell count performed. In the case of cells cultured in the serum-free media, a trypsin inhibitor such as soya bean trypsin inhibitor was used to inactivate the trypsin. The required number of cells was transferred to a new labeled flask containing pre-warmed medium, and the cell line was incubated at 37°C. This process was repeated as demanded by the growth characteristics of the cell line.

3.7.2.3.5. Viable Cell Count

Trypan blue exclusion staining assay provides a method for cell counting and distinguishing between viable (unstained) and nonviable (stained) cells in suspension. The following protocol was considered, according to Darling and Morgan (1994).

The Vero cells were harvested by trypsinization and suspended in an appropriate volume of prewarmed growth medium to give a cell density of10^6 cells/mL. Next, about 200 µL of the cell suspension was transferred into a 1.5 mL microcentrifuge tube, followed by the addition of 300 µL of PBS and 500 µL of 0.4% trypan blue solution (creating a dilution factor of 5). The content was mixed thoroughly and allowed to stand for 20 minutes. With a coverslip in place, 20 µL of the trypan blue cell suspension was transferred to a chamber of a hemocytometer by carefully touching the edge of the coverslip with the pipette tip

and allowing the chamber to fill by capillary action. All the cells were counted in the 1 mm center square and the four corner squares. Nonviable cells would be stained blue, whereas viable cells would remain opaque. If there were less than 50 or more than 200 cells per large square, the procedure was repeated adjusting to an appropriate dilution factor. The cell count was repeated using the other chambers of the hemocytometer. Each square of the hemocytometer (with coverslip in place) represents a total volume of 0.1 mm^3. Since 1 cm^3 is equivalent to 1 mL, the subsequent cell concentration per mL (and the total number of cells) was determined by using the following calculations:

Number of cells per mL = the average cell count per square X dilution factor X 10^4.

Total cell number = number of cells per mL X the original volume.

3.7.2.4. Infection of Vero cells with *Chamydia abortus*

Monolayers of Vero cells were grown in 25 mL tissue culture flasks at 2X 10^6 cells/ mL. Several culture flasks, often three to four, were inoculated with each sample to permit fixing and staining at various intervals. Confluent monolayers (70–80%) were achieved 24 hours after incubation at 37°C with 5% CO_2 with Dulbecco's Modified Eagle medium supplemented with 10% fetal bovine serum (Labiran, 2014). On the day of infecting the cells, the necks of flasks were wiped with 70% ethanol to prevent contamination. The medium in the flask was removed, and cells were washed twice for one minute with 5 mL PBS (pH 7.4). The frozen stored suspension (10% cell suspension in SPG) was thawed and added to Vero cell cultures at a concentration of 25 µL for each flask. Tissue

culture flasks were incubated for one hour at 37°C in a saturated humidity environment and 5% CO_2. After incubation, DMEM, supplemented with 10% fetal bovine serum and gentamicin (20 μg/mL), was added, and tissue culture flasks were again incubated at 37°C for 48–72 hours. This was considered as time zero of infection. After 72 hours, the inclusions were large enough to be visualized under an inverted microscope and the extent of infection could also be determined visually (Campos-Hernández *et al.*, 2014).

3.7.2.5. Harvesting of *Chlamydia abortus* Isolates

Vero cells were harvested three days after infection with *C. abortus*. For this purpose, a cell scraper was used to detach the cells from the plastic of the flask. The sample was then centrifuged at 2850×g for 10 minutes, the supernatant was discarded, and the pellet was resuspended in 1:10 cold phosphate buffer saline (PBS). The infected cells were harvested from these cultures by high-speed centrifugation (30,000×g for 45 minutes). *Chlamydiae* were released from the intracellular vacuoles by ultra-sonication and centrifuged again at low speed (500×g for 10 minutes) to separate the bacteria from cell debris. The supernatant was centrifuged at high speed (30,000×g for 60 minutes) to harvest the bacteria and then kept at -70°C. (Pantchev *et al.*, 2010).

3.7.2.6. Staining the Cell Culture

Staining of the isolated *C. abortus* from cell cultures started by the removal of the culture medium and fixation of infected cell culture with a mixture of 50% acetone and 50% methanol for 10 minutes. The cells were stained with Giemsa solution and incubated at 37°C for 15 minutes. After that, the cells were

washed gently with PBS until the dye was removed. The cells were observed under an inverted microscope at 400X magnification microscope. The EBs of *Chlamydia* appeared as small cocci occurring singly and in clusters in the cytoplasm of the infected cells (Andersen and Vanrompay, 2008).

3.8. Molecular detection of some virulence factors of *Chlamydia abortus* proteins

3.8.1. Propagation of *Chlamydia abortus* in Mice

Eighteen female and three male albino mice of *Mus musculus* species, BALB/c strain, were used in the study (Silva-Zacarias *et al.*, 2009). The mice were 6–8 weeks old, and their weight ranged between 25 and 30 grams. Mice were allowed to acclimate for one week before starting the experiment. They were accommodated in plastic cages, maintained under standard laboratory conditions (25 ± 3°C temperature, white fluorescent light photoperiod, and 40–45% humidity) and provided with *ad libitum* access to a standard food pellet diet and water. All mice-involving procedures were performed humanely according to the Guide for the Care and Use of Laboratory Animals and the ethical agreement that was achieved from the Ethics Committee at the College of Veterinary Medicine, University of Sulaimani. The female mice were divided into two equal groups as follows: Group 1 of pregnant mice (Clinical case), and group 2 of nonpregnant mice (Control). The female mice in each group were injected intraperitoneally with 0.2 µL of the supernatant result from the isolation of *C. abortus* in Vero cells (kept at -70°C).

Injection of *C. abortus* was conducted after one week of pregnancy in Group 1. Started to take samples by dividing the duration of pregnancy into three stages to detect the expression of three bacterial genes by extraction of RNA from the organs (Silva-Zacarias *et al.*, 2009) as follows:

1. Stage one, ten days (First period of pregnancy): It was after three days of injection and involved euthanizing three mice from each group.
2. Stage two, 15 days (Middle of the pregnancy period): it involved euthanizing three mice from each group after eight days of injection.
3. Stage three, 20 days (Last period of pregnancy): it involved euthanizing three mice from each group after 13 days of injection.

3.8.2. Animal Euthanasia

All mice were euthanized at the end of each stage by an intraperitoneal injection of a mixture of xylazine (16 mg/kg) and ketamine (100 mg/kg), followed by cervical dislocation (Schoell *et al.*, 2009).

The xylazine and ketamine mixture was prepared as follows: Ketamine (50 mg/mL), 2 mL (100 mg), and xylazine (20 mg/mL), 0.8 mL (16 mg) Sterile distilled water, 7.2 mL. The intraperitoneal injection dosage of the mixture was 0.1 mL/10 grams of mouse body weight.

3.8.3. Sample Collection and RNA Extraction

Following the euthanasia of the mice, the external surface was cleaned and disinfected with 70% ethanol, and then the abdomen was opened under aseptic

conditions. Fragments were resected from the liver, spleen, lung, kidney, placenta, and fetus (Silva-Zacarias *et al.*, 2009) .

The Total RNA isolation Mini Kit (Tissue, RT05O), as shown in Table 3.10, was used to isolate the RNA from tissues (Van Lent *et al.*, 2016). Total RNA was extracted and prepared, following the manufacturer's guidelines. The procedure for RNA extraction is illustrated in the following paragraphs.

Table 3.10. Contents of the total RNA isolation Mini Kit from tissue.

Buffer	**Volume (mL)**	**Origin / Cat. No.**
RB Buffer	30	GeneAid, Taiwan (RT05O)
Wash1 Buffer	30	
Wash Buffer	25	
RNase- free water	6	

Small pieces of tissue were cut from each collected organ, pooled, and then minced with a micro pestle until homogenization. About 25 mg of the homogenized tissue was transferred into a 1.5-mL capacity microcentrifuge tube. After that, about 400 μL of RB buffer and four μL of β-mercaptoethanol were added. The sample mixture was incubated at room temperature for three minutes. And transferred to a filter column, fixed on to a 2-mL collection tube. The tube was centrifuged at 1,000×g for 30 seconds. Then, the filter column was discarded. The filtrated sample was transferred to a new 1.5-mL microcentrifuge tube. About 400 μL of 70% ethanol (prepared in DEPC water) was added and then mixed well by up- and down pipetting.

The sample mixture was transferred to the RB column. The RB column was placed onto a 2-mL collection tube, and centrifuged at 15,000×g for 1 to 2 minutes. Then, the flow-through was discarded, and the RB column was placed on a new collection tube. A volume of 400 µL from W1 buffer was added to each RB column and centrifuged at 15,000×g for 30 seconds. After centrifugation, the flow-through was discarded, and each RB column was transferred to a new 2-mL collection tube.

The volume of 600 µL of wash buffer was added to each RB column and centrifuged at 15,000×g for 30 seconds. After centrifugation, the flow-through was discarded, and each RB column was transferred to a new 2-mL capacity collection tube. Later, 600 µL of the wash buffer was added to each RB column and centrifuged at 15,000×g for 30 seconds. After centrifugation, the flow-through was discarded, and the RB column was reassembled with its collection tube. Once more, it was centrifuged at 15,000×g for 3 minutes, to obliterate the ethanol, rest completely. The RB column was transferred onto a new 1.5-mL tube for elution. 50 µL of RNase- free water was added to each RB column and left for two minutes at room temperature. The RNA was eluted by centrifugation at 15,000×g for 60 seconds.

3.8.4. RNA Quantification

RNA measurement was performed using a Nanodrop, ND-8000 (8-Sample spectrophotometer, U.S.A). Briefly, small amounts of samples (1 µL) were pipetted onto Tray Cell which is a fiber-optic ultra-micro cell designed for analysis of DNA/RNA and built in the Nanodrop, and the absorbance was measured. RNA sample absorbance was measured at 230 nm, 260 nm, and 280

nm. The 260/280 ratio can be used to evaluate the purity of RNA. A ratio of ~2.0 is generally accepted as "pure" for RNA. If these ratios are appreciably lower, it may indicate the presence of co-purified contaminants (Wheelhouse *et al.*, 2009).

