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**Molecular characterisation of twelve *Chlamydomonas reinhardtii* polymorphic  
membrane protein genes**

Ross Harley <sup>1\*</sup>, Alan Herring <sup>1</sup>, Kathy Egan <sup>1</sup>, Pam Howard <sup>1</sup>, Tim Gruffydd-Jones <sup>1</sup>,  
Yoshinao Azuma <sup>2</sup>, Mutsumori Shirai <sup>2</sup>, & Chris Helps <sup>1</sup>

<sup>1</sup>School of Clinical Veterinary Science, University of Bristol, Langford House, Langford,  
Bristol BS40 5DU, United Kingdom.

<sup>2</sup>Department of Microbiology, Yamaguchi University School of Medicine, 1-1-1,  
Minamikogushi Ube, Yamaguchi 755-8505, Japan

\*Corresponding author:

University of Bristol, School of Clinical Veterinary Science, Division of Pathology,  
Infection and Immunity, Langford House, Langford, Bristol BS40 5DU, United Kingdom

Tel. +44 (0) 117 928 9280

Fax +44 (0) 117 928 9505

Email: ross.harley@bristol.ac.uk

1 **Abstract**

2 A group of genes thought to encode members of the unique chlamydial polymorphic  
3 membrane protein (pmp) family were recently described in the *Chlamydophila felis*  
4 genome. This study aimed to commence characterisation of a subset of 12 of these  
5 putative *pmp* genes by developing and using gene-specific real-time (Q)PCR assays to  
6 confirm their presence in a wide range of *C. felis* field isolates and laboratory strains, and  
7 to look for *pmp* mRNA expression during *in vitro* infection. Sequencing of 534-698 base  
8 pair regions of *pmp* genes 7, 9-11, 13-20 for two laboratory strains of *C. felis* and  
9 alignment with the published Fe/C-56 sequence found only a single nucleotide  
10 polymorphism present in *pmp9*. Following the development of gene-specific (Q)PCR  
11 assays, analysis of genomic DNA extracted from 40 *C. felis* field isolates and 4  
12 laboratory strains found that all 12 *pmp* genes were represented in all cases. Reverse  
13 transcription (RT)-QPCR analysis of RNA extracted from cell cultures at 24 and 48 hrs  
14 post-inoculation with one of 5 different strains of *C. felis* detected transcripts for all 12  
15 *pmp* genes at both time points. Analysis of the relative levels of *pmp* gene transcription  
16 suggested that down-regulation of the expression of multiple *C. felis pmp* genes occurs  
17 between 24 and 48 hrs post inoculation. This study provides the first evidence that 12 of  
18 the putative *pmp C. felis* genes are transcribed during *in vitro* infection, and shows that  
19 these genes are present in a large range of *C. felis* field isolates and multiple passage  
20 laboratory grown strains.

## 1 **Introduction**

2 Sequencing studies have shown that genes that encode for a family of surface proteins  
3 known as polymorphic membrane proteins (pmp) are a prominent feature of all  
4 chlamydial genomes (Stephens et al., 1998; Kalman et al., 1999; Read et al., 2000; Shirai  
5 et al., 2000), (Read et al., 2003), (Azuma et al., 2006). *Pmp* genes have high levels of  
6 intra- and inter-species heterogeneity in their amino acid sequences and size, but they can  
7 be identified by the presence of conserved amino acid motifs, GGA(I, L, V) and FXXN,  
8 repeated within the N-terminal region (Stephens et al., 1998) and a C-terminal domain  
9 with predicted structural similarities to the translocator domain of the autotransporter  
10 family of proteins (Henderson and Lam, 2001; Thomson et al., 2005). The number of  
11 *pmp* genes identified in different chlamydial species ranges from 9-21 (Stephens et al.,  
12 1998; Kalman et al., 1999; Read et al., 2000; Shirai et al., 2000; Read et al., 2003; Azuma  
13 et al., 2006). The large number of *pmp* genes present within chlamydial genomes  
14 suggests that they are important to chlamydial biology. The function of pmps is poorly  
15 understood, however they have been implicated in various aspects of chlamydial  
16 infection and disease, such as invasion, tissue tropism, disease pathogenesis, and  
17 induction of host immune responses (Stothard et al., 2003; Wehrl et al., 2004; Gomes et  
18 al., 2006).

