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Expression Level of the *mip*, *pmp18D*, and *ompA* Genes in *Chlamydia abortus* Isolated from Aborted Ewes

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A b s t r a c t

In this manuscript, we report the proteins macrophage infectivity potentiator (*mip*, CAB080), major outer membrane protein (*momp*, CAB048), and polymorphic outer membrane protein (*pmp18D*, CAB776) that are expressed in different times of pregnancy in mice infected with *Chlamydia abortus*. Enzootic abortion of ewes (EAE) by *C. abortus*, an obligate intracellular pathogen, is a critical zoonotic disease-causing significant economic loss to livestock farming globally. This study was carried out for the detection and characterization of macrophage infectivity potentiator (*mip*, CAB080), major outer membrane protein (*momp*, CAB048), and polymorphic outer membrane protein (*pmp18D*, CAB776) using RT-qPCR. These proteins are believed to be expressed as virulence factors in *C. abortus* isolated from aborted ewes. BALB/c mice (pregnant and nonpregnant) were used as an animal model to be injected intraperitoneally with *C. abortus* culture in Vero cells since the endometrial lymphoid tissues of these animals resembles that of ewes. Also, the short duration of pregnancy in mice makes them a suitable animal model for obstetric studies. Tissue samples were taken from the mice after 10, 15, and 20 days of pregnancy to compare the expression of the genes *mip, pmp18D*, and *ompA*. Transcription level was quantified using RT-qPCR, the GAPDH transcription quantification, as a normalization signal. Abortion occurred in pregnant mice, and apparent differences between the transcriptional levels of the *mip, pmp18D*, and *ompA* genes in the samples taken during different time intervals of pregnancy were not observed ($p > 0.05$). The result indicated that the three bacterial genes, *mip*, *pmp18D*, and *ompA*, play a role as virulence factors in abortion and are differentially expressed in pregnant and nonpregnant animals. Inactivation of the genes is suggested to confirm the hypothesis.

K e y w o r d s : *Chlamydia abortus*, RT-qPCR, macrophage infectivity potentiator (*mip*), polymorphic membrane protein (*pmp*), major outer membrane protein (*momp*)

Introduction

Enzootic abortion of ewes (EAE) induced by *Chlamydia abortus* is a significant cause of reproductive failures in most sheep-producing countries (Borel et al. 2018). The disease in sheep is characterized by abortion in the later stages of pregnancy, after three months of gestation, when *Chlamydiae* begin to invade placentomes. The infection causes a diffuse inflammatory response, thrombotic vasculitis, and tissue necrosis.

The birth of a vital lamb may occur, but these lambs usually cannot survive for more than two days (Spičic et al. 2015). Mice have been widely used and considered helpful animal models for *C. abortus* infections and evaluating new vaccine candidates to reduce economic losses from chlamydial infections (Kerr et al. 2005; Caro et al. 2009). Abortion in experimentally infected mice occurs in the final stages of pregnancy (about day 20), whether *C. abortus* is inoculated on day seven or day 11 (Rodolakis et al. 1998).

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Chlamydia species are obligate intracellular microorganisms that occur in two distinct forms during their lifecycle, switching between an extracellular infectious phase and an intracellular replicative (noninfectious) phase (McClure et al. 2017). Elementary bodies (EBs) are environmentally stable and can infect epithelial cells. Reticulate bodies (RBs) cannot infect cells but are active metabolically and can replicate (Brunham and Rey-Ladino 2005).