3.8.5. qRT-PCR Program

The primers used in the present study were designed by the primer3 software (http://biotools.umassmed.edu/bioapps/primer3_www.cgi). The primers were designed to span exon-exon junction to avoid any genomic DNA amplification, in which the primers bind to two consecutive exons and cannot bind if there are intronic sequences present. The publicly available mRNA sequences of the genes were taken from NCBI (http://www.ncbi.nlm.nih.gov/pubmed/) and were blasted against the Refseq mRNA database using the free online available service provided by NCBI at http://www.ncbi.nlm.nih.gov/tools/primerblast/index. Primers for *mip*,*pmp18D,* and *ompA* genes were previously designed by Forsbach-Birk *et al.* (2013) and Wheelhouse *et al.* (2009), respectively (Table 3.11).

Table 3.11. Sequences of the forward and reverse primers of four genes used in real-time PCR *(GAPDH, mip, pmp18D,* and *ompA*).

Target gene	**Primer Sequence 5' to 3'**	**Size (bp)**	**Ref.**
GAPDH	Forward: GGGGTCCCAGCTTAGGTTCA	95	Bio-Lab.
	Reverse: ACGGCCAAATCCGTTCACA		
mip	Forward: AAGAAAACCTCTCCCTAGCC	139	(Forsbach-Birk *et al.*, 2013)
	Reverse: CTGAAGGTTTCCCTGATATTG		
pmp18D	Forward: TCCACTGGGATGATCACCAATA	81	(Wheelhouse *et al.*, 2009)
	Reverse: GCATAGAAAGCGTATCGAGAATCAC		
ompA	Forward: GCGGCATTCAACCTCGTT	85	**(**Wheelhouse *et al.*, 2009)
	Reverse: CCTTGAGTGATGCCTACATTGG		

Profiling was performed using a real-time PCR-based array, and the housekeeping gene (Bio-lab. Design) *GAPDH* (Glyceraldehyde-3-phosphate dehydrogenase) was employed to measure the expression level of mRNA of target genes, which were *mip, pmp18D*, and *ompA* genes. Briefly, a volume 500 ng of total RNA extracted from mice tissue samples was reverse-transcribed to cDNA, and qRT- PCR was performed using SYBR Master Mix and specific primers. SYBR green qRT-PCR assay was used to quantify the amount of mRNA product of a specific gene. In this study, the Add RT-PCR SYBR kit/ Korea (Cat. number-71301), which is a single tube real-time one-step RT-PCR (Table 3.12), was used. Furthermore, q-PCR was undertaken in an IQ5 Multicolor Real-time PCR system (BIO-RAD, U.S.A.).

Table 3.12. Contents of the Add RT-PCR SYBR kit.

Buffer	**Composition**	**Origin/Cat. No.**
20x AddScript Enzyme Solution	100 µL Enzyme solution With thermostable MMLV RTase, (RNase), hot-start Taq DNA Polymerase and RNase inhibitor.	AddBio, Korea (71301)
2.5x Reaction Buffer	1.0 mL Reaction buffer with dNTPs mixture and SYBR Green 1	

3.8.6. Optimization of PCR Reactions

Each reaction was performed in a final volume of 20 µL containing 500 ng of the RNA, 1 µL of each primer and 1× SYBR Green PCR Master mix (add Bio/ Korea). The amplification profile was 50°C for 20 minutes (RT phase to make a cDNA). Then, denaturation at 95°C for 10 minutes was followed by 40 cycles of 95°C for 30 seconds, and 60°C for 30 seconds. At the end of the PCR cycles,

melting curve analyses were performed to validate the specific generation of the expected PCR product.

3.8.7. Data Analysis and mRNA Expression

The qRT-PCR data obtained from the above reaction were analyzed using the comparative CT (2-ΔCT) method, which is a convenient way to analyze the relative changes in gene expression from real-time quantitative PCR experiments. The Ct values for each gene were first obtained through the BIO-RAD (IQ5) RT-PCR machine used in this study. These values were then imported into a Microsoft Excel file for further calculation and analysis.

The cycle threshold (Ct) is defined as the number of cycles required for the fluorescent signal to cross the threshold in qPCR. The expression levels of mRNAs were normalized to mice *GAPDH*. mRNA expression was quantified as ΔCt values for both pregnant and nonpregnant mice separately. Where Ct = threshold cycle and ΔCt = (Ct target gene - average Ct of *GAPDH*). The expression level of all mRNAs was measured in all normal tissue samples after comparing it with the pooled sample. The ΔΔCt values, which are expressed as ΔCt target gene - ΔCt of the pooled sample, were used to quantify mRNA expression of *mip, pmp18D,* and *ompA* genes in infected pregnant mice compared to infected nonpregnant mice. The equation 2-ΔΔCt was used to calculate the fold change of the gene. Pooled samples were taken by mixing an equal amount of RNA extracted from each of three infected mice at three intervals during pregnancy, and the same was done for the infected nonpregnant mice.

3.8.8. Statistical Analysis

The data were imported into a Microsoft Excel Spreadsheet, and they were transferred into SPSS (Statistical Package for Social Science-version 20) software program for statistical analysis. The result of qRT-PCR included the comparison of the Ct of each gene analyzed (*mip, pmp18D* and *ompA)* and using the signed-rank test of Wilcoxon. *P-values* < 0.05 were considered significant.

Chapter Four

RESULTS

4.1. Detection by Direct Microscopic Examination

Microscopic examination of direct smears taken from the liver of aborted fetuses and vaginal swabs of aborted ewes using Giemsa stain showed that one out of the 30 tissue samples (3.33%) was positive for *Chlamydia* only from Kalar district. However, the vaginal swabs had given a negative. The diagnosis was based on the manifestation of elementary bodies of *Chlamydia* (Figure 4.1).

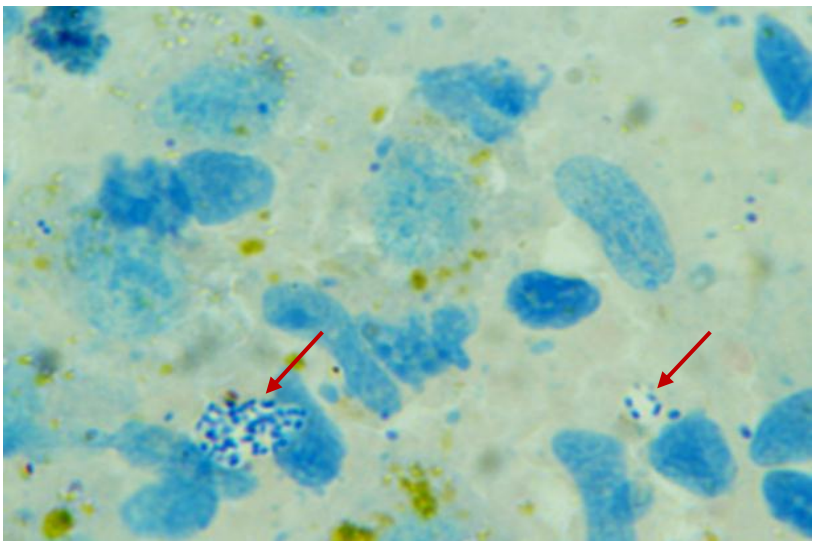

Figure 4.1. Direct impression smears from the liver of an aborted fetus (Giemsa staining).

Giemsa stain revealed *Chlamydia* elementary bodies as small purple-stained cocci, occurring individually and clusters, in the cytoplasm of hepatocytes of the aborted fetus. To confirm the diagnosis, PCR was used for all samples.

4.2. Molecular Detection of *Chlamydia abortus*

Genomic DNA was successfully extracted from fetal samples and vaginal swabs of aborted ewes using the DNA extraction kit. The genus-specific primers of *ompA* gene that encode cell surface protein of *C. abortus* species were successful in amplifying the 1058 bp amplicon (Figure 4.2). A total of 80 samples (30 aborted fetuses and 50 vaginal swabs) were taken from different flocks in three districts of Slemani provinces. The districts were Kalar, Said Sadiq, and Chamchamal, in which abortions had been observed. One out of 15 aborted fetuses from Kalar, only one (6.66%) gave a positive result of tissue samples. One out of 30 aborted fetuses from different flocks in three districts of Slemani, one (3.33%) gave a positive result from tissue samples (Table 4.1), and all of the remaining 29 samples (96.66%) were found positive to *Brucella abortus* (Figure 4.3). However, *C. abortus* and *Brucella abortus* DNA could not identify from the vaginal swab samples.

Table 4.1. Detection of *Chlamydia abortus* and *Brucella abortus* from different herds of sheep in three districts in Slemani province by PCR.

Name of district	**PCR result**		
	No. of Exam.	***C. abortus* (%)**	***B. abortus* (%)**
Kalar	15	1 (6.66)	14 (93.33)
Said Sadiq	10	0	10 (100)
Chamchamal	5	0	5 (100)
Total	30	1 (3.33)	29 (96.66)

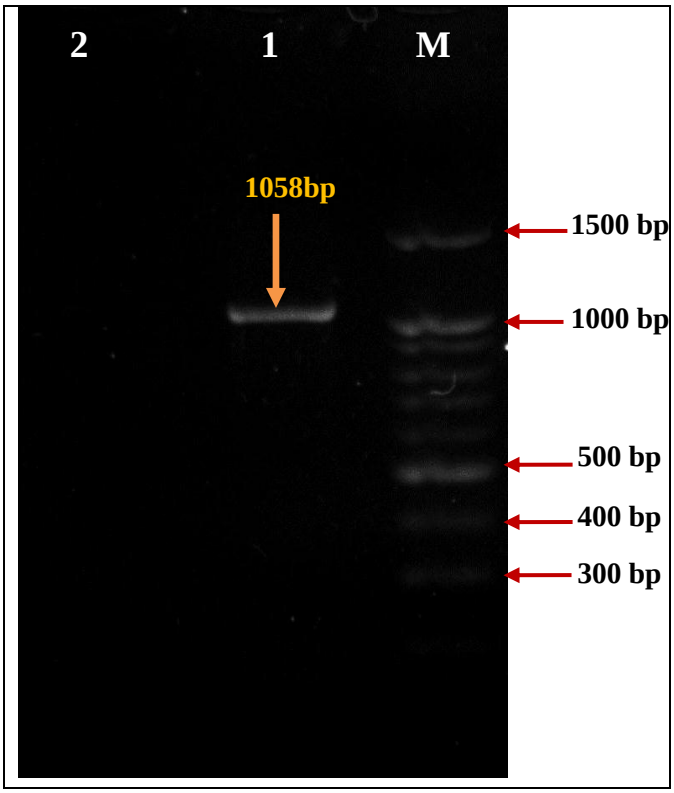

Figure 4.2. PCR amplification of *Chlamydia* DNA from the tissue of an aborted fetus. Lane M = Molecular weight marker (100 bp DNA ladder); lane 1 = Positive result of *Chlamydia abortus* (1058bp); and lane 2 = Negative sample.

