Polymorphic Membrane Protein-13G Expression Variation Demonstrated between *Chlamydia abortus* Culture Conditions and Strains

by
Emaan M. Khan

A THESIS

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Oregon State University

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degree of

Honors Baccalaureate of Science in Microbiology
(Honors Associate)

Presented May 18, 2017
Commencement June 2017

AN ABSTRACT OF THE THESIS OF

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Abstract approved: _____
Daniel Rockey

Chlamydiae encode a family of proteins named the polymorphic membrane proteins, or Pmps, whose role in infection and pathogenesis is unclear. The Rockey Laboratory is studying polymorphic membrane protein expression in *Chlamydia abortus,* a zoonotic pathogen that causes abortions in ewes. *C. abortus* contains 18 *pmp* genes, some of which carry internal homopolymeric repeat sequences (poly-G) that may have a role in controlling protein expression within infected cells.

The goal of this project was to elucidate the role of Pmps in the pathogenesis of *C. abortus* by evaluating patterns of Pmp expression and of the length of a poly-G region within *pmp13G*. Previous research in the Rockey Laboratory showed a variation in Pmp13G expression and suggested that expression of the *pmp13G* gene is required under certain culture conditions and not in others. It was hypothesized that this variation was due to changes in culture conditions and may have been linked to the necessity of the *pmp13G* gene only under certain stages of growth or certain culture conditions. Our approach integrated PCR-based Sanger sequencing results with both qualitative and quantitative data collected via fluorescence microscopy.

A variation in the length of the poly-G region within *pmp13G* was demonstrated between *C. abortus* samples, and also between culture conditions via passaging of a single sample. Fluorescence microscopy showed Pmp18D was uniformly present among the strains analyzed, while detection of Pmp10G and Pmp13G differed between strains. Pmp13G presence was shown to vary between *C. abortus* strains, as well as between culture conditions via passaging of a single strain.

Key Words: *Chlamydia abortus*, polymorphic membrane protein, genomics

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I understand that my project will become part of the permanent collection of Oregon State University, Honors College. My signature below authorizes release of my project to any reader upon request.

_____

Emaan Khan, Author

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Introduction

Chlamydiae are gram-negative, obligate intracellular bacteria that threaten human and animal health. *Chlamydia abortus* is a zoonotic pathogen that causes diseases concerned with abortions in small ruminants, such as Ovine Enzootic Abortion (OEA), and has a large impact on both animal health and the farming industry. In addition, pregnant women working in close contact with infected animals are at risk of becoming infected as well.

Members of the *Chlamydia* genus share a unique bi-phasic developmental cycle, consisting of an infectious elementary body (EB) and a replicative reticulate body (RB). The cycle begins with an EB attaching to and entering a host cell. The EB then differentiates into an RB, which replicates inside of a vacuole called an inclusion. These RBs accumulate and differentiate back into EBs, and are then released to infect new cells.

All chlamydial species contain polymorphic membrane proteins (Pmps), though the number of genes that encode for these proteins differs between species. *C. abortus* contains 18 *pmp* genes, some of which carry internal homopolymeric repeat sequences (poly-G regions) that may have a role in controlling protein abundance within infected cells. Pmps resemble classical autotransporters, also known as the type V secretion system. These contain an N-terminal sequence, a C-terminal β-barrel domain with a phenylalanine at the end, and a central passenger domain (1, 2). These parts, respectively, are for sec-dependent translocation, suggestive of outer membrane localization of Pmps, and responsible for the protein's function being surface-localized or secreted (1, 2). All members of the Pmp protein family have multiple

repeats of GGA and FxxN motifs (7) (Figure 1). *Pmps* encompass more than 5% of the coding capacity of the chlamydial genome and are unique to *Chlamydiaceae*, which suggests they are of importance and might be related to virulence factors (3). Chlamydia has a reduced genome structure, but the group of genes that encode for Pmp proteins has been significantly expanded, further suggesting they play an important role in chlamydial function (13). There is still much that is not known about the function of Pmps. Some Pmps are located on the surface of infectious EBs and form a bridge between the pathogen and the infected cell (1, 2, 4, 5, 6). This also suggests that they may play a role in chlamydial virulence. Pmp6, Pmp20, and Pmp21 have been shown to act as adhesins in *C. pneumoniae* infection (7) and PmpA and PmpH are important in pathogenesis and infection immunity in *C. psittaci* (8).