19

20 The recent identification of 39 putative *pmp*/outer membrane protein (*omp*) genes, 20 of  
21 which were identified as putative *pmp* genes, in the genome sequence from a single strain  
22 of *C. felis* by Azuma et al. (2006) makes possible the study of *pmp* genes within the *C.*  
23 *felis* population and investigation of their biological properties. The purpose of this study

1 was to undertake the first steps in characterising a group of 12 of the *C. felis pmp* genes.  
2 First we performed partial sequence analysis of the *pmp* genes from two *C. felis* strains to  
3 identify conserved sequence regions for the design and development of *pmp*-specific real-  
4 time PCR assays. These assays were then used to determine the prevalence of the 12  
5 target genes in a wide range of *C. felis* isolates and strains. *In vitro* infection studies were  
6 then performed to confirm the transcription of each of these *pmp* genes by multiple  
7 different strains of *C. felis*.

8

## 9 **Materials and Methods**

### 10 ***C. felis* isolates and strains**

11 Purified DNA samples from 40 *C. felis* field isolates were obtained from diagnostic  
12 samples processed by Langford Veterinary Diagnostics, University of Bristol, UK, and  
13 identified positive for *C. felis* using a multiplex real-time PCR assay as previously  
14 described (Helps et al., 2003). DNA used for sequence analysis of *pmp* genes was  
15 extracted from stocks of *C. felis* strains K2487 (see below) and egg-grown Cello held by  
16 the School of Clinical Veterinary Science, University of Bristol. Five *C. felis* strains were  
17 used during the infection studies: Strain 905 was obtained by culture of Purevax RCPCh  
18 vaccine (Merial Animal Health Ltd, Harlow, Essex, UK). Strains K2490, 1497V, and  
19 K2487 were obtained by culture of diagnostic samples submitted to Langford Veterinary  
20 Diagnostics. Strain K2487 was subsequently cultivated in embryonated hen eggs by the  
21 Moredun Research Institute (Pentlands Science Park, Penicuik, Scotland) prior to use in  
22 the infection studies. The *C. felis* Cello strain was a kind gift from Ian Clarke (University  
23 of Southampton, United Kingdom). This was used for culture as the *C. felis* egg-grown

1 Cello stock used for DNA analysis (above) was found to be nonviable following  
2 prolonged storage at -20°C.

3

#### 4 **Culture of *C. felis* strains**

5 Mouse McCoy cells (mouse fibroblasts) were maintained and grown in MEM with  
6 glutamax (Gibco, Paisley, Scotland), gentamicin (50 µg/ml), streptomycin (50 µg/ml),  
7 and 10% foetal calf serum. *C. felis* strains were cultured in McCoy cell monolayers  
8 prepared on glass coverslips (Wills et al., 1984). The cell monolayers were inoculated  
9 with *C. felis*, centrifuged at 2000 x g for 30 mins and incubated for up to 48 hours in  
10 medium (as above) with 5% foetal calf serum. Infection was monitored by fluorescence  
11 microscopy (Leitz Diavert, Wetzlar, Germany) using x 20 and x 40 objective lenses after  
12 staining methanol-fixed monolayers with a 1:1000 dilution of convalescent serum from  
13 an infected cat followed by FITC-conjugated Goat-anti-cat IgG antibody diluted 1:70 in  
14 PBS (Sigma, Poole, Dorset, UK) (Wills et al., 1984).

15

#### 16 **Nucleic acid extraction, RNA analysis, and reverse transcription**

17 *C. felis* genomic DNA was extracted from infected McCoy cells (24 and 48 hrs post  
18 infection), culture supernatants, reconstituted lyophilised vaccine, and egg-grown  
19 chlamydia material using the Nucleospin Blood extraction kit (ABgene, Epsom, Surrey,  
20 UK). A total of 100µl of sample was mixed with 100µl PBS, then 200µl of buffer B3  
21 and 25µl proteinase K was added and incubated at 70°C for 10-15 mins. Subsequent steps  
22 were as given in the manufacturer's instructions.