Chlamydiae possess a cell wall and are bordered by an outer membrane (OM) and a cytoplasmatic inner membrane (IM) (Elwell et al. 2016). The EB outer membrane contains phospholipids, lipids, lipooligosaccharides (LOS), and proteins, while the cell wall contains a small amount of peptidoglycan (Singh et al. 2020). A part of the *Chlamydia* cell wall is insoluble in sarkosyl, and this fraction is known as the *Chlamydia* outer membrane complex (COMC). This complex mainly consists of the polymorphic membrane proteins (*pmps*), major outer membrane protein (*momp*), and two cysteine-rich proteins (CRP): EnvA or OmcA and EnvB or OmcB (Stephens and Lammel 2001). *Momp* is a cysteine-rich protein of about 41.9 kDa (Longbottom et al. 2019) that covers more than half of the EB outer membrane and almost all RB (Hatch and McClarty 1998). *Pmps* are only present in Chlamydiales and were first detected at the surface of *C. abortus* S26/3 (Longbottom et al. 1996). *Pmps* are ordinarily responsible for virulence and immune response avoidance (Tan et al. 2006). Different studies have shown that the macrophage infectivity potentiator (*mip*) is a solid immunoreactive protein (Forsbach-Birk et al. 2013; Hagemann et al. 2016).

Diagnosis of EAE can be made with DNA- or protein-based assays (Essig and Longbottom 2015). Lately, conventional and real-time PCRs have mainly been used to detect *C. abortus* in clinical samples. PCR methods are based on amplifying the chlamydial OMP genes *ompA*, *omp1*, *omp2*, the polymorphic membrane gene *pmp*, genes encoding 16S rRNA and helicase, and the 16S-23S rRNA intergenic interval (Berri et al. 2009). Speedy and dependable diagnostic assays are essential to rapid disease control.

The expression and role of the *mip*, *pmp18D*, and *ompA* genes of *C. abortus* in abortion in ewes have not been adequately elucidated. Hence, this work aimed to identify and purify *C. abortus* from ovine abortion samples and characterize three proteins expressed as virulence factors in *C. abortus* isolated from aborted ewes using RT-qPCR. The studied genes were the macrophage infectivity potentiator (*mip*, CAB080), major outer membrane protein (*momp*, CAB048), and polymorphic outer membrane protein (*pmp*, CAB776). The genes' expression level was also compared in experimentally infected pregnant and nonpregnant mice with *C. abortus*.

Experimental

Materials and Methods

Bacterial culture in Vero cell line. *C. abortus*, isolated from an aborted bovine fetus in Sulaimani province, north Iraq (Arif et al. 2020), was used in this study. Vero cells were also provided as a gift by the Cell Culture Unit at the College of Medicine/Babylon University, Iraq.

Monolayers of Vero cells were grown in 25 ml tissue culture flasks at 2×10^6 cells/ml. Confluent monolayers (70–80%) were achieved 24 hours after incubation at 37°C with 5% CO_2 with Dulbecco's Modified Eagle medium (DMEM) supplemented with 10% fetal bovine serum (Labiran 2014). About 25 µl of a frozen stored suspension of an aborted ewe's placental tissue was thawed in SPG (0.25 M sucrose, 10 mM sodium phosphate, and 5 mM L-glutamic acid) and added to Vero cell cultures. 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 the tissue culture flasks were incubated again at 37°C for 48–72 hours. After 48 hours, *Chlamydia* inclusions were large enough to be visualized under an inverted microscope (Campos-Hernández et al. 2014).

Vero cells were harvested two days after infection with *C. abortus* by high-speed centrifugation ($30,000 \times g$) for 45 minutes. *Chlamydiae* were released from the intracellular vacuoles by ultra-sonication and centrifuged again at low speed ($500 \times g$ for 10 minutes) to separate the bacteria from cell debris (Labiran 2014).

C. abortus cells were stained with Giemsa solution and observed under an inverted microscope at $400 \times$ magnification. The EBs of *Chlamydia* appeared as small cocci occurring singly and in clusters in the infected cells' cytoplasm (Arif et al. 2020).