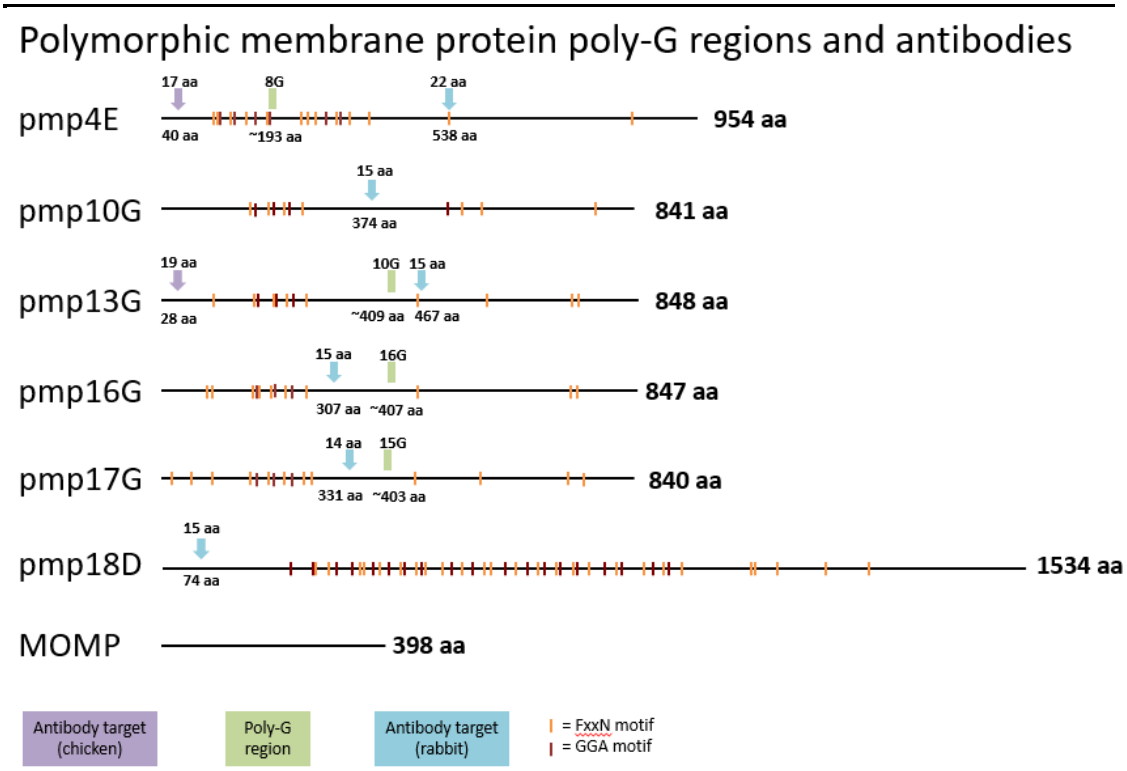

Figure 1: Polymorphic Membrane Proteins 4E, 10G, 13G, 16G, 17G, and 18D Poly-G Regions, FxxN and GGA repeating motifs, and Antibodies

Ovine Enzootic Abortion is a disease associated with infection of pregnant ewes by *Chlamydia abortus*. Using culture-independent technologies, The Rockey Laboratory isolated and whole-genome sequenced 12 *Chlamydia abortus* strains extracted from infected placenta tissues collected at farms in the Western United States. Parallel samples were obtained via culturing in the laboratory. Analysis of genomic DNA sequence reads suggests that selected genes may be differently activated or inactivated in different culture conditions. This previous research in the Rockey Laboratory found that, for chlamydia taken from embryonated egg culture, a single chlamydial gene (*pmp13G*) was translationally intact in over 80% of the sequence reads. In contrast, in all but a single sample from any other culture condition (i.e. within sheep or in mammalian cell culture) this gene was intact in only ~10% of the reads. This suggests the complete, intact *pmp13G* gene product is required under certain culture conditions and not in others.