23

1 Total RNA was extracted from *C. felis* infected and non-infected McCoy cells using a 2  
2 column, triple DNase procedure. Samples were first processed using the Nucleospin  
3 RNA II kit (ABgene) following the manufacturer's instructions except during the on-  
4 column DNase step when 5 units RQ1 RNase free DNAase in 50µl 1x reaction buffer  
5 (Promega, Madison, Wisconsin) were used in place of the manufacturer's DNase. RNA  
6 was eluted in 80µl RNase free water. A total of 10 units RQ1 RNase free DNAase and  
7 10µl 10x reaction buffer was added to each sample prior to incubation at room  
8 temperature for 15-20 minutes. RNA was re-extracted using a second Nucleospin RNA II  
9 kit as above and the RNA was eluted in 50µl RNase free water. RNA quality, total RNA  
10 concentration and total chlamydial 16S and 23S ribosomal RNA (rRNA) concentrations  
11 were evaluated using the Experion Automated RNA Electrophoresis System (BioRad).  
12 Reverse transcription of 0.3 – 1.0 µg total RNA was performed using ImProm II Reverse  
13 Transcription System (Promega) and random hexamers in a reaction volume of 20µl. The  
14 cDNA was diluted to a final working volume of 50µl.

15

### 16 **Conventional PCR and sequencing**

17 *C. felis pmp* sequence data was obtained from strain Fe/C-56 genome sequence (Azuma  
18 et al., 2006) (GenBank Accession no. AP006861). *Pmp*-specific primers (Table 1) were  
19 designed using Primer 3 (Rozen and Skaletsky, 2000). Secondary structure predictions  
20 were made using M-fold (Zuker, 2003). PCR was performed using a 'PTC-200 DNA  
21 Engine' (MJ Research, Waltham, Massachusetts). The PCR mixture comprised 12.5µl  
22 2x HotStartaq mastermix (Qiagen, Crawley, UK), 0.75µl 50mM MgCl<sub>2</sub>, 200 nM final  
23 concentration each of the sense and antisense *pmp*-specific primers, 2µl genomic (g)DNA

1 from *C. felis* strain Cello or K2487, and water made up to a final volume of 25 $\mu$ l. The  
2 PCR conditions were 95°C for 15 minutes, then 35 cycles of 20s at 95°C, 20s at 60°C, and  
3 60s at 72°C, with a final extension for 15 minutes at 72°C. PCR products were visualised  
4 on a 1% agarose gel stained with ethidium bromide. The bands were excised and the  
5 DNA recovered using the NucleoSpin Extraction Kit II (ABgene). Sequencing reactions  
6 were performed by The Sequencing Service (School of Life Sciences, University of  
7 Dundee, Scotland). Sequencing (sense and antisense) of Cello *pmp9* PCR products was  
8 performed on products from three separate PCRs. All other *pmp* PCR products were  
9 sequenced (sense and antisense) from a single PCR. Sequences were submitted to the  
10 GenBank database (Accessions DQ652568 - DQ652591).

11

### 12 **Real-time RT-PCR assays for *C. felis pmp* genes and mouse glyceraldehyde-3-** 13 **phosphate dehydrogenase (G3PDH)**

14 *Pmp*-specific primer pairs and Taqman probes (Table 2) were designed as described  
15 above. The annealing temperature of the fluorescently quenched probe (FAM  
16 reporter/TAMRA quencher) was designed to be 10°C higher than that of the primer pairs.  
17 Real-time PCRs were performed using an iCycler IQ (Bio-Rad Laboratories Ltd, Hemel  
18 Hempstead, United Kingdom). The PCR mixture comprised 12.5 $\mu$ l 2x HotStartaq  
19 mastermix (Qiagen), 1.5  $\mu$ l 50mM MgCl<sub>2</sub>, 200 nM final concentration each of the sense  
20 and antisense primers, 100 nM final concentration of Taqman probe, 2  $\mu$ l template, and  
21 water to a final volume of 25  $\mu$ l. The real-time PCR conditions for each assay were 95°C  
22 for 15 minutes, then 45 cycles of 10s at 95°C and 15s at 60°C with fluorescence detected  
23 at 530-nm following excitation at 490-nm. In each run of each *pmp* assay, controls