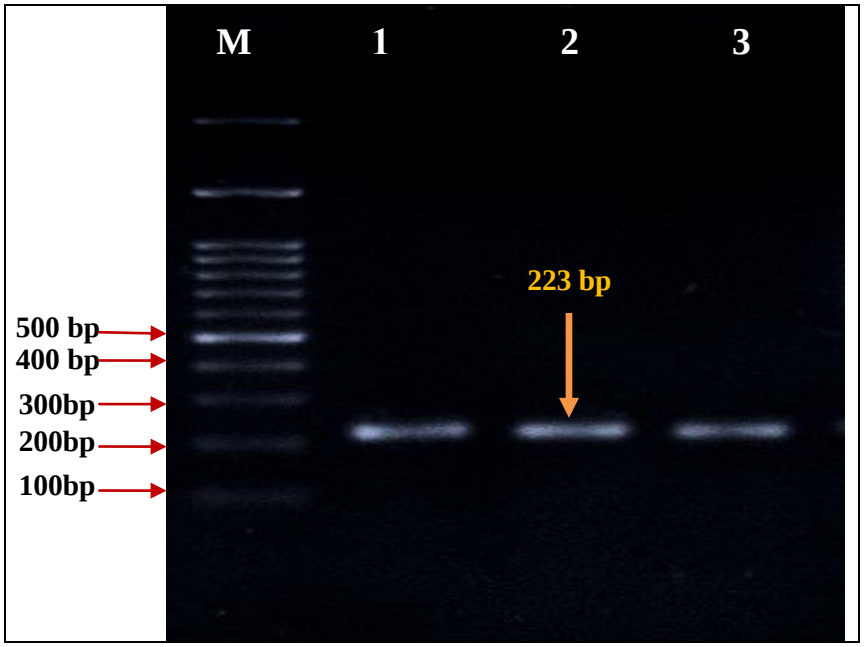

Figure 4.3. PCR amplification of *Brucella* DNA from the tissue of an aborted fetus. Lane M= Molecular weight marker (100 bp DNA ladder); lanes 1, 2, and 3= Positive result of *Brucella abortus* (223bp).

4.3. Sequence Analysis

4.3.1. Nucleotide Sequences

C. abortus was carrying the *ompA* gene, based on agarose gel electrophoresis, which demonstrated the expected amplicon of about 1058bp **(**Figure 4.2). The result was confirmed by sequencing the PCR product, as shown in Appendix 1, and the sequence received the accession no. MK643153 in NCBI GenBank and was named *C. abortus* strain Sul/2017.

4.3.2. Sequence Alignment

The sequences of the partial *ompA* gene of *C. abortus* strain Sul/2017 showed alignment 99.89% homology with two isolates of *C. abortus* from the UK with the accession no. LN554882 and LN554883. However, the isolate in our study demonstrated 99.79% homology with a previous isolate from Sulaimani (Sul/014, accession no. KY399850), which was isolated in the same province. Sequence alignment of Sul/2017 with 100 isolates of *C. abortus* depicted that the Iraqi strain had a single nucleotide polymorphism (SNP, A59050) that caused amino acid substitution A92G protein in the *ompA* gene. However, the strain Sul/2017 differed from Sul/2014 by two amino acid substitutions at E115K and K259N.

4.3.3. Phylogenetic Analysis

The phylogenetic tree, based on the *ompA* gene, showed that Sul/2017 *Chlamydia* from Iraq belonged to *Chlamydia abortus (*Figure 4.4). The phylogenetic tree disclosed that Sul/2017 has a mutual ancestor with isolates of

sheep in Iraq and Tunisia with accession no.KY399850 and HQ62243, respectively. The partial *ompA* gene of Sul /2017 was compared with 75 sequences of *Chlamydia* that were published in GenBank and MLST website for Chlamydiales (http://pubmlst.org/chlamydiales/). According to the phylogenetic tree, the isolates were divided into seven clusters. Each cluster represented specific species of *Chlamydia* (Figure 4.4). The cluster of *C. abortus* was separated into three groups. The topology of the phylogenetic tree hinted that the field isolate Sul/2017 had a common ancestor with Sul/2014, CAAB7, 1H, and Krauss-15, which were located in group 2 of *Chlamydia abortus*.

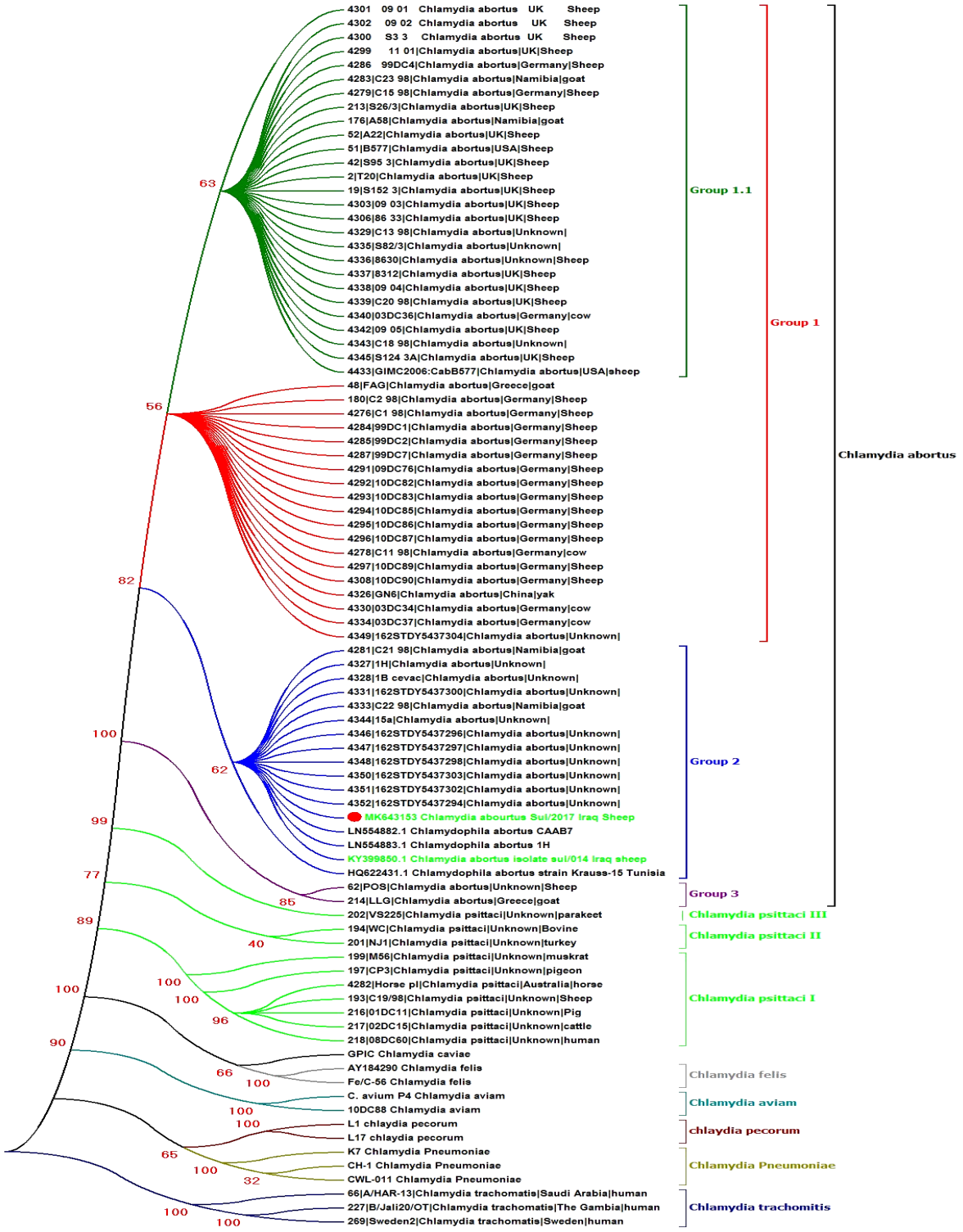

Figure 4.4. Phylogenetic trees based on the *ompA* gene. The tree showed that the Sul/2017 *chlamydia* from Iraq belonged to *C. abortus* and revealed that Sul/2017 has a common ancestor. Partial *ompA* gene of Sul/2017 was compared with 75 sequences of *Chlamydia* that were published in GenBank and MLST websites for Chlamydiales (http://pubmlst.org/chlamydiales/). The tree shows that Sul/2017 has a common ancestor with isolates of sheep in Iraq and Tunisia with accession numbers KY399850 and HQ622431 and with Sul/2014, CAAB7, 1H and Krauss-15 isolates that were located in group 2 of *C. abortus*.

4.4. Isolation of *Chlamydia abortus*

4.4.1. Inoculation of Embryonated Chicken Eggs

The isolation of *C. abortus* in embryonated chicken eggs was positive. Infection with *C. abortus* revealed the death of chick embryo 4–5 days after inoculation. The infected yolk sacs were thin-walled, and their blood vessels were severely congested .Yolk appeared as a bright-colored liquid. The growth of the embryo was stunted. Embryos were suffering from curled toes, and their bodies were covered with hemorrhage (Figure 4.5).