The goal of this project was to elucidate the role of Pmps in *C. abortus* by evaluating patterns of polymorphic membrane protein expression. Our approach integrated PCR-based Sanger sequencing results with both qualitative and quantitative data collected on protein abundance via fluorescence microscopy. *C. abortus* strains used in this study include both laboratory strains and primary isolates collected from Oregon and Idaho sheep producers. Primary isolate samples were also passaged and observed. Fluorescence microscopy detected *pmp18D* abundance was consistent among the strains analyzed, while *pmp10G* and *pmp13G* detection differed between strains. In contrast to *pmp10G* and *pmp18D, pmp13G* contains a poly-G region, which may contribute to the control of this gene's expression. The length of

the poly-G region of *pmp13G* was characterized in the *C. abortus* samples mentioned above via Sanger sequencing. The number of guanines seen in this region varied from 9 to 10 between samples. It was hypothesized that this poly-G region is a control mechanism for the expression of *pmp13G* and that both the poly-G region length and the *pmp13G* detection in fluorescence microscopy vary between samples based on culture conditions and the necessity of this gene only at certain stages of growth.

Thesis Statement

The goal of this project is to gather information on polymorphic membrane proteins, a group of proteins that encompass a significant proportion of the chlamydial genome, but whose functions are poorly characterized. We will address this problem by examining polymorphic membrane protein 13G (Pmp13G) detection in *Chlamydia abortus* via both Sanger sequencing assays and cell culture-based fluorescence microscopy assays. We hypothesize that the poly-G region in *pmp13G* varies between 9 and 10 base pairs in length because it is a control mechanism for a gene that is required at only certain stages of development.

Materials and Methods

Cell Culture and Chlamydial Infection

McCoy Cell Maintenance and Chlamydial Strains Origins

McCoy cells were maintained at 37°C and 5% CO_2 in Dulbecco's Minimum Essential Medium (DMEM) supplemented with fetal bovine serum (10%) and L-glutamine (5 mM).

C. abortus samples were isolated from aborted sheep placenta samples from farms in Oregon and Idaho. Laboratory strains were also used. These strains were passaged to represent in-vivo and in-vitro culture conditions.

Table 1: Chlamydial Strains

Chlamydia abortus uncultured strains
- Idaho placental samples— IPE, IPF, IPG, IPC/IPD
- Oregon placental samples— OP1, OP2, OP3, OP4, OP5, OP6, OP7
- Eastern Oregon placental samples— EOP1, EOP5, EOP6

Chlamydia abortus strains passaged in cell culture
- Oregon placental Isolate 2 (OP2) passage 3
- Oregon placental Isolate 5 (OP5) passages 1, 3, and 4
- Idaho placental Isolate E passage 3
- LW203 clones 1, 2, 3 and 6

Chlamydia abortus egg propagated strains
- LW 203
- V2901
- MSV139.1, MSV139.2

Others
- SV139B
- LW508
- Colorado Serum Company (CSC) + CSC Vaccine
- 577

Chlamydial Infection

McCoy cells were grown on glass coverslips to 20% confluency in 24-well tissue culture plates. These cells were then inoculated with *C. abortus* strains. After centrifugation at room temperature for 1 hour at 20,000 g, inoculum was removed and replaced with DMEM with 1 µM gentamicin/amphotericin B and 1 µM vancomycin. After 55 to 60 hours, medium was aspirated and cells were fixed in 100% methanol for 10 minutes at room temperature. Cells were then washed two times with Dulbecco's Phosphate-Buffered Saline (1x DPBS).

Passaging of Chlamydial Strains

To passage strains, McCoy cells were set to 70% confluency in 6-well tissue culture plates. Cells were infected with antibiotic treated and filtered (using a 0.45 µM membrane syringe filter) 20% suspension of placental tissues as described above. After incubation, infected cells were frozen once at -80°C and pipetted vigorously after thawing at room temperature. Then, 200 uL of the cell lysate was used to infect cells and allowed to grow for 72 hours. If successful growth occurred, this was considered the next passage of the strain. After 72 hours, trays were placed in a -80°C freezer overnight. Samples were then thawed and used to infect new McCoy cells again set to 70% confluency in 6-well tissue culture plates. The process was repeated and each repeat was considered one passaging.

Fluorescence Microscopy

Methanol fixed chlamydial infected cells were washed two times with 1×DPBS. They were then labeled with antibody to the chlamydial 60 kDa heat shock protein (PMID: 1375196) to illuminate chlamydial inclusions and with chosen anti-Pmp (Table 2) antibody to show Pmp-expression. VectaShield (Vector Laboratories) DAPI was used to visualize host cell nuclei.