1 included positive (*C. felis* Cello gDNA) and negative (water in place of template)  
2 controls. In gDNA assays, DNA extracted from an ocular swab taken from a cat, that was  
3 submitted to Langford Veterinary Diagnostics and found to be negative for *C. felis* by  
4 PCR (see above), was included as a negative control. In transcription assays, a sample  
5 lacking RT was included for each corresponding cDNA sample to detect any *C. felis*  
6 gDNA that had not been completely eliminated by the on-column digestion.

7

8 Real-time PCR for mouse G3PDH was performed as a positive control for DNA  
9 extraction and RT in each sample using an assay originally designed for the detection of  
10 feline G3PDH (Nguyen Van et al., 2006). The primers and probes (Table 2) are 100%  
11 complementary for the mouse G3PDH sequence (GenBank Accession BC083080) and a  
12 single PCR product of the expected size was generated when McCoy cell cDNA was  
13 used as template (data not shown). Except for the annealing temperature, which was  
14 64°C, the PCR mixture and reaction conditions were as described above. Control samples  
15 comprised no-RT samples and positive and negative controls.

16

### 17 **Calculation of *pmp* gene expression levels**

18 The efficiency of each *pmp*-specific real-time PCR assay was calculated from 10-fold  
19 serial dilutions of a sample of *C. felis* strain Cello gDNA using the iCycler IQ optical  
20 system software v3.0 (Biorad). The amount of each *pmp* target template present in each  
21 sample was calculated relative to a standard sample of *C. felis* Cello gDNA included in  
22 all batches of the assay. The standard was assigned an arbitrary value of 1 gDNA  
23 equivalent (gEQ) and was assumed to contain an equal number of copies of each of the

1 *pmp* genes. The difference in  $C_t$  value ( $\Delta C_t$ ) between the standard and sample was  
2 calculated ( $\Delta C_t = \text{standard } C_t - \text{sample } C_t$ ) for each *pmp*. The amount of specific *pmp*  
3 template in the sample relative to the copy number present in the standard was then  
4 calculated using the efficiency of the relevant real-time PCR assay (E) raised to the  
5 power of  $\Delta C_t$  and expressed as gEQ. Finally, to permit comparison between samples, the  
6 results were normalized relative to the amount of total chlamydial rRNA within each  
7 sample RT reaction calculated from the Experion profile of the RNA.

8

### 9 **Statistical analysis**

10 Statistical analysis was performed using SPSS 12.0 for Windows (SPSS Inc, Chicago,  
11 Illinois). The Shapiro-Wilk normality test was used to analyse the distribution of data  
12 sets. Paired *t*-tests were used to compare the levels of expression of each *pmp* gene at 24  
13 and 48 hrs post inoculation (PI). Correlations between strains in the change in *pmp*  
14 expression levels between 24 and 48 hrs PI were calculated using Pearson's method.  
15 Results were considered significant where  $P < 0.05$ .

16

### 17 **Results**

#### 18 **Sequencing of *pmp* genes**

19 Based upon the *C. felis* strain Fe/C-56 genome sequence (Azuma et al., 2006), twelve  
20 putative *pmp* genes were selected for analysis: *pmp7*, *9-11*, *13-20* (Genbank Accession  
21 AP006861, locus tags CF0725, CF0727, CF0728, CF0729, CF0731, CF0732, CF0733,  
22 CF0735, CF0736, CF0737, CF0801, CF0802 respectively). All of these genes are

1 predicted to encode proteins containing conserved N-terminal GGA(I, L, V) and FXXN  
2 motifs (Table 3).