Experimental infection of mice. Thirty-six female and six male albino mice of *Mus musculus* species, BALB/c strain, were used in the study. The mice, 6–8 weeks old, between 25 and 30 grams, were obtained from the Research Center at the College of Veterinary Medicine/University of Sulaimani. All mice-involving procedures were executed humanely according to the Guide for the Care and Use of Laboratory Animals, and the Ethics Committee at the College of Veterinary Medicine, University of Sulaimani, approved the experimental protocol (Approval number AUP-20/9). The 36 female mice were divided into two equal groups; Group 1 of pregnant mice (clinical case) and Group 2 of nonpregnant mice (control). The mice were injected intraperitoneally with 0.2 ml of the supernatant collected from the isolation of *C. abortus*

in Vero cells (kept at –70°C). The solution contained 1,000 inclusion-forming units (IFU) of *C. abortus* in 0.9% saline solution, following a previously described method (Livingstone et al. 2009). Injection of *C. abortus* was conducted after one week of pregnancy in Group 1.

Animal euthanasia and sample collection. The duration of pregnancy was divided into three stages. The first stage was on the 10th day of pregnancy, while stages two and three were after 15 and 20 days. In each stage, six mice were euthanized from each group by intraperitoneal injection of a mixture of xylazine (16 mg/kg) and ketamine (100 mg/kg), followed by cervical dislocation (Schoell et al. 2009).

Following the mice's euthanasia, the external surface was cleaned and disinfected with 70% ethanol, and the abdomen was opened using aseptic procedures. Fragments were resected from the liver, spleen, lung, kidney, placenta, and fetus. The organ fragments were ground and pooled together. The samples were subjected to RNA extraction using a tissue RNA extraction kit, RT05O (Gene aid, Taiwan). The procedure was conducted following instructions provided by the manufacturer.

RNA quantification and RT-qPCR program. RNA measurement was performed using a Nanodrop, ND-8000 (8-sample spectrophotometer, USA). RNA sample absorbance was measured at 230 nm, 260 nm, and 280 nm.

The primer for the *mip* gene was previously designed by Forsbach-Birk et al. (2013), the primers of *pmp18D* and *ompA* genes were designed by Wheelhouse et al. (2009) (Table I), while the *GAPDH* primer was designed for this study. Profiling was executed using a real-time PCR-based array and *GAPDH* (glyceraldehyde-3-phosphate dehydrogenase) transcript as a normalization signal. The AddRT-qPCR SYBR kit (Add Bio, Korea), a single tube real-time one-stepRT-qPCR, was used in this study. Furthermore, q-PCR was undertaken in an IQ5 Multicolor Real-time PCR system (BIO-RAD, USA).

Optimization of PCR reactions, data analysis, and mRNA expression. Each reaction was run in a final volume of 20 μl containing 500 ng of the RNA, 1 μl (10 pmol) of each primer, and 10 μl SYBR Green PCR Mastermix (Add Bio/Korea). The amplification profile was 50°C for 20 minutes. Then, at 95°C for 10 minutes, denaturation was followed by 40 cycles of 95°C for 30 seconds and 60°C for 30 seconds.

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

The expression levels of mRNAs were normalized to *GAPDH*. mRNA expression was quantified as ΔCt values for both pregnant and nonpregnant separately, where Ct = threshold cycle and ΔCt = Ct target gene – average Ct of *GAPDH*. After comparing it with the pooled sample, all mRNAs' expression levels were measured in standard tissue samples. The ΔΔCt values, which are expressed as ΔCt target gene – ΔCt of the pooled sample, were used to quantify mRNA expression of the *pmp18D, ompA*, and *mip* genes in infected pregnant mice compared to infected nonpregnant mice. The equation 2-ΔΔCt was used to calculate the fold change of the gene.

Results

Isolation in cell culture. *C. abortus* organisms were successfully cultivated into Vero cells monolayer. Giemsa staining showed the *Chlamydial* inclusions in the cytoplasm of Vero cells after 48 hours of inoculation. The DNA was detectable from the infective Vero cells.

mRNA expression. RT-qPCR data were obtained using the ΔΔ Ct method (fold change mean), normalizing to the reference *GAPDH*. The effect of different time intervals of pregnancy (10 days, 15 days, and 20 days) was assessed by the relative expression of the *mip*,

Table I
Sequences of the forward and reverse primers of four genes used
in Real-time PCR.