Antibodies against chlamydial heat shock protein 60 (HSP60) were diluted 1:1 in 2% bovine serum albumin in PBS (FA block) and antibodies for Pmp proteins were diluted 1:5000 in FA block. After 300µL of diluted primary antibody was added to the cells, they were incubated for 45 minutes at room temperature on a rocker. Primary antibody was then removed and the cells were washed twice with 1×DPBS. Secondary antibodies were diluted 1:1000 and 300µL were added to each well before incubation in the dark at room temperature for 30 minutes. Secondary antibodies were removed and cells were washed twice with 1×DPBS. Coverslips were inverted and placed over 1.5 µL 4′,6′-diamidino-2-phenylindole (DAPI) in Vectashield (Vector Laboratories). Images were collected at both low power (40×) and oil immersion (100×) magnification with a Leica fluorescence microscope and a PC-based digital camera imaging system (Qimaging Co, Surrey BC, Canada).

Table 2: Antibody targets

Target	Source	Peptide Sequence
Pmp4E	Rabbit	Cys-PKIWLDPDDTSKENAITENEPK
Pmp4E	Chicken	CLAKDKTTSSSYTLSAS
Pmp10G	Rabbit	DQTSSIKVQENVDIK
Pmp13G	Chicken	SLYSEEPDQKTLTSAHSYN
Pmp13G	Rabbit	SGSSGSITKPTTNLE
Pmp16G	Rabbit	ANTGGSTEIELNKTE
Pmp17G	Rabbit	EEKARAENLASTFN
Pmp18D	Rabbit	EKPIHAQGPKKGETD
HSP60		

PCR & Sanger Sequencing

Chlamydial DNA Extraction

DNA was extracted from placenta samples and from laboratory strains using a Qiagen DNeasy Blood and Tissue kit, with an addition of 10 mM DTT after the addition of buffer ATL in step 1. Some of these samples were also passaged in the laboratory (described above) to represent *in-vivo* vs *in-vitro* culture conditions, and then the same Qiagen DNeasy Blood and Tissue Kit procedure with the addition of DTT was followed.

Polymerase Chain Reactions and Sanger Sequencing

A primer set was designed surrounding the poly-G region in *pmp13G* (Table 3). This provided a 224-bp PCR product that allowed observations to be made regarding the length of the *pmp13G* poly-G region (9 or 10 G) in *C. abortus*.

Table 3: *pmp13G* gene based primers

Primer	**Sequence 5' → 3'**
Pmp13G Forward	GCATTGCCATTGTCATCGAC
Pmp13G Reverse	ATCCTTCACGCAAACAGAGG

PCR reactions were run with total volumes of 25 µL. Each PCR reaction had 12.5 µL Phusion High Fidelity PCR MasterMix with HF buffer (New England BioLabs), 1.25 µL each of the forward and reverse *pmp13G* primers (Table 3), approximately 50 ng DNA, and deionized water to 25 µL total reaction added. The amplification program was run at 1 minute at 98°C, followed by 30 cycles of 10 seconds at 98°C, 15 seconds at 58°C, and 30 seconds at 72°C, with a final extension of 5 minutes at 72°C. *Pmp13G* primers produced a 224-bp product with the poly-G region in the center.

The PCR product was analyzed via gel electrophoresis in a 1% agarose gel. DNA from the PCR product was then extracted using either a Qiagen gel extraction kit or a Qiagen PCR clean-up kit. These samples were prepped for sequencing with 50ng DNA, 1.2 µL forward or reverse primer (Table 3), and DI water up to 12 µL total sample, before being sent to the Oregon State University Center for Genome Research & Biocomputing (OSU CGRB) to be analyzed via Sanger Sequencing.

Sequencing data were analyzed using Geneious software. The *pmp13G* poly-G region length was determined and the quality of sequence reads assessed.

Results

Primary isolate strains of *C. abortus* and the passaged equivalents of these strains were characterized by Sanger sequencing and observed by fluorescence microscopy. The poly-G region in *pmp13G* was characterized and compared between strains and culture conditions. The abundance of Pmps 10G, 13G and 18D was observed via fluorescence microscopy.

Polymorphic Membrane Protein 10G, 13G, & 18D abundance patterns between strains

Fluorescence microscopy was used to obtain both qualitative and quantitative data (Tables 4 and 5) on the *C. abortus* strains used in this study, which include the laboratory strains and isolates mentioned above (Table 1). Fluorescence microscopy demonstrated that Pmp18D was consistently present amongst the strains analyzed. Detection of Pmp10G differed between strains, with no strains observed showing all on or all off Pmp10G expression. These strains showed a mix with some inclusions expressing the protein and others not.