3

4 As studies of other chlamydial organisms have shown inter-strain sequence  
5 polymorphisms exist in some *pmp* genes, regions of each of the 12 selected *C. felis pmp*  
6 genes that encompassed the annealing sites for the *C. felis pmp*-specific real-time PCR  
7 primers and probes were analysed for evidence of inter-strain sequence variation. Using  
8 primers pairs specific for all 12 selected *pmp* genes (Table 1), single PCR products  
9 ranging from 534-698 bp were generated using DNA extracted from *C. felis* strains Cello  
10 or K2487 as template. The PCR products were sequenced and aligned with strain Fe/C-  
11 56 *pmp* nucleotide sequences. Poly-C tracts present in *pmp15* and *pmp17* prevented  
12 complete sequencing of both strands. Except for *pmp9*, the region sequenced in each of  
13 the *pmp* genes was identical in all three strains of *C. felis*. For *pmp9* in the *C. felis* Cello  
14 strain, a non-synonymous single nucleotide polymorphism (SNP) was found that mapped  
15 to position 847211 of the *C.felis* Fe/C-56 genome (Genbank AP006861). A C > T  
16 transition was found, which is predicted to code for a change from threonine (T) to  
17 isoleucine (I).

18

### 19 **Development of pmp-specific real time PCR assays and detection of pmp genes in *C.*** 20 ***felis* isolates and strains**

21 Each of the *pmp*-specific real time PCR assays produced a single amplicon of the  
22 expected size using DNA extracted from *C. felis* Cello strain as template (FIG 1).

1 Analysis of serial dilution curves showed the reaction efficiencies ranged from 91% to  
2 98%.

3  
4 To look for variation in the presence of *pmp* genes between different field isolates and  
5 strains of *C. felis*, DNA samples extracted from 40 field isolates from different cats  
6 (including strain 1497V) and *C. felis* strains 905 and K2490 were screened using the *pmp*  
7 real-time PCR assays. All of the 12 *pmp* genes were detected in all of the *C. felis* isolates  
8 and strains examined.

9

#### 10 **Detection and analysis of *pmp* mRNA transcription**

11 To analyse *pmp* mRNA transcription during infection, total RNA was extracted from  
12 McCoy cells infected with *C. felis* strain Cello, 905, K2487, K2490 or 1497V and  
13 incubated for 24h or 48h (FIG 2). Following reverse transcription, real time PCR was  
14 performed using the 12 *pmp*-specific assays. Transcripts for all 12 *pmp* genes were  
15 detected at both 24h and 48h PI with each of the five strains of *C. felis*. The sample  $C_t$   
16 values ranged from 21.6 to 29.8. Control samples amplified without reverse-transcription  
17 were either negative or indicated that any residual gDNA within each of the reverse-  
18 transcribed sample contributed less than 0.2% to the *pmp*  $C_t$  value. cDNA from samples  
19 of uninfected McCoy cells were negative for all of the *pmp* genes and were positive for  
20 G3PDH.

21

22 Figure 3 shows the levels of *pmp* gene expression relative to the total chlamydial rRNA  
23 concentration in the sample at 24 and 48 hrs PI for each of the five strains of *C. felis*. In

1 each strain, *Pmp* 14, 17 and 20 tended to be the most abundantly expressed of the 12 *pmp*  
2 genes examined, whereas *pmp9* always had the lowest level of expression within each  
3 sample (FIG 3). A significant decrease in the expression of *pmp9*, 11, 14, 16, 17 & 19  
4 relative to input chlamydial rRNA was observed between 24 and 48 hrs PI (FIG 3). To  
5 look for evidence that different strains of *C. felis* display similar changes in *pmp*  
6 expression, correlations were performed using the changes in *pmp* expression between 24  
7 and 48 hrs PI with each strain (data not shown). The changes in *pmp* expression observed  
8 in strains 905 and 1497V, and strains 905 and Cello, were not found to be significantly  
9 correlated. However, in each of the remaining 8 different possible strain pairings, the  
10 changes in *pmp* expression were significantly correlated.

11

## 12 **Discussion**

13 The detection of each of the *pmp* genes analysed (*pmp7*, 9-11, 13-20) in all of a wide  
14 range of field isolates and laboratory adapted strains of *C. felis*, including a strain (*C. felis*  
15 905) used in a current feline chlamydia vaccine, suggests that there is no gross genomic  
16 inter-strain variation in the occurrence of these *pmp* genes. This is consistent with studies  
17 indicating that the genomes of some chlamydial species are highly conserved between  
18 strains (Read et al., 2000; Shirai et al., 2000; Carlson et al., 2005).