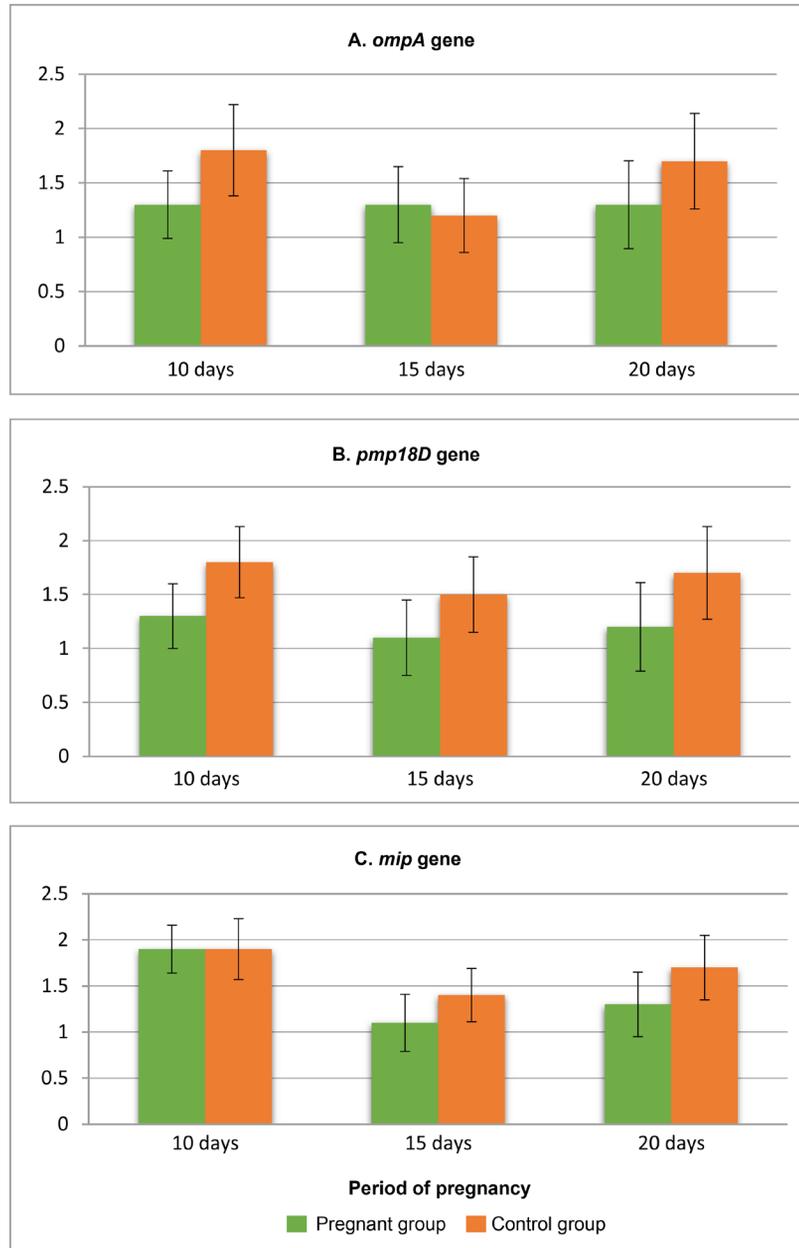| Target gene	Primer sequence (5′ to 3′)	Size (bp)
GAPDH	Forward: GGGGTCCCAGCTTAGGTTCA Reverse: ACGGCCAAATCCGTTCACA	95
mip	Forward: AAGAAAACCTCTCCCTAGCC Reverse: CTGAAGGTTTCCCTGATATTG	139
ompA	Forward: GCGGCATTCAACCTCGTT Reverse: CCTTGAGTGATGCCTACATTGG	85
pmp18D	Forward: TCCACTGGGATGATCACCAATA Reverse: GCATAGAAAGCGTATCGAGAATCAC	81

Fig.1. mRNA expression levels of the *ompA, pmp18D*, and *mip* genes as fold change in pregnant
and nonpregnant mice tissues at different pregnancy times by RT-qPCR. Data are presented
as means of values for six mice (columns) ± SEM (error bars).