Fluorescence microscopy also shows a clear variation in the detection of Pmp13G between samples. In contrast to *pmp10G* and *pmp18D*, *pmp13G* contains a poly-G region, which may contribute to the control of this gene's expression. Certain strains showed a mix in Pmp13G abundance with some inclusions showing protein detection and others not (Figures 2 and 3), some showed all inclusions expressing Pmp13G (Figure 4), and some showed no inclusions expressing the protein (Figure 5 and 6) (Tables 4 and 5). These results also suggested a variation in the abundance of Pmp13G between a sample directly from placenta (Oregon Placenta 5) and a parallel

sample that has been passaged (Oregon Placenta 5 passage 4), representing different culture conditions (Figures 4-5). However, sequencing results from both of these samples showed a poly-G length of 9.

Polymorphic Membrane Protein 13G Poly-G region varies in length between 9 and 10

The poly-G region of *pmp13G* was observed via Sanger sequencing in *C. abortus* laboratory strains, primary isolates collected from Oregon and Idaho sheep producers, and parallel passaged samples of the primary isolates. The number of guanines characterized in this region varied from 9 to 10 between samples (Tables 4 to 7). Sequencing results show a variation in the length of the poly-G region between *C. abortus* strains, with some strains containing a 9G poly-G region and others containing a 10G poly-G region. These results also suggest there is a variation in the poly-G length between a sample directly from placenta and a parallel sample that has been passaged, representing varying culture conditions. The placental isolate (Idaho Placenta E) contains a 10–G region and the parallel passaged sample (Idaho Placenta E Passage 3) contains a mixed population with both 9G and 10G regions, meaning some chlamydia in the sample contain a 9G region and some contain a 10G region (Table 6).

Table 4: Laboratory Strain Samples
Length of the poly-G region in *pmp13G* for laboratory strains of *Chlamydia abortus* and fluorescence microscopy results for select strains.

		Pmp13G	Fluorescence Microscopy Results		
Strain	**Passage**	**poly-G region**	**PMP10**	**PMP13**	**PMP18**
LW203	Egg	10	0.40	0.72	1
	Clone 2	10			
	Clone 3	10			
	Clone 6	10			
	Clone 1	10	0 < 1	0 < 1	1
V2901	Egg	10	0.77	0.93	1
MSV139.1	Egg	10			
MSV139.2		10			
SV139B		10			
LW508		10			
CSC		10			
CSC Vaccine	Vaccine	10			
577		10			

Table 5: Oregon Placenta Samples
Length of the poly-G region in *pmp13G* of *Chlamydia abortus* for placenta strains from a farm in Oregon and fluorescence microscopy results for select strains.

		Pmp13G	Fluorescence Microscopy Results		
Strain	**Passage**	**poly-G region**	**PMP10**	**PMP13**	**PMP18**
OP1	Placenta	9			
OP2	Placenta	10	ON	ON	ON
	Passage 3- lineage 1	10			
	lineage 2	10			
	lineage 3	10			
OP3	Placenta	10			
OP4	Placenta	10			
OP5	Placenta	9		1	1
	Passage 1		0 < 1	0	1
	Passage 3	9	0.32	0	1
	Passage 4	9	0 < 1	0	1
OP6	Placenta	9			
OP7	Placenta	9			

Table 6: Idaho Placenta samples
Length of the poly-G region in *pmp13G* of *Chlamydia abortus* for placenta strains from a farm in Idaho.

Strain	Passage	Pmp13G poly-G region	Fluorescence Microscopy Results		
			PMP10	PMP13	PMP18
IPE (14-0162-1)	Placenta	10			
	Passage 3-lineage 1	9/10			
	lineage 2	9/10			
	lineage 3	9/10			
IPF (14-0162-2)	Placenta	10			
IPG (14-0162-3)	Placenta	10			
IPC/IPD (14-0074)	Placenta	10			

Table 7: Eastern Oregon Placenta Samples
Length of the poly-G region in *pmp13G* of *Chlamydia abortus* for placenta strains from a farm in Eastern Oregon.