19

20 Although only portions (~600-700 bases) of each of the *pmp* genes from two strains of *C.*  
21 *felis* (Cello and K2487) were sequenced and aligned with the published strain Fe/C-56  
22 genome sequence data (Azuma et al., 2006), the high degree of identity between the *pmp*  
23 nucleotide sequences is striking and only one, non-synonymous, SNP in *pmp9* was found.

1 In view of the fact that the *pmp* sequences analysed within this study came from *C. felis*  
2 isolates originating from the UK (K2487), USA (Cello) (Cello, 1971) and Japan (Fe/C-  
3 56) (Azuma et al., 2006), the results suggest that at least for the regions analysed *C. felis*  
4 *pmp* genes are highly conserved between strains from different geographic locations. This  
5 observation is consistent with studies of other *C. felis* genes, and would lend further  
6 support the suggestion that the high level of genetic identity between *C. felis* strains  
7 implies that they are likely to be clonal in origin (Sayada et al., 1994).

8

9 In contrast to the present findings in *C. felis*, studies of whole gene sequences in *C.*  
10 *trachomatis* strains have found that SNPs are common in some *pmp* genes and that the  
11 genetic variants correlate with serovar tissue tropisms (Stothard et al., 2003; Carlson et  
12 al., 2005; Gomes et al., 2006). These latter findings imply that the *pmps* may constitute  
13 virulence factors that determine the observed tissue tropisms. Unlike *C. trachomatis*, *C.*  
14 *felis* is considered to comprise a single serovar (Andersen, 1991). Moreover, from a  
15 clinical perspective, *C. felis* is principally considered to be an ocular pathogen (Cello,  
16 1971; Wills et al., 1984) and *C. felis* strain-specific tissue tropisms or pathologies are not  
17 recognised. It is possible that the uniformity of the clinical disease associated with *C.*  
18 *felis* infection may be due, at least in part, to the apparent lack of genetic diversity of *pmp*  
19 genes between different strains of *C. felis*. Moreover, evidence that *C. felis* can cause  
20 conjunctivitis in man (Hartley et al., 2001) suggests that this tissue tropism is preserved  
21 in zoonotic infections, as is the case with *C. abortus* which can cause abortion in sheep  
22 and women, and *C. psittaci* which can cause respiratory disease in birds and humans  
23 (Longbottom and Coulter, 2003).

1  
2 Evidence of transcription of the putative *C. felis pmp* genes provides the first step in  
3 establishing if these genes encode for functional proteins. RT-(Q)PCR analysis of *C. felis*  
4 infected cell cultures detected transcripts for all 12 of the *pmp* genes analysed at 24 and  
5 48 hrs PI using 5 different strains of *C. felis*. This is consistent with studies of other  
6 chlamydial species that have shown all of the species-specific *pmp* genes are transcribed  
7 (Grimwood et al., 2001). The use of RT-(Q)PCR assays permitted analysis of the level of  
8 expression of each of the *pmp* genes. The level of expression of each *pmp* gene was  
9 normalised relative to the sample chlamydial rRNA concentration. Chlamydial ribosomal  
10 genes have been shown to be constitutively expressed during the developmental cycle  
11 (Engel and Ganem, 1987; Nicholson et al., 2003) and chlamydial rRNA expression is  
12 often used as a surrogate for the number of chlamydial organisms to permit  
13 standardisation between samples (Wilson et al., 2004; Gomes et al., 2005). Although  
14 detailed analysis of the developmental cycle of the different strains of *C. felis* was not  
15 performed in this study, it is likely that at 24 and 48 hrs PI all of the strains would be in  
16 the late stages of the development cycle (Shaw et al., 2000). The results suggest that  
17 down-regulation of expression of multiple *pmp* genes occurs between 24 and 48hr PI and  
18 that this trend is a feature common to different strains of *C. felis*. Nonetheless, the  
19 changes in the level of expression of the *pmp* genes were, in general, relatively small, and  
20 only *pmp11* showed a greater than four fold change in expression in all of the strains.  
21 Microarray analysis of gene expression during *C. trachomatis* infection found that *pmp*  
22 genes were maximally expressed by 18-24 hrs PI and thereafter the level of expression  
23 remained relatively stable to 36hrs PI (Nicholson et al., 2003). The results of this study