pmp18D, and *ompA* gene transcripts in murine tissues as a fold change mean using the RT-qPCR analysis with normalizing to the reference *GAPDH*. Abortion occurred only after 20 days of pregnancy, and Fig. 1A–1C indicate apparent differences between the transcriptional levels of the *mip, pmp18D*, and *ompA* gene among different time intervals of pregnancy. The RT-qPCR analysis revealed that the mRNA level of *mip* increased in both the pregnant and nonpregnant groups by 1.9-fold change on day 10 (Fig. 1A,

Table II), and there was no statistically significant difference ($p = 0.593$).

Moreover, the RT-qPCR analysis also revealed an increase in *pmp18D* mRNA expression level by 1.2 times in the pregnant group on day 20. Compared with the nonpregnant group, which increased on the tenth day by 2.2 folds, the difference was insignificant statistically (Fig. 1B, Table II).

The *ompA* mRNA level increased in the pregnant group by 1.3-fold on day 15. However, compared with

Table II
Wilcoxon signed-rank test analysis of the *mip, pmp18D*, and *ompA* genes expression between the pregnant groups in comparison to the nonpregnant groups at each time interval of pregnancy.

Group	Gene type	Period of pregnancy (days)	*p*-values
Pregnant group – nonpregnant	*mip*	10	0.593
		15	0.655
		20	0.655
Pregnant group – nonpregnant	*pmp18D*	10	0.109
		15	0.593
		20	0.593
Pregnant group – nonpregnant	*ompA*	10	0.285
		15	1.000
		20	0.655

its expression in the nonpregnant group, the difference was insignificant ($p = 0.285$) (Fig. 1C, Table II).

According to the statistical analyses, there was no statistically significant difference (p-values > 0.05) in the fold change means of these three genes at each sample time point by using Wilcoxon signed-rank test, as shown in Table II. These results imply that different time intervals do not affect the expression of each *mip, pmp18D*, and *ompA* in the pregnant group compared to the nonpregnant.

Discussion

Chlamydia isolation from aborted tissues is the standard for definitive diagnosis. Nevertheless, bacterial isolation requires collecting samples in optimal conditions; the samples must be fresh, with little or no contamination, and contain a suitable amount of live and viable microorganisms (Li et al. 2015). *Chlamydia* cultivation in cell culture is considered the gold standard. However, this lengthy technique is only applicable to certain cultivable strains. Besides, many strains are challenging to grow, and not all labs have the required facilities to grow the microorganism. The odds for diagnosing *Chlamydia* have considerably increased since the introduction of DNA-based techniques. For example, PCR allows direct identification from clinical samples and species differentiation (Hailat et al. 2018; Saeed et al. 2019).

In this study, the culturing of *C. abortus* was followed by Giemsa staining, and the DNA of *Chlamydia* could be detected from the infected Vero cells. The sample was diagnosed as positive for *C. abortus* after the microscopic examination of Giemsa-stained smears. This outcome implies that *C. abortus* growth may be related to the number of bacteria in the inoculum or their developmental cycle. Like contamination, storage circumstances, and tissue homogenization, other factors may affect *C. abortus* elementary bodies' viability and cause slow growth and appearance in culture (Zhang et al. 2015).

The current study investigated and quantified the expressions of *mip, pmp18D*, and *ompA* in *C. abortus* using RT-qPCR. *C. abortus* naturally infects ewes, but mice can be infected via parenteral administration, such as the intraperitoneal or intravenous routes (Kerr et al. 2005).

This method has not been adopted in the Kurdistan and Iraq so far. Combining three gene transcriptomes by RT-qPCR detects virulence factors that might show a substantial role in the pathogenesis of *C. abortus*. Besides, this method may increase the chances of better identifying the abortion mechanisms in sheep caused by *Chlamydia* and help diagnose disease in the future.