Strain	Passage	Pmp13G poly-G region	Fluorescence Microscopy Results		
			PMP10	PMP13	PMP18
EOP 1	Placenta	9/10			
EOP 5	Placenta	9			
EOP 6	Placenta	9			

Images of LW203 Clone 1- Stained for Pmp13G

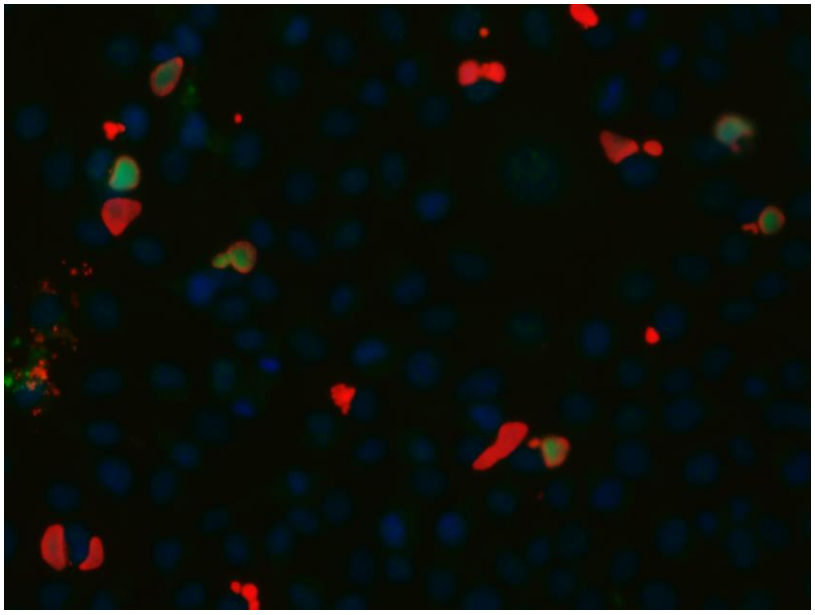

Figure 2: McCoy cells infected with *C. abortus* sample LW203 Clone 1 fixed at 55-60hpi (40×). Total DNA is stained blue with DAPI. HSP60 is labeled with red, illuminating chlamydial inclusions. Pmp13G is labeled with green.

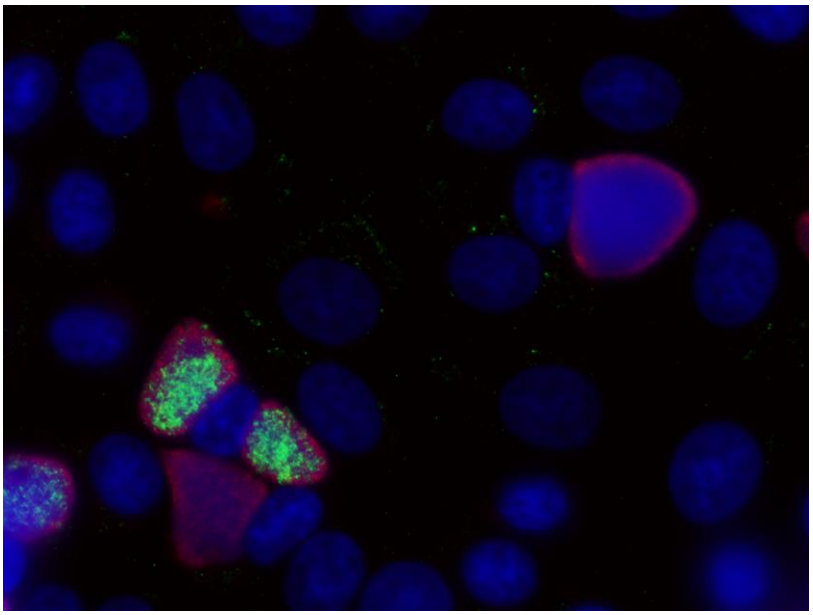

Figure 3: McCoy cells infected with *C. abortus* sample LW203 Clone 1 fixed at 55-60hpi (100×). Total DNA is stained blue with DAPI. HSP60 is labeled with red, illuminating chlamydial inclusions. Pmp13G is labeled with green.