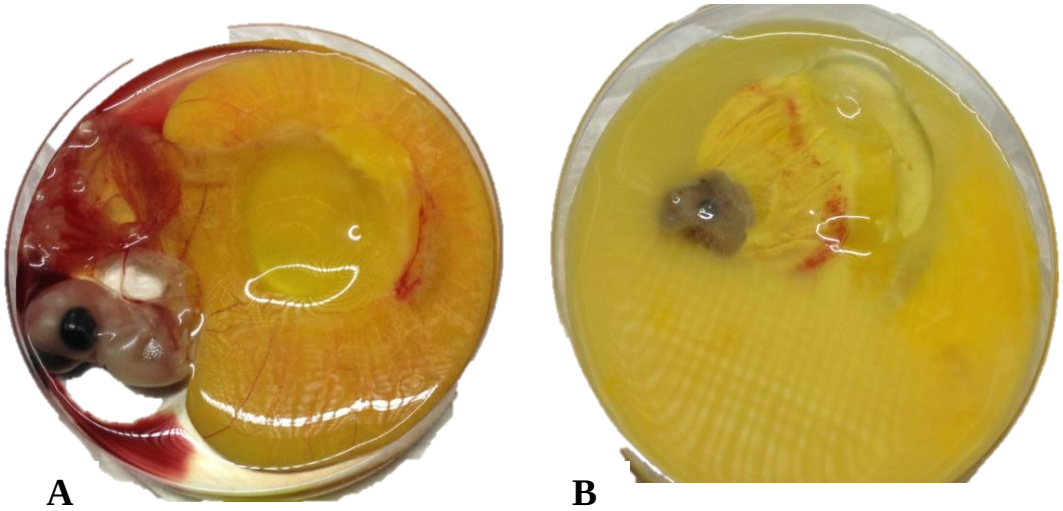

Figure 4.5. Embryonated chicken egg infected by a suspension of *C. abortus*. A: Yolk appeared as mucous, and the growth of the embryo was normal. B: A dead chick embryo five days after inoculation; The infected yolk sacs were thin-walled, and their blood vessels were severely congested; Yolk appeared as a bright-colored liquid, and the growth of the embryo was stunted.

The DNA of *C. abortus* was detected from the membrane of infective yolk sac using genus-specific primers of the *ompA* gene. This gene encodes the cell surface protein of *C. abortus* with an amplicon size of 1058 bp (Figure 4.6).

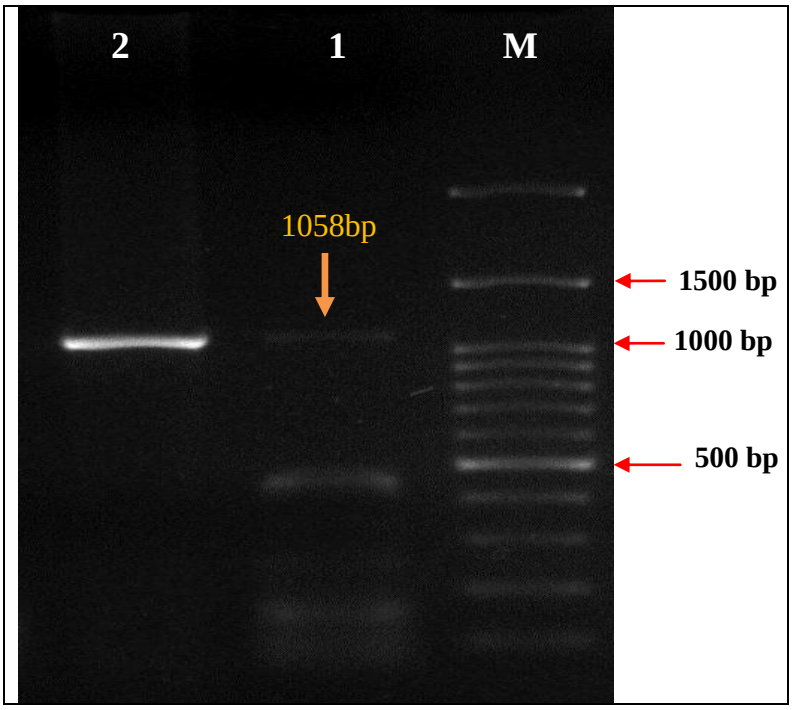

Figure 4.6. PCR amplification of *Chlamydia* DNA from an infectious embryonated chicken egg inoculated with a sample of tissue. Lane M = Molecular weight marker (100 bp DNA ladder); Lane 1 = Band sample; Lane 2 = (control) *C. abortus* (1058bp).

4.4.2. Cell Culture

The PCR-positive sample was successfully cultivated in Vero cells, followed by Giemsa staining. *C. abortus* DNA was detectable from the infective Vero cells. (Figure 4.7) showed the Chlamydial inclusions in the cytoplasm of Vero cells after 48 hours of inoculation.

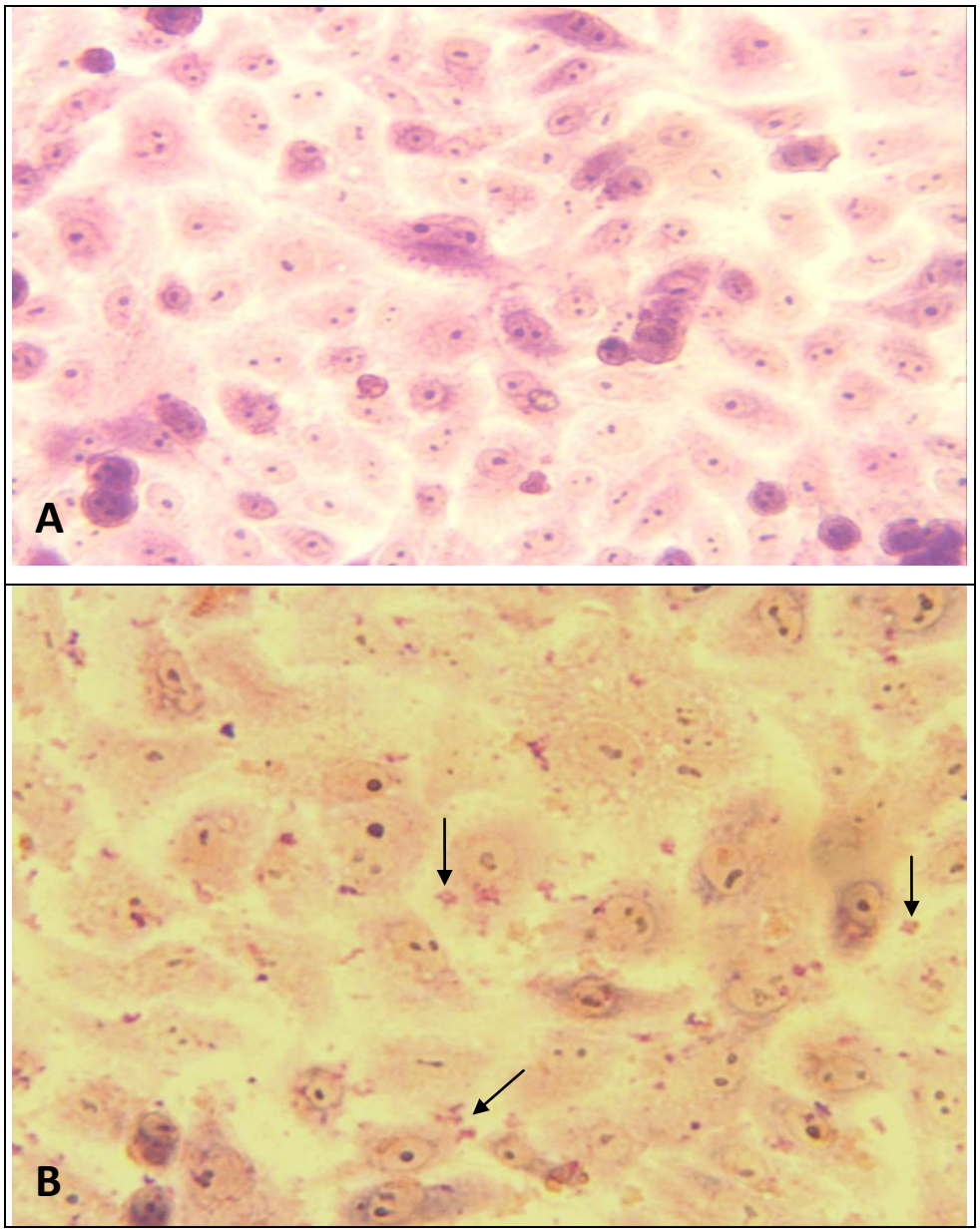

Figure 4.7. Vero cells stained with Giemsa. A: Normal Vero cells. B: Vero cells infected with *C. abortus*. Arrows indicate perinuclear Chlamydial inclusions in the cytoplasm of cells 400X.

4.5. qRT- PCR

4.5.1. Measurement of the Quality and Quantity of mRNA

Total RNA was extracted from 18 infected pregnant and nonpregnant mice tissue samples. All samples had a 260/280-ratio ranged between 2.10 and 2.30, as shown in Appendix 2. After that, a qRT- PCR assay was developed to measure the

expression of mRNAs, in which relative quantification was applied. Thus, *GAPDH* was used as an appropriate internal normalization control to normalize sample-to-sample variations.

4.5.2. mRNA Expression

This study screened mRNA levels of *mip, pmp18D,* and *ompA* to identify aberrantly expressed mRNAs in 18 mice tissues among the different time intervals of pregnancy. All assays were done in triplicate and the Ct value of all targets in all samples with SD less than 1.0 between replicates Ct value, as shown in Appendix 3, 4, and 5. q RT-PCR data were obtained using the ΔΔ Ct method (fold change mean), with normalization to the reference *GAPDH*.

The effect of different time intervals of pregnancy (10 days, 15 days, and 20 days) were assessed by the relative expression of *mip, pmp18D,* and *ompA* gene transcripts in mice tissues as a fold change mean by using the RT-PCR analysis with normalization to the reference *GAPDH*. The our results, as shown in Figure 4.8, reveal apparent differences between the transcriptional levels of the *mip, pmp18D,* and *ompA* genes among different time intervals of pregnancy.