Chlamydia species express a wide range of virulence factors. Some of these factors are presumed to be involved in processes associated with the regulation of host adaptation. Our results revealed that *mip* mRNA expression increased in both the pregnant and nonpregnant groups at the same gestation period (1st stage, 10th day) without any significant difference between the two groups (p-values > 0.05). The results also showed no measurable increase in mRNA *pmp18D* expression in the pregnant group at the third stage of gestation (20th day) compared to its nonpregnant group expression. Furthermore, a significant difference between the two groups was not detected (p-values > 0.05). The results showed that the mRNA level of *ompA* expression increases in the pregnant group at the second stage of gestation (15th day) compared to its nonpregnant group expression. Still, the difference between the two groups was not statistically significant.

Chlamydial pathogens have evolved sophisticated mechanisms to elude lytic damage in the host cell (Fields and Hackstadt 2002). As a survival method and equivocation 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 reaction.

This difference in the expression of these genes at different time intervals of pregnancy in mice might be due to various causes, for example, technical reasons, sample size, and criteria for evaluating the result. In addition to the bacteria's virulence characteristics, the host-mediated immune response's quality and intensity could be responsible for the host specificity of individual species and strains of *Chlamydia* (Braukmann et al. 2012).

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 pregnant mouse uterus' granulate material gland cells (Chavan et al. 2016). Our results showed that the different time intervals of pregnancy do not affect the expression of *mip*, *pmp18D*, and *ompA* in pregnant mice. Inactivation of the genes is suggested to confirm the hypothesis.

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Author contributions

E.A., N.S., and S.R. conceived and designed the experiments; E.A. and H.D. drafted and revised the manuscript; E.A. and P.R. performed the experiments and analyzed the data.

Conflict of interest

The authors do not report any financial or personal connections with other persons or organizations, which might negatively affect the contents of this publication and/or claim authorship rights to this publication.

Literature

Arif ED, Saeed NM, Rachid SK. Isolation and identification of *Chlamydia abortus* from aborted ewes in Sulaimani province, Northern Iraq. Pol J Microbiol. 2020 Mar 25;69(1):65–71. https://doi.org/10.33073/pjm-2020-009

Berri M, Rekiki A, Boumedine K, Rodolakis A. Simultaneous differential detection of *Chlamydophila abortus*, *Chlamydophila pecorum* and *Coxiella burnetii* from aborted ruminant's clinical samples using multiplex PCR. BMC Microbiol. 2009;9(1):130. https://doi.org/10.1186/1471-2180-9-130

Borel N, Polkinghorne A, Pospischil A. A review on chlamydial diseases in animals: still a challenge for pathologists? Vet Pathol. 2018 May;55(3):374–390. https://doi.org/10.1177/0300985817751218

Braukmann M, Sachse K, Jacobsen ID, Westermann M, Menge C, Saluz HP, Berndt A. Distinct intensity of host-pathogen interactions in *Chlamydia psittaci*- and *Chlamydia abortus*-infected chicken embryos. Infect Immun. 2012 Sep;80(9):2976–2988. https://doi.org/10.1128/IAI.00437-12

Brunham RC, Rey-Ladino J. Immunology of *Chlamydia* infection: implications for a *Chlamydia trachomatis* vaccine. Nat Rev Immunol. 2005 Feb;5(2):149–161. https://doi.org/10.1038/nri1551

Campos-Hernández E, Vázquez-Chagoyán JC, Salem AZM, Saltijeral-Oaxaca JA, Escalante-Ochoa C, López-Heydeck SM, de Oca-Jiménez RM. Prevalence and molecular identification of *Chlamydia abortus* in commercial dairy goat farms in a hot region in Mexico. Trop Anim Health Prod. 2014 Aug;46(6):919–924. https://doi.org/10.1007/s11250-014-0585-6

Caro MR, Buendía AJ, Del Rio L, Ortega N, Gallego MC, Cuello F, Navarro JA, Sanchez J, Salinas J. *Chlamydophila abortus* infection in the mouse: A useful model of the ovine disease. Vet Microbiol. 2009 Mar;135(1–2):103–111. https://doi.org/10.1016/j.vetmic.2008.09.029