1 suggest that some *pmp* genes may become down-regulated during the very late stages of  
2 the development cycle at 48hr PI. This might be due to completion of membrane  
3 assembly and reduced functional requirement for *pmp* expression during conversion of  
4 reticulate bodies to elementary bodies at the end of the life cycle. Further studies are  
5 required to corroborate these findings and determine the functional significance, if any, of  
6 these temporal changes in *pmp* gene expression.

7

8 In conclusion, we have developed gene specific real-time (Q)PCR assays for 12 of the  
9 putative *pmp* genes recently identified in the Japanese *C. felis* Fe/C-56 strain genome  
10 (Azuma et al., 2006). Using these assays, we have confirmed the presence of each these  
11 *pmp* genes in multiple laboratory adapted strains of *C. felis* and a wide range of *C. felis*  
12 clinical isolates from cats within the UK, indicating that these genes are highly conserved  
13 within the *C. felis* genome. Furthermore, we have shown that all 12 *pmp* genes are  
14 transcribed during *in vitro* infection with different strains of *C. felis*. Future studies will  
15 aim to extend these findings to the remaining *pmp* genes and look for evidence of  
16 translation of *C. felis pmp* genes prior to investigating their biological function and how  
17 they interact with the feline immune system.

18

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23 Clarke at Southampton University for providing the Cello strain.

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- 9  
10

1

## FIGURE LEGENDS

2

3 **FIG. 1. Each *pmp*-specific real-time PCR assay generates a single product band.**

4 Real-time PCR assays were run using the primer pairs shown in Table 2 with genomic  
5 DNA from *C. felis* Cello strain as template. 10 $\mu$ l of PCR product was run out on a 2%  
6 agarose gel containing 0.1 $\mu$ g/ml ethidium bromide. Single product bands of the expected  
7 size for each *pmp*-specific assay (Table 2) were visualised. M = molecular weight  
8 markers.

9

10 **FIG. 2 (A & B) Electropherograms of total RNA extracted from (A) uninfected and**

11 **(B) *C. felis* infected McCoy cells.** Panel A shows a representative electropherogram of  
12 total RNA extracted from uninfected McCoy cells. 5S, 18S and 28S ribosomal (r)RNA  
13 peaks are apparent. LAM = lower alignment marker. Panel B shows a representative  
14 electropherogram of total RNA extracted from McCoy cells 24 hrs PI with *C. felis* strain  
15 905. Peaks representing chlamydial 16S and 23S rRNA are present in addition to the  
16 mammalian 5S, 18S and 28S rRNA peaks.

17

18 **FIG. 3 *Pmp* transcription levels at 24 and 48 hours post inoculation of cell cultures**

19 The relative transcription levels for *pmps* 7, 9-11, 13-20 were determined at 24 and 48  
20 hours PI of McCoy cells with one of 5 different strains of *C felis*: K2490 (filled circle),  
21 1497V (filled square), 905 (open triangle), Cello (cross), K2487 (open diamond).

22 \* P < 0.05; \*\*P $\leq$  0.002 (paired *t*-test 24 hr vs 48 hr).