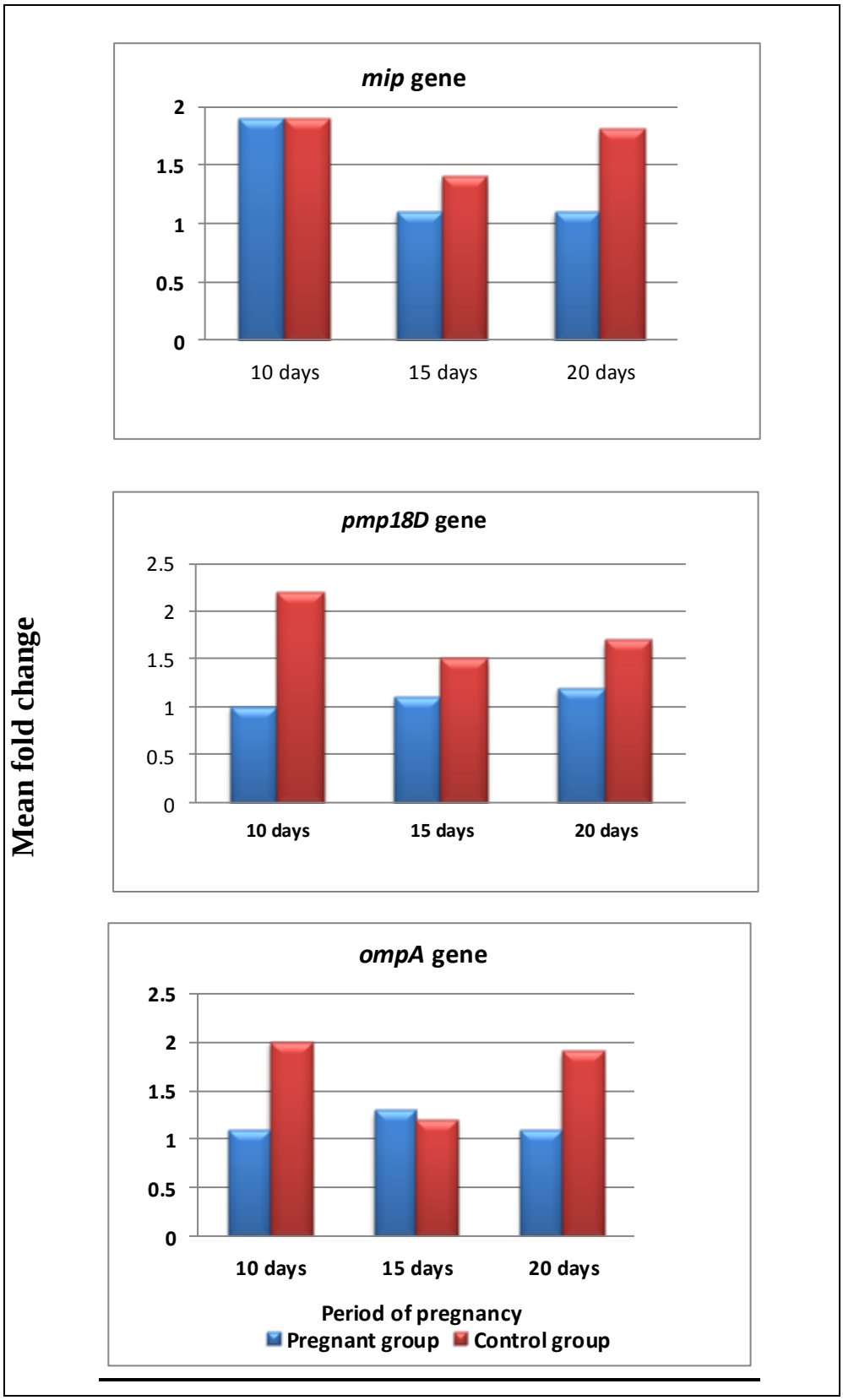

Figure 4.8. mRNA expression levels of *mip*, *pmp18D*, and *ompA* genes as fold change in tissues of pregnant and nonpregnant mice at different times of pregnancy by qRT-PCR.

According to statistical analysis, there was no significant difference (P-values > 0.05) in fold change means of these three genes at each sample time point by using Wilcoxon signed-rank test, as shown in Table 4.2. These results imply that different time intervals of pregnancy do not affect the expression of *mip, pmp18D,* and *ompA* in the pregnant group compared to the nonpregnant and without notice of the presence of abortion.

Table 4.2. Wilcoxon signed-rank test analysis of *mip, pmp 18D,* and *ompA* genes expression between the pregnant groups in comparison to the nonpregnant groups and among different time intervals of pregnancy.

Group	**Gene Type**	**Period of Pregnancy**	**P-values > 0.05**
Pregnant–nonpregnant	*mip*	10 days	0.593
		15 days	0.655
		20 days	0.655
Pregnant–nonpregnant	*pmp18D*	10 days	0.109
		15 days	0.593
		20 days	0.593
Pregnant–nonpregnant	*ompA*	10 days	0.285
		15 days	1.000
		20 days	0.655

The qRT-PCR analysis revealed that the mRNA level of *mip* increased in both the pregnant and nonpregnant groups by 1.9-fold change on day 10 (Figure 4.8, Table 4.2), and there was no statistically significant difference (p = 0.593).

Moreover, the analysis of qRT-PCR also revealed an increase in *pmp18D* mRNA expression level by 1.2 times in the pregnant group on day 20. When compared with the nonpregnant group, which increased on the tenth day by 2.2 folds and decreased on the twentieth day to 1.7 times (Figure 4.8, Table 4.2), the difference was not significant (p=0.593). The *ompA* mRNA level increased in the pregnant group by 1.3-fold on day 15. However, compared with its expression in the nonpregnant group (Figure 4.8, Table 4.2), the difference was not significant (p=1.000).

Chapter Five

DISCUSSION

Ovine enzootic abortion caused by *Chlamydia abortus* is an infectious disease characterized by placentitis and abortion; it has a negative effect on sheep breeding in many countries (Rodolakis and Mohamad, 2010).

It was observed that serological detection of chlamydial infection in animals is of a little value due to cross-reaction with other chlamydial species (Selim, 2016).

C. abortus infections in Iraq have been reported serologically by ELISA, in Slemani and Duhok provinces (Mahmood, 2016), Duhok province (Mikaeel *et al.*, 2016), Fallujah city (Fahad and Salman, 2017), and Baghdad city (Salman *et al.*, 2020). Besides, studies on pathogen isolation and detection by molecular methods and studied virulence factors of *C. abortus* proteins are relatively rare in Slemani province.

The current study showed positive and negative results of the direct examination methods for the demonstration of *Chlamydia* from different samples. The microscopic examination of direct smears taken from the liver of aborted fetuses and vaginal swabs of aborted ewes using Giemsa stain showed that one out of the 30 tissue samples (3.33%) was positive for *Chlamydia* only from Kalar district (Figure 4.1). In contrast, all vaginal swabs gave a negative result.

This result was in agreement with (Barhoom, 2015), who found similar bodies in smear taken from organs of aborted fetuses in North Palestine, while

inclusion bodies were not seen in 24 smears taken from the liver of aborted fetuses in Slemani and Duhok provinces (Mahmood, 2016). On the other hand, our result was in agreement with Elberbawy and Elkhabaz (2014). They could not detect intracytoplasmic inclusion bodies in 92 direct smears stained with Giemsa swabs taken from previously aborted ewes in Assiut Governorate. Also, inclusion bodies were not seen in 33 stained smears with Giemsa swabs taken from aborted ewes in Slemani and Duhok province (Mahmood, 2016).

In the current study, difficulty was seen in obtaining chlamydial inclusion bodies in some stained smears. This might be due to some problem in preparing direct smears such as an error in the staining process, or as Brown and Newman (1989) mentioned, the stained smear technique has low sensitivity because it identifies only elementary bodies. In addition to the sampling time from aborted animals.

Tissues from aborted ewes were investigated by PCR for the detection of *Chlamydia*. This method was previously used by many studies (Soomro *et al.*, 2012; Kalender *et al.*, 2013; Spičic *et al.*, 2015; Alem *et al.*, 2017). The genus-specific primers of *Chlamydia ompA* genes used in the current study are used by other studies (Arshi *et al.*, 2011; Yang *et al.*, 2011).

As shown in Table 4.1, the result shows that one of 30 sheep herds (3.33%) with abortion cases was infected with *Chlamydia* by using the genus-specific primers of the *ompA* gene that encode cell surface protein of *C. abortus* species (Figure 4.2).

The occurrence of *C. abortus* in our study was much lower than the result of Arshi *et al.* (2011), who reported that *Chlamydia* caused 13 out of 50 abortion cases in sheep (26%) northwest of Iran by using the same primers. Other investigators also reported higher incidences of chlamydial infections. For example, Kalender *et al.* (2013) reported an incidence of 9.4%, and Alem *et al.* (2017) reported a prevalence rate of 4.4% in aborted ewes. Also, Spičic *et al.* (2015) reported that *Chlamydia* caused 25 out of 32 abortion cases in ewes (78.1%).

The lower rates of outcomes in our samples may be due to many factors. These factors include the study's geographical location, the sampling time of aborted animals, the size and type of sample taken, and the animal's breed. Other factors may also be involved, such as the type of study performed on aborted or healthy animals, virulence of chlamydial strains, and possibly innate immunity amongst animals.

The result of this study showed that *Brucella* caused abortion in 29 out of 30 (96.66%) sheep herds by using the genus-specific *(bcsp31)* gene primers (Figure 4.3). This result is higher than Faik and Habeb (2013), who reported that *Brucella* infected 9 out of 15 (60%) herds in Baghdad with abortion. Also, Talib (2016) reported that 15 out of 17 (88.23 %) herds were infected with abortion in Slemani by *Brucella* using the same primers. However, Mukherjee *et al.* (2007) reported that *Brucella* caused abortion in 19 out of 19 (100%) farms in India using the same primers.

The high rates of *Brucella* in our samples may be due to several variables. These variables include the *Brucella* strains' virulence, the study's geographical

location, unvaccinated animals, grazing methods, poor management, nutritional deficiency, and uncontrolled animal movement restriction from the infected area. The sampling time of aborted animals can be correlated with this finding (Dağ *et al.*, 2012).

Vaginal swabs are another type of sample used in this study to detect *Chlamydia* in aborted ewes by PCR. There are certain advantages to using vaginal swab samples for diagnosis. The risk of human contagion is reduced considerably due to lack of direct contact with the abortion material, and a minimal amount of sample is sufficient. (Marsilio *et al.*, 2005).

The current study could not identify *C. abortus* DNA from the 50 vaginal swab samples. Our results are also close to other researchers' findings using the same sampling method and could not detect *Chlamydia* in vaginal swab samples taken from aborted ewes (Elberbawy and Elkhabaz, 2014; Mahmood, 2016).

In our study, vaginal swab samples were taken from the endocervix, not from the placenta. Also, samples were collected a few days and sometimes 1–2 weeks after the abortion, not from a recent abortion. Mandeep and Andersen (2000) reported that smears made from cervical swabs of ewes aborted within the previous 24 hours contain fewer organisms than placental smears. There are reports of large amounts of pathogens in the vaginal exudate from a few days before the abortion until a short time after that (Marsilio *et al.*, 2005). This explains the high rates of negative results in our study.