Images of Oregon Placenta 5- Stained for Pmp13G

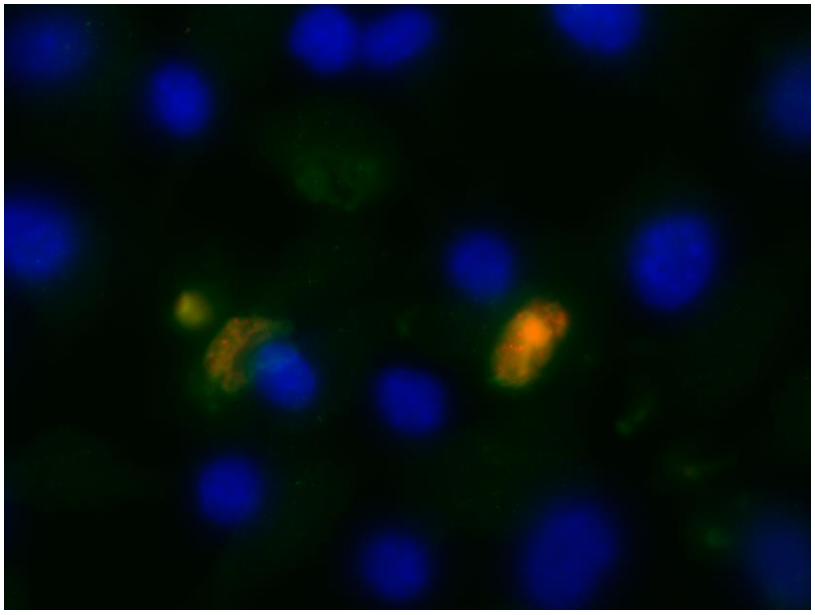

Figure 4: McCoy cells infected with *C. abortus* Oregon placenta 5 (OP5) fixed at 55-60hpi (100×). Total DNA is stained blue with DAPI. HSP60 is labeled with red, illuminating chlamydial inclusions. Pmp13G is labeled with green.

Images of Oregon Placenta 5 Passage 4- Stained for Pmp13G

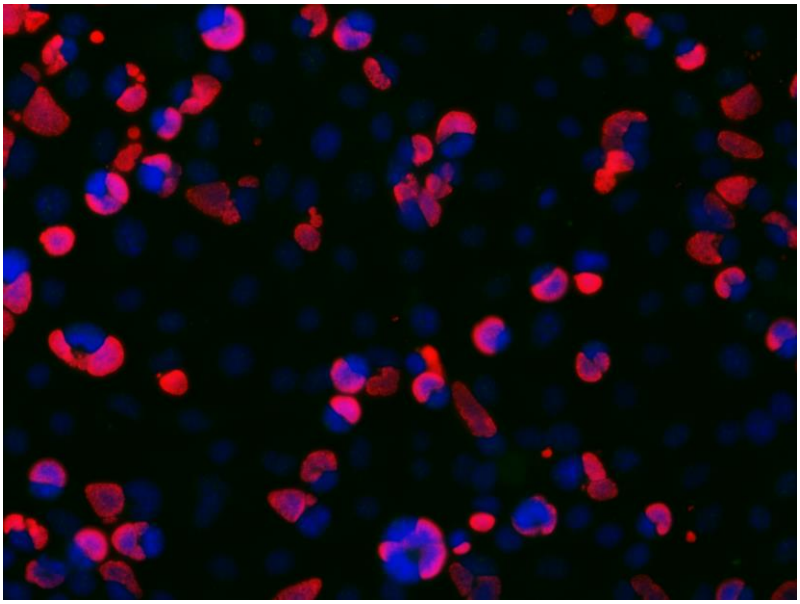

Figure 5: McCoy cells infected with *C. abortus* Oregon placenta 5 (OP5) passage 4 fixed at 55-60hpi (40×). Total DNA is stained blue with DAPI. HSP60 is labeled with red, illuminating chlamydial inclusions. Pmp13G is labeled with green.