Chavan AR, Bhullar BAS, Wagner GP. What was the ancestral function of decidual stromal cells? A model for the evolution of eutherian pregnancy. Placenta. 2016 Apr;40:40–51. https://doi.org/10.1016/j.placenta.2016.02.012

Chun T, Spitznagel JK, Shou H, Hsia R, Bavoil PM. The polymorphic membrane protein gene family of the *Chlamydiaceae*. In: Bavoil PM, Wyrick PB, editors. *Chlamydia*: genomics and pathogenesis. Wymondham (UK): Horizon Bioscience; 2006. p. 195–218.

Elwell C, Mirrashidi K, Engel J. *Chlamydia* cell biology and pathogenesis. Nat Rev Microbiol. 2016 Jun;14(6):385–400. https://doi.org/10.1038/nrmicro.2016.30

Essig A, Longbottom D. *Chlamydia abortus*: new aspects of infectious abortion in sheep and potential risk for pregnant women. Curr Clin Microbiol Rep. 2015 Mar;2(1):22–34. https://doi.org/10.1007/s40588-015-0014-2

Fields KA, Hackstadt T. The chlamydial inclusion: escape from the endocytic pathway. Annu Rev Cell Dev Biol. 2002 Nov;18(1):221–245. https://doi.org/10.1146/annurev.cellbio.18.012502.105845

Forsbach-Birk V, Foddis C, Simnacher U, Wilkat M, Longbottom D, Walder G, Benesch C, Ganter M, Sachse K, Essig A. Profiling antibody responses to infections by *Chlamydia abortus* enables identification of potential virulence factors and candidates for serodiagnosis. PLoS One. 2013 Nov 15;8(11):e80310. https://doi.org/10.1371/journal.pone.0080310

Hagemann JB, Simnacher U, Longbottom D, Livingstone M, Maile J, Soutschek E, Walder G, Boden K, Sachse K, Essig A. Analysis of humoral immune responses to surface and virulence-associated *Chlamydia abortus* proteins in ovine and human abortions by use of a newly developed line immunoassay. J Clin Microbiol. 2016 Jul;54(7):1883–1890. https://doi.org/10.1128/JCM.00351-16

Hailat N, Khlouf S, Ababneh M, Brown C. Pathological, immunohistochemical and molecular diagnosis of abortions in small ruminants in Jordan with reference to *Chlamydia abortus* and *Brucella melitensis*. Pak Vet J. 2018 Mar 01;38(01):109–112. https://doi.org/10.29261/pakvetj/2018.022

Hatch GM, McClarty G. Cardiolipin remodeling in eukaryotic cells infected with *Chlamydia trachomatis* is linked to elevated mitochondrial metabolism. Biochem Biophys Res Commun. 1998 Feb; 243(2): 356–360. https://doi.org/10.1006/bbrc.1998.8101

Kerr K, Entrican G, McKeever D, Longbottom D. Immunopathology of *Chlamydophila abortus* infection in sheep and mice. Res Vet Sci. 2005 Feb;78(1):1–7. https://doi.org/10.1016/j.rvsc.2004.08.004

Labiran C. Molecular epidemiology of *Chlamydia trachomatis*: valuation, implementation and development of high resolution genotyping [PhD Thesis]. Southampton (UK): University of Southampton, Faculty of Medicine, Division of Clinical and Experimental Science; 2014.