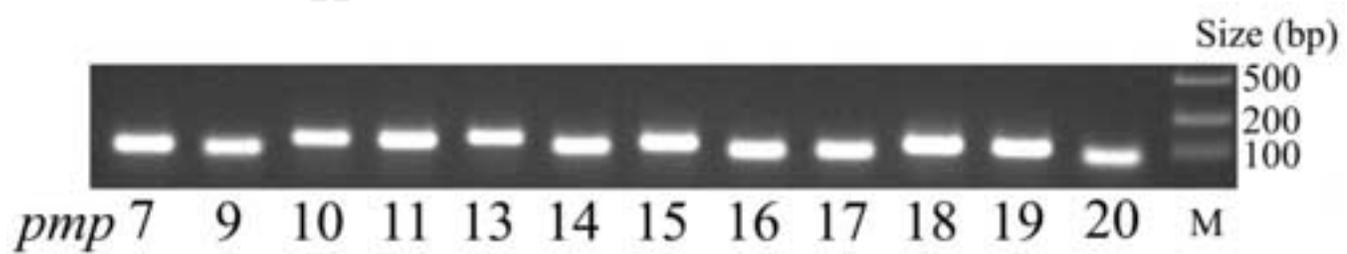


FIG 1

FIG. 2

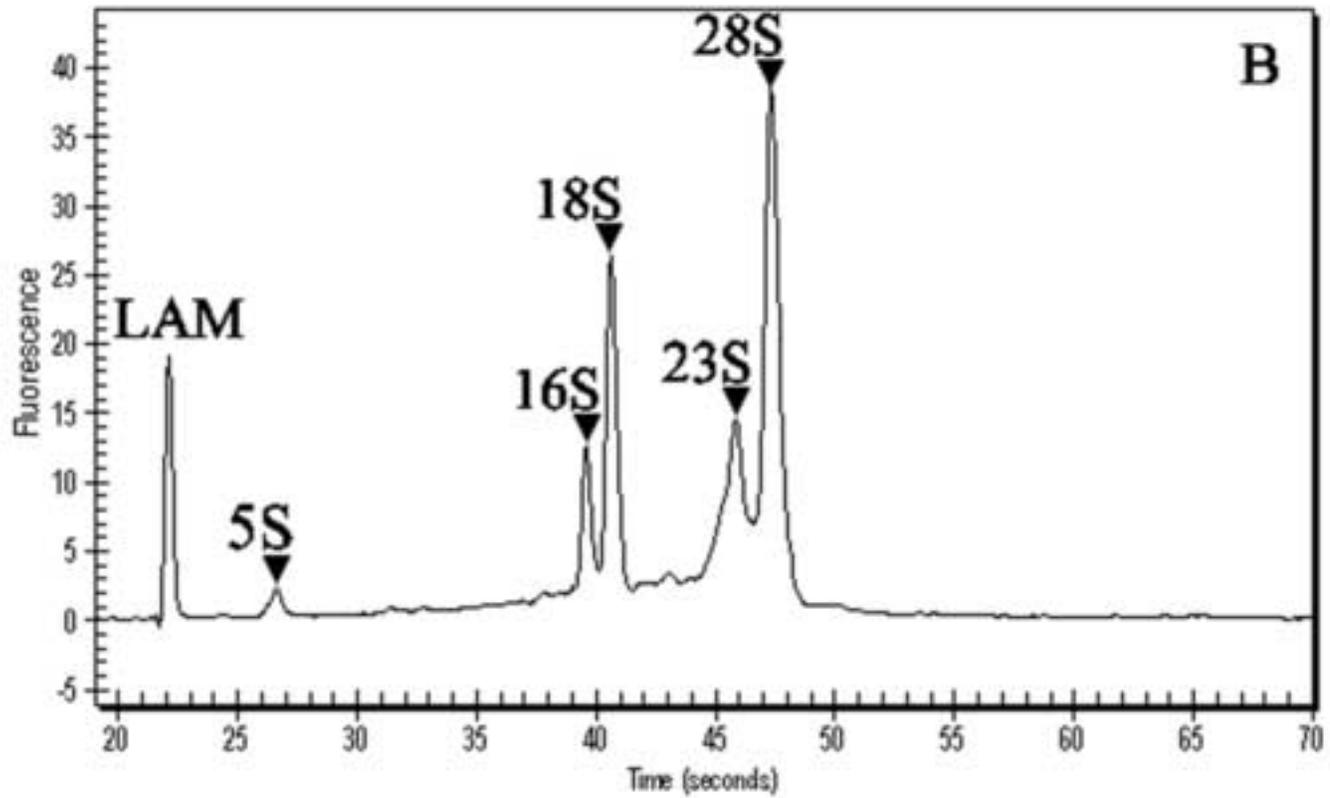