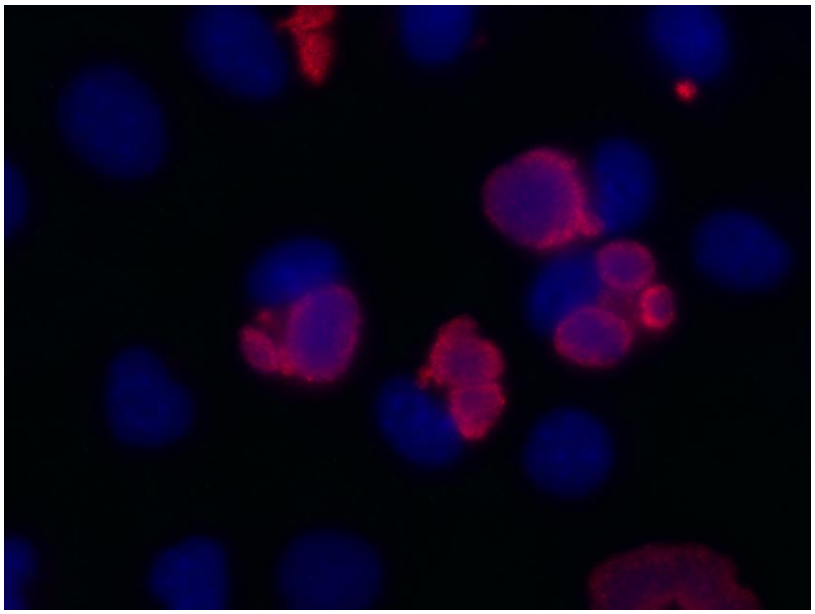

Figure 6: McCoy cells infected with *C. abortus* Oregon placenta 5 (OP5) passage 4 fixed at 55-60hpi (100×). Total DNA is stained blue with DAPI. HSP60 is labeled with red, illuminating chlamydial inclusions. Pmp13G is labeled with green.

Discussion

There is not much known about the function of polymorphic membrane proteins (Pmps), which encompass a significant portion of the Chlamydial genome and may play a role in infection and pathogenesis. The abundance of the *pmp* genes and their presence in all chlamydial species suggests they play an essential role in chlamydial biology (Becker et al., 2014). The goal of this project is to elucidate the role of Pmps in the pathogenesis of *C. abortus* by evaluating patterns of Pmp abundance and of the length of a poly-G region within *pmp13G*. A variation in *pmp13G* expression was observed previously, which suggested that expression occurred only under certain culture conditions. This led to the hypothesis that this variation in Pmp13G is due to changes in culture conditions and may be linked to the necessity of the *pmp13G* gene during certain stages of growth.

Understanding how culture conditions affect Pmp abundance may elucidate their role in chlamydia. Pmp18D has been shown to be consistently present in all *C. abortus* strains observed, while the presence of Pmp10G and Pmp13G varies. Different members of the Pmp family may have different roles within the chlamydial developmental cycle. Some (Pmp18D) may be necessary throughout the cycle, while the heterogeneity of expression of others (Pmp13G) may aid in antigenic variation (Wheelhouse, et al. 2012). *Pmp13G* also contains a poly-G region that varies between 9 and 10 Gs between strains and possibly between culture conditions, so it is hypothesized this region may act as a genetic switch for the expression of this protein.

Variation in Pmp abundance and the *pmp13G* poly-G region length is seen between culture conditions, but this does not occur in every case. Oregon placenta 5

presented Pmp13G (Figure 4), but no longer presented the protein after passaging (Figures 5 and 6), suggesting the expression was turned off after a change in culture conditions. However, the same pattern did not occur after the passaging of other placental samples. Similarly, Idaho placenta E contained a 10G *pmp13G* poly-G region, which switched to a mixed population of 9G and 10G poly-G regions after passaging, but this pattern was not observed in all passaged samples (Table 6).

Chlamydia abortus is a zoonotic pathogen that causes abortions in ewes and has a large impact on the health of these animals and the economic productivity of the farms they live on. *C. abortus* contains 18 *pmp* genes that may play a role in the pathogenicity of this organism. Additional research is being planned to better predict polymorphic membrane protein abundance through sequencing and manipulation of cell culture conditions. Further data must be collected on Pmp 10G, 13G, and 18D in *C. abortus* strains from placental isolates, cell-cultured strains, and egg-cultured strains, as well as on additional polymorphic membrane proteins. The poly-G region may play a role in controlling the expression of *pmp13G*, but *pmp10G* does not contain a poly-G region so determination of potential genetic switches for *pmp10G* expression is of value. These findings may lead to a more complete understanding of the pathogenicity of *C. abortus* and other members of the chlamydiae. These proteins may play a role in maintaining niche-specific pathogenesis-related functions and may provide antigenic diversity when chlamydiae face the adaptive immune response of the host (Tan et al., 2010). Further knowledge on Pmps and their antigenic diversity may lead to vaccines that are more effective than current options and thus lead to a decrease in Ovine Enzootic Abortion through better preventative measures.

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