Li Z, Cao X, Fu B, Chao Y, Cai J, Zhou J. Identification and characterization of *Chlamydia abortus* isolates from yaks in Qinghai, China. Biomed Res Int. 2015;2015:658519. https://doi.org/10.1155/2015/658519

Livingstone M, Wheelhouse N, Maley SW, Longbottom D. Molecular detection of *Chlamydophila abortus* in post-abortion sheep at oestrus and subsequent lambing. Vet Microbiol. 2009 Mar; 135(1–2):134–141. https://doi.org/10.1016/j.vetmic.2008.09.033

Longbottom D, Livingstone M, Aitchison KD, Imrie L, Manson E, Wheelhouse N, Inglis NF. Proteomic characterisation of the *Chlamydia abortus* outer membrane complex (COMC) using combined rapid monolithic column liquid chromatography and fast MS/MS scanning. PLoS One. 2019 Oct 24;14(10):e0224070. https://doi.org/10.1371/journal.pone.0224070

Longbottom D, Russell M, Jones GE, Lainson FA, Herring AJ. Identification of a multigene family coding for the 90 kDa proteins of the ovine abortion subtype of *Chlamydia psittaci*. FEMS Microbiol Lett. 1996 Sep;142(2–3):277–281. https://doi.org/10.1111/j.1574-6968.1996.tb08443.x

McClure EE, Chávez ASO, Shaw DK, Carlyon JA, Ganta RR, Noh SM, Wood DO, Bavoil PM, Brayton KA, Martinez JJ, et al. Engineering of obligate intracellular bacteria: progress, challenges and paradigms. Nat Rev Microbiol. 2017 Sep;15(9):544–558. https://doi.org/10.1038/nrmicro.2017.59

Rockey DD, Scidmore MA, Bannantine JP, Brown WJ. Proteins in the chlamydial inclusion membrane. Microbes Infect. 2002 Mar; 4(3):333–340. https://doi.org/10.1016/S1286-4579(02)01546-0

Rodolakis A, Salinas J, Papp J. Recent advances on ovine chlamydial abortion. Vet Res. 1998 May–Aug;29(3–4):275–288.

Saeed NM, Talib SM, Dyary HO. Molecular detection of outer membrane protein 2 gene in isolated brucella from sheep and goats in Sharazor/Kurdistan region of Iraq. Pak Vet J. 2019 Jul 01;39(03): 383–388. https://doi.org/10.29261/pakvetj/2019.043

Schoell AR, Heyde BR, Weir DE, Chiang PC, Hu Y, Tung DK. Euthanasia method for mice in rapid time-course pulmonary pharmacokinetic studies. J Am Assoc Lab Anim Sci. 2009 Sep;48(5): 506–511.

Singh R, Liechti G, Slade JA, Maurelli AT. *Chlamydia trachomatis* oligopeptide transporter performs dual functions of oligopeptide transport and peptidoglycan recycling. Infect Immun. 2020 Apr 20; 88(5):e00086–e20. https://doi.org/10.1128/IAI.00086-20

Spičic S, Račić Ivana, Andrijanić M, Duvnjak S, Zdelar-Tuk M, Stepanić M, Cvetnić Z. Emerging cases of chlamydial abortion in sheep and goats in Croatia and Bosnia and Herzegovina. Berl Munch Tierarztl Wochenschr. 2015 May-Jun;128(5–6):183–187.

Stephens RS, Lammel CJ. *Chlamydia* outer membrane protein discovery using genomics. Curr Opin Microbiol. 2001 Feb;4(1):16–20. https://doi.org/10.1016/S1369-5274(00)00158-2

Valdivia RH. *Chlamydia* effector proteins and new insights into chlamydial cellular microbiology. Curr Opin Microbiol. 2008 Feb; 11(1):53–59. https://doi.org/10.1016/j.mib.2008.01.003

Wheelhouse N, Aitchison K, Spalding L, Livingstone M, Longbottom D. Transcriptional analysis of *in vitro* expression patterns of *Chlamydophila abortus* polymorphic outer membrane proteins during the chlamydial developmental cycle. Vet Res. 2009 Sep;40(5):47. https://doi.org/10.1051/vetres/2009030

Zhang Q, Huang Y, Gong S, Yang Z, Sun X, Schenken R, Zhong G. *In vivo* and *ex vivo* imaging reveals a long-lasting chlamydial infection in the mouse gastrointestinal tract following genital tract inoculation. Infect Immun. 2015 Sep;83(9):3568–3577. https://doi.org/10.1128/IAI.00673-15