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Molecular analysis reveals that *C. abortus* Sul/2017 strain was the causative agent of abortion in the sheep herd. Before this study, there was not enough genetic information about *C. abortus* in Iraq. Genetic analyses indicated that the present study's strain differed from the previous strain in the region, which was deposited in the GenBank database. The number of *C. abortus* isolates worldwide were 6,718 within the complete phylogeny (Seth-Smith *et al.*, 2017). On the other hand, there were 17,163 variable sites recognized within the phylogeny of *C. trachomatis* (Harris *et al.*, 2012), and 47,710 variable sites were identified within *C. psittaci* strains (Read *et al.*, 2013). This variation was evident in our study, in which each species of *Chlamydia* was in a different cluster (Figure 4.4). Because of low diversity in *C. abortus* (Seth-Smith *et al.*, 2017), there were no apparent differences among the strains according to the geographical region or host range within the *C. abortus* cluster groups. Hence, Sul/2017 shares a common ancestor with a wide range of strains from different hosts and countries. The grouping within the *C. abortus* was not clear due to a low rate of diversity in *C. abortus*. Therefore, to make the phylogram more prominent, we used cutoff value with the condensed tree option in the phylogenic tree constriction.

In this study, we depended only on the positive sample of *C. abortus* DNA extracted from an infected fetal organ. The result of isolation in embryonated chicken eggs was positive. It revealed the death of chick embryo 4–5 days after inoculation, the infected yolk sacs were thin-walled, their blood vessels were severely congested, and yolk appeared as a bright-colored liquid (Figure 4.5). Our result was in agreement with the finding of Kalender *et al.* (2013) and Soomro *et al.* (2012), who isolated *C. abortus* DNA using embryonated egg inoculation. Besides, to confirm isolation, the *C. abortus* DNA was detected from the

membrane of the yolk sac embryonated chicken egg by PCR (Figure 4.6). Due to contamination of *Chlamydia* DNA isolated from the embryonated chicken egg with other bacteria, another technique, cell culture, was used to obtain a pure sample.

For *C. abortus*'s isolation from embryonated chicken eggs, specific-pathogen-free (SPF) chicken eggs should be used. However, local chicken eggs were used in our study due to the unavailability of SPF chicken eggs. This explains why *Chlamydia* isolated from embryonated chicken eggs has been contaminated with other bacteria in our study.

In this study, *Chlamydia* responsible for abortion in ewes was isolated after 48 hours of inoculation of infected fetal organ extract in Vero cells. Culturing of *C. abortus* was followed by Giemsa staining (Figure 4.7), and Giemsa-stained smears showed the *Chlamydial* inclusions in the cytoplasm of Vero cells after 48 hours of inoculation. The pure DNA of *Chlamydia* was detected from the infective Vero cells. Our result was in agreement with other studies results (Osman *et al.*, 2011; Soomro *et al.*, 2012), which isolated *C. abortus* by the cell culture.

In the present study, *C. abortus* was incubated into embryonated chicken eggs 4-5 days from inoculation and in Vero cells for 48 hours. The sample was diagnosed as positive for *C. abortus* after microscopic examination of Giemsa-stained smears. This outcome suggests that the speed of growth of *C. abortus* may be related to the number of bacteria present in the inoculum or stage of their developmental cycle. Many other factors, such as contamination, storage conditions, and homogenization of tissue, may affect the viability of *C. abortus*

elementary bodies and cause slow growth and appearance in culture (Andersen, 1991).

The expressions of *mip, pmp18D,* and *ompA* in *C. abortus* was investigated and quantified using qRT- PCR in the current study. The idea of combining three gene transcriptomes by qRT-PCR is to detect the virulence factors that may play a significant role in the pathogenesis of *C. abortus*. Besides, this method may increase the chances of better identifying the mechanism of abortion in sheep caused by *Chlamydia* and could help in disease diagnosis in the future.

Chlamydia species express a wide variety of potential virulence factors, some of which are assumed to be involved in processes linked to host adaptation (Hagemann *et al.*, 2016).

Our results revealed that *mip* mRNA expression increased in both the pregnant and nonpregnant groups at the same period of gestation (1^{st} stage, 10^{th} day) and without any significant difference between the two groups (P-values > 0.05). The results also showed an apparent increase in mRNA pmp18D expression in the pregnant group at the third stage of gestation (20th day) compared to its expression in the nonpregnant group. Furthermore, a significant difference between the two groups was not detected (P-values > 0.05).

The results showed that mRNA level *ompA* expression increases in the pregnant group at the second stage of gestation (15^{th} day) compared to its expression in the nonpregnant group. However, no significant difference was observed between the two groups (P-values > 0.05).

This difference of expression for these genes in different time intervals of pregnancy in mice might be due to various causes such as technical reasons, sample size, and criteria of evaluation of the result. In addition to the bacterial virulence properties, the host-mediated immune response's quality and intensity could be accountable for the observed variations in host specificity of individual *Chlamydia* species and strains (Braukmann *et al.*, 2012).

For their intracellular survival, chlamydial pathogens have developed sophisticated mechanisms to evade lytic damage along the intracellular pathway in the host cell (Fields and Hackstadt, 2002). As a mechanism of survival and evasion of elimination, chlamydial virulence-associated factors are integrated into the inclusion membrane (Rockey *et al.*, 2002) or secreted into the host cell cytoplasm (Valdivia, 2008), which can modulate the host immune response. It cannot be concluded that the mouse model results are similar to sheep since there are substantial differences in the type of placenta and local immune response. However, it is well known that lymphoid cells in sheep endometrial tissues are morphologically and functionally analogous to the granulate material gland (GMG) cells of pregnant mouse uterus (Rodolakis *et al.*, 1998).

Chapter Six

CONCLUSIONS AND RECOMMENDATIONS

6.1. Conclusions

Within the limitation of this study, the following conclusions can be drawn:

1. The *C. abortus* is not the leading cause of abortion in sheep in the Slemani province.
2. *C. abortus* can be propagated on embryonated chicken egg and Vero cells.
3. PCR technique is an important method for the diagnosis of *C. abortus.*
4. There are apparent differences between the transcriptional level of the *mip, pmp18D,* and *ompA* genes in different time intervals of pregnancy.
5. Different time intervals of pregnancy do not affect the expression of *mip, pmp18D,* and *ompA* in the pregnant mice.

6.2. Recommendations

Further studies need to include:

1. To isolate and characterize *C. abortus* from placentae, fetuses, and vaginal discharges of aborting ewes and does in order to confirm the causes of abortion in small ruminants in the study area.
2. Detection and characterization of other virulence factors of *C. abortus*, contributing to abortion in animals.
3. The whole-genome analysis of all *Chlamydia* strains in Iraq.
4. Study of the genetic correlation between different *Chlamydia* species isolated from animals and humans.

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APPENDICES

Appendix 1.The complete nucleotide sequence (1058bp) of *ompA* gene of *Chlamydia abortus*.

TANGGNNTNNNNNATNCGATNTGAGCNTTCGTATAAGTATAGAATGTGA
AGATCTAAGCTGTAGTTCTCCTAATAGGTTGGGTTCCATGTGGTCAAGTT
AACACAGCAGCAGCAAATTTAGGTGAGCGATAGGATAGGTCAGTTATAC
TGCTCGTGTCTAGTTTACGCCAATATAAGGACAAGCATGTGCATCGATAA
AGAGCGCTAACCCACTTGCCATTCGTGGTATTAAATTGTAGCCGACTTAC
GTCAGTTGCCTGAGCAGTACCAGCTGTTAGAGGTTGAGGAAATGCTGGT
CTTGTATCCTCTTGGCTTGTGAACCACAGATTGTGCTGGCTGGAGACTAC
TCACATTTCAATTTTAGGATTAGACTGAGCGTATTGGGAACTCTGCTCCT
AAGTCGCACAACCACACTCCTCATAAAGCTCCGCGTGCACATACACTCC
GAGAATGGTGTATCTNTATACATTCAACGATTCCTTGAGTCATGCCTACT
TGGGAAGCTGATCAGCTGCTATCGAGGATCCTTTAACACCAATCANACC
ACTAGCTTGAATGCCGCAGCAACTAGCTTTGAGAGTACCCCATTAGTAG
CTGCCTGGATGTGCAGNAAGATATTCATAGCTGATCGCCAGGATGATTC
TGGCGAGGGAAAGCTTGCATTTGGGTGAACCCAATTCGGGCGTCTTGGT
AAGTTNTTGCCGGTAAGCGATGGTTGGGNCTATCCGTTAGGAAGTNTTT
AATTAGCCTGCCTGGCGGCTTCCTGTAGGNACTGCCACCCATGCCCGGG
AANAAGNNTTNTTTCACANCNANTTTNAATANACGATCGNAANATGAN
CCGTAGGNATCNGCGCGGGATNNNNNAGCATCNNNNNNGNNNAGCCAG
NTCNNNNNNCNNCNNNGCACNNNCAAGGCACGATNNNANNNNNTCAGG
GNNNC.

Appendix 2. A representative image showing RNA concentration and purity by Nano drop, ND-1-8000 spectrophotometer.

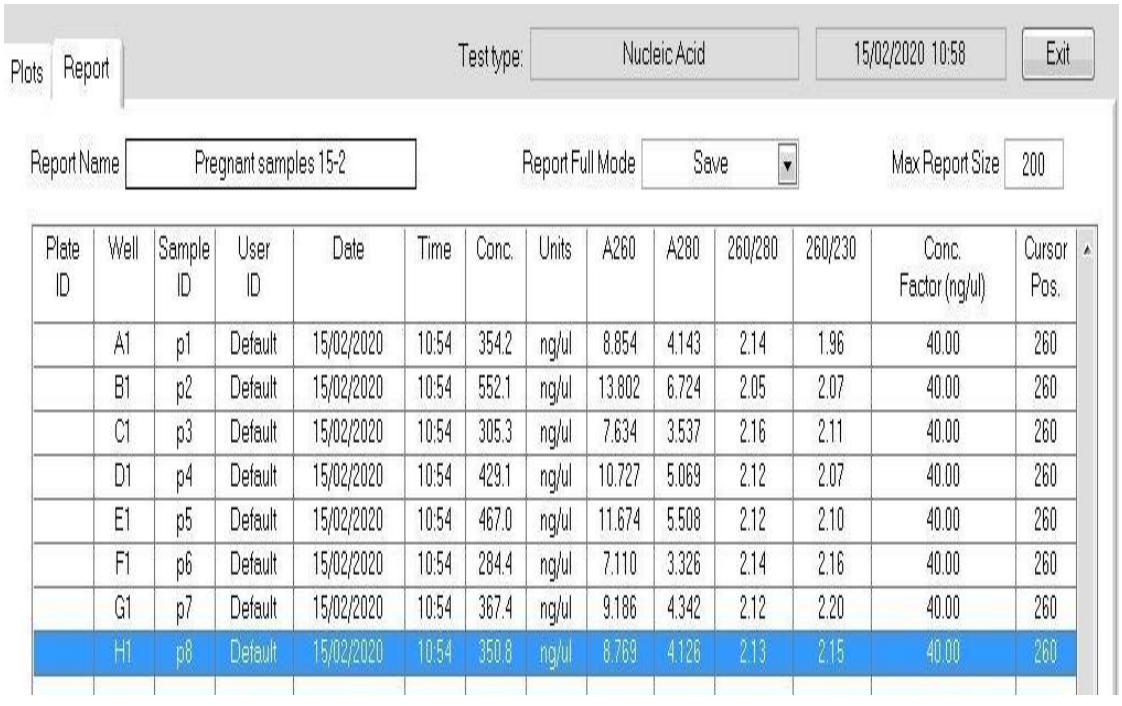
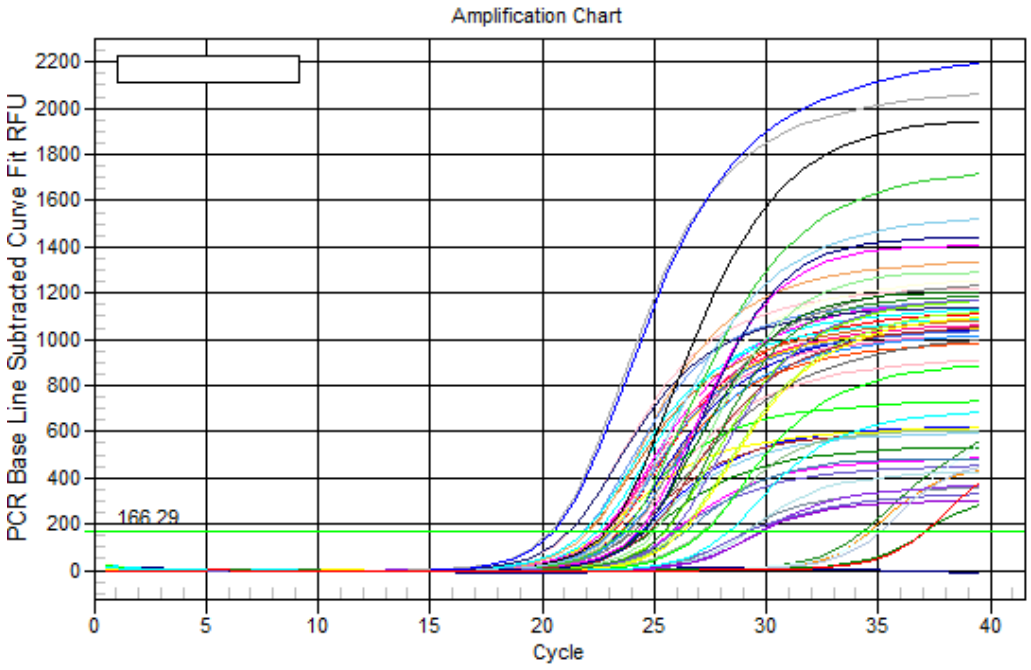
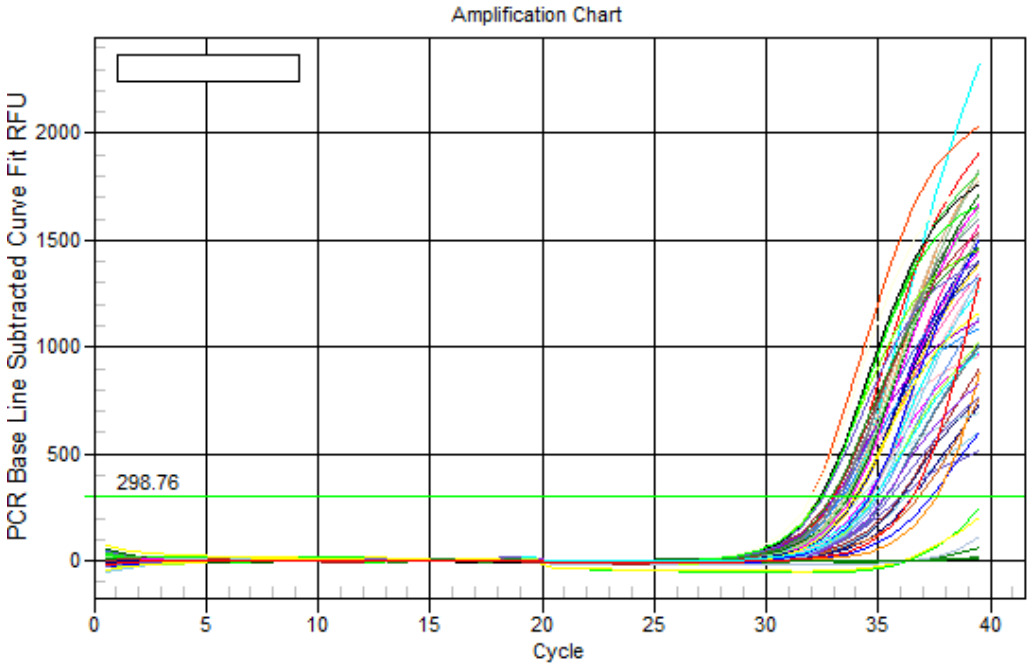


Plate ID	Well	Sample ID	User ID	Date	Time	Conc.	Units	A260	A280	260/280	260/230	Conc. Factor (ng/ul)	Cursor Pos.
	A1	p1	Default	15/02/2020	10:54	354.2	ng/ul	8.854	4.143	2.14	1.96	40.00	260
	B1	p2	Default	15/02/2020	10:54	552.1	ng/ul	13.802	6.724	2.05	2.07	40.00	260
	C1	p3	Default	15/02/2020	10:54	305.3	ng/ul	7.634	3.537	2.16	2.11	40.00	260
	D1	p4	Default	15/02/2020	10:54	429.1	ng/ul	10.727	5.069	2.12	2.07	40.00	260
	E1	p5	Default	15/02/2020	10:54	467.0	ng/ul	11.674	5.508	2.12	2.10	40.00	260
	F1	p6	Default	15/02/2020	10:54	284.4	ng/ul	7.110	3.326	2.14	2.16	40.00	260
	G1	p7	Default	15/02/2020	10:54	367.4	ng/ul	9.186	4.342	2.12	2.20	40.00	260
	H1	p8	Default	15/02/2020	10:54	350.8	ng/ul	8.769	4.126	2.13	2.15	40.00	260

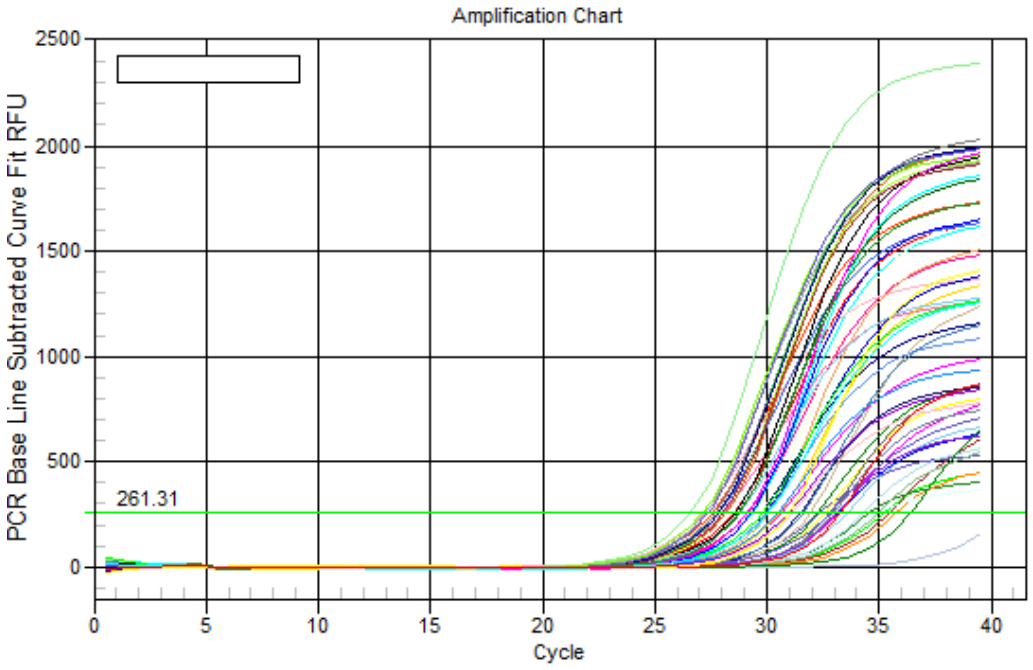
Appendix 3. Show amplification of *mip* gene using qRT-PCR against cycle number in 18 tissues of the mice.



Appendix 4. Show amplification of *pmp18D* gene using qRT-PCR against cycle number in 18 tissues of the mice.



Appendix 5. Show amplification of *ompA* gene using qRT-PCR against cycle number in 18 tissues of the mice.



BIOGRAPHY OF STUDENT

Eman Dhahir Arif is a lecturer in the Microbiology Department at the College of Veterinary Medicine, University of Sulaimani, Iraq, from 2005 till now. Eman was born and raised in Mosul city, Iraq in 1976. She got a Bachelor of Veterinary Science in 1998-1999 from the University of Mosul. After that, she held a master's degree with excellence in Veterinary Microbiology in 2003 from the University of Mosul. Later at the end of 2004, she traveled in order to stay and live in Sulaimani city, located on the Northern side of Iraq. Her Ph.D. dissertation research focuses on the detection and characterization of some proteins expressed as virulence factors in C*hlamydia abortus*, like *mip, pmp18D* and *ompA* using qRT-PCR. This study was considered the first study in the Kurdistan region and Iraq in general. During her Ph.D. study, she participated in several conferences and workshops. Also, she published nine researches during her scientific life. She published one research from this dissertation with Clarivate Web Science an impact factor of 0.897.

LIST OF PUBLICATIONS

Eman Dhahir Arif, Nahla Muhammad Saeed, and Shwan Kamal Rachid. (2020). Isolation and identification of *Chlamydia abortus* from aborted ewes in Sulaimani Province, Northern Iraq. *Polish Journal of Microbiology*, 69(1): 65-71. https://doi.org/10.33073/pjm-2020-009.

الخلاصة

الإجهاض المتوطن للنعاج بسبب الاصابة بكتريا مجبرة داخل الخلايا *Chlamydia abortus* هو مرض حيواني المنشا يؤدي إلى خسائر اقتصادية كبيرة في جميع أنحاء العالم . الإجهاض عادة ما يحدث في الأسابيع ٢–٣ الأخيرة من الحمل مع ظهور حملان ميتة والتهاب مشيمي ظاهر عيانيا . أجريت هذه الدراسة لعزل وتحديد بكتريا *C. abortus* في النعاج المجهضة بواسطة الزرع الخلوي وتفاعل البلمرة المتسلسل PCR، ولتوصيف البروتينات Mip (CAB080) و Pmp18D (CAB776) و Momp (CAB048) في بكتريا *C. abortus* . تم جمع ٣٠ عينة من أنسجة الأجنة المجهضة و ٥٠ مسحه مهبلية من قطعان الأغنام من حالات الإجهاض خلال مواسم الحمل من شهر تشرين الاول ٢٠١٧ إلى شهر حزيران ٢٠١٨ وذلك في اقضية كل من كلار وسيد صادق وجمجمال التابعة لمحافظة سليمانية . اظهر الفحص المجهري للطخات المباشرة المأخوذة من كبد الأجنة المجهضة والمسحات المهبلية من النعاج المجهضة باستخدام صبغة كيمزا أن عينة واحدة فقط من بين ٣٠ عينة نسيجية (٣،٣٣٪) كانت إيجابية لبكتريا الكلاميديا ومن قضاء كلار . بينما أعطت المسحات المهبلية نتيجة سلبية . أظهر الكشف الجزيئي ان عينة واحدة فقط من ٣٠ عينة نسيجية (٣،٣٣٪) كانت إيجابية لبكتريا *C. abortus* ومن قضاء كلار والعينات ٢٩ المتبقية كانت موجبة لبكتريا *Brucella abortus* (٩٦،٦٦٪) ولم يتم الكشف عن بكتريا *C. abortus* من المسحات المهبلية. تم إيداع تسلسل جين *ompA* من بكتريا *C. abortus* في بنك الجينات NCBI برقم الانضمام MK643153 وتحت مسمى *C. abortus* strain Sul/ 2017 . عزلت بكتريا *C. abortus* من خلال الحقن بالبيض الدجاج المحضن وتنقيتها بزرعها في خلايا فيرو. بعد عزلها وتنقيتها تم الكشف عن الأجسام الأولية للكلاميديا من خلال تصبيغها بصبغة كيمزا. أيضا ، تم حقن ثمانية عشر من الفئران من نوع BALB/c (حامل وغير حامل) في منطقة البريتون براشح أعد من زرع بكتريا *C. abortus* في خلايا فيرو وذلك لكشف عن تعبير الجينات *mip* و*pmp18D* و*ompA* خلال مراحل الحمل المختلفة باستخدام qRT-PCR . أظهرت النتائج وجود اختلافات واضحة بين مستويات نسخ الجينات في العينات المأخوذة خلال فترات زمنية مختلفة من الحمل . واشارت النتائج أن هذه الفترات الزمنية المختلفة للحمل لا تؤثر على تعبير الجينات *mip* و *pmp18D* و *ompA* في الفئران الحاملة . اقترحت الدراسة عزل وتوصيف بكتريا *C. abortus* من المشيمة والأجنة والإفرازات المهبلية للنعاج المجهضة وذلك من اجل تأكيد أسباب الإجهاض في المجترات الصغيرة في منطقة الدراسة .

التحري الجزيئي لبعض عوامل ضراوة *Chlamydia abortus* المعزولة من النعاج المجهضة في محافظة السليمانية

اطروحة مقدمة الى

مجلس كلية الطب البيطري في جامعة السليمانية

كجزء من متطلبات نيل شهادة الدكتوراه في

الطب البيطري/ علم الجراثيم

من قبل

ايمان ظاهر عارف عبدالله البرزنجي

بأشراف

الأستاذ المساعد
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محرم، ١٤٤٢هـ

پوختە

بەراویتەبوونی ناوچەگیر لە مەڕدا Enzootic abortion of ewes بە هۆی تووشبوون بە بەکتریای ناوخانەیی *Chlamydia abortus* نەخۆشیەکی ناوچەگیرە کە ئەبێتە هۆی لەدەستدانی ئابووری گەورە لە جیهاندا. بەراویتەبوون بە شێوەیەکی نمونەیی لە ٢–٣ هەفتەی کۆتایی ئاووسیدا ڕوودەدات لەگەڵ دەرکەوتنی بەرخی بە مردوویی لە دایکبوو و هەوکردنی وێڵاش. ئەم توێژینەوەیە ئەنجامدرا بۆ جیاکردنەوە و ناسینەوەی *C. abortus* لە مەڕی بەراویتەبوو بە هۆی چاندن لە ناو خانەو بەکارهێنانی PCR و بۆ دەرخستنی سیفەتی پرۆتینەکانی Mip (CAB080) و Pmp18D (CAB776) وMomp (CAB048) لەو بەکتریایەدا. لە وەرزی زاینی مەڕدا لە مانگەکانی نیوان ئۆکتۆبەری ٢٠١٧ و حوزەیرانی ٢٠١٨، ٣٠ نموونە لە شانەکانی کۆرپەلە بەراویتەبووەکان و ٥٠ نموونە لە سڕاوەی ڕووکەشی ناو زێ کۆکرایەوە لە چەند مێگەلێک لە ناوچەکانی کەلار، سەیدسادق، و چەمچەماڵ کە سەربە پارێزگای سلێمانین. پشکنینی مایکرۆسکۆپی بۆ ئەو سلایدانەی کە لە جگەری کۆرپەلە بەراویتەبووەکان و سڕاوەی ناو زێی مەڕە بەرخستووەکان وەرگیراون و ڕەنگکراون بە بۆیەی گیمزا دەریان خست کەوا کلامیدیا تەنیا یەکێک لە ٣٠ نموونەکەی شانە وەرگیراوەکان (٣،٣٣٪) پۆزەتیڤ بوو بۆ *C. abortus* لە قەزای کەلار. بە پێچەوانەوە، نموونەکانی وەرگیراو لە زێ نێگەتیڤ بوون . پشکنینی مۆلیکولی دەریخست کەوا تەنیا یەکێک لە ٣٠ نموونەکەی شانە وەرگیراوەکان (٣،٣٣٪) پۆزەتیڤ بوو بۆ *C. abortus* لە قەزای کەلار و ٢٩ نموونەکەی تر پۆزەتیڤ بوون بۆ *Brucella abortus* (٩٦،٦٦٪) و هیچ کام لە سڕاوەکانی زێ *C. abortus* یان تیادا بەدی نەکرا. زنجیرەی جینی پرۆتینی *ompA* لە داتابەیسی جینبانک ژمارەی پەیوەندیکردنی MK643153 وەرگرت لە ژێر ناوی *C. abortus* strain Sul/2017 .*C. abortus* جیاکرایەوە بە بەکارهێنانی کۆرپەلەی مریشک لە ناو هێلکەی پیتاودا و پووختەکردنی بە چاندن لەسەر خانەی Vero. پاش جیاکردنەوەو پوختەکردن، تەنە سەرەتاییەکانی کلامیدیا دەرکەوتن بە ڕەنگکردنیان بە بۆیەی گیمزا. هەروەها، هەژدە مشکی ئاوس و قەسر لە جۆری BALB/c لە ڕێگەی پێریتۆنەوە گیراوەیەکیان لە چاندراوی *C. abortus* لەسەر خانەی Vero لێدرا بۆ دەرخستنی جینەکانی *mip* و *pmp18D* و*ompA* لە قۆناغە جیاوازەکانی ئاوسیدا بە بەکارهێنانی تەکنیکی qRT-PCR. ئەنجامەکان جیاوازی بەرچاویان دەرخست لە ئاستی پیشاندانی جینە دەرخراوەکان لە قۆناغە جیاوازەکانی ئاوسیدا. درێژی ماوەی ئاوسبوون کاریگەری نیە لەسەر ئاستی پیشاندانی *mip* و *pmp18D* و *ompA* لە مشکی ئاوسدا. پێشنیاز دەکرێت کەوا *C. abortus* لە وێڵاش،

کۆرپەلە، و دەردراوی زێی مەڕ و بزنی بەراویتەبوو جیابکرێتەوە و سیفاتەکانی دیاری بکرێت بۆ پشتڕاستکردنەوەی هۆکارەکانی بەراویتەبوون لە کاوێژکەرە بچووکەکاندا لەو ناوچەیەی ئەم توێژینەوەی تیادا ئەنجامدرا.

لێکۆڵینەوەی گەردیلەیی لە هەندێ سڵە هۆکاری *Chlamydia abortus*ی جیاکراوە لە مەڕی بەراویتەبوو لە پارێزگای سلێمانی

ئەم نامەیە پێشکەشە بە کۆلیجی پزیشکیی ڤێترنەری زانکۆی سلێمانی وەک بەشێک لە پێداویستیەکانی بەخشینی بڕوانامەی دکتۆرا لە بواری پزیشکیی ڤێترنەری/ بەکتریۆلۆجی

لەلایەن

ایمان ظاهر عارف عبدالله البرزنجي

بە سەرپەرشتی

پرۆفیسۆری یاریدەدەر

د. نهلة محمد سعيد

پرۆفیسۆر

د. شوان كمال رشيد

ڕەزبەر، ٢٧٢٠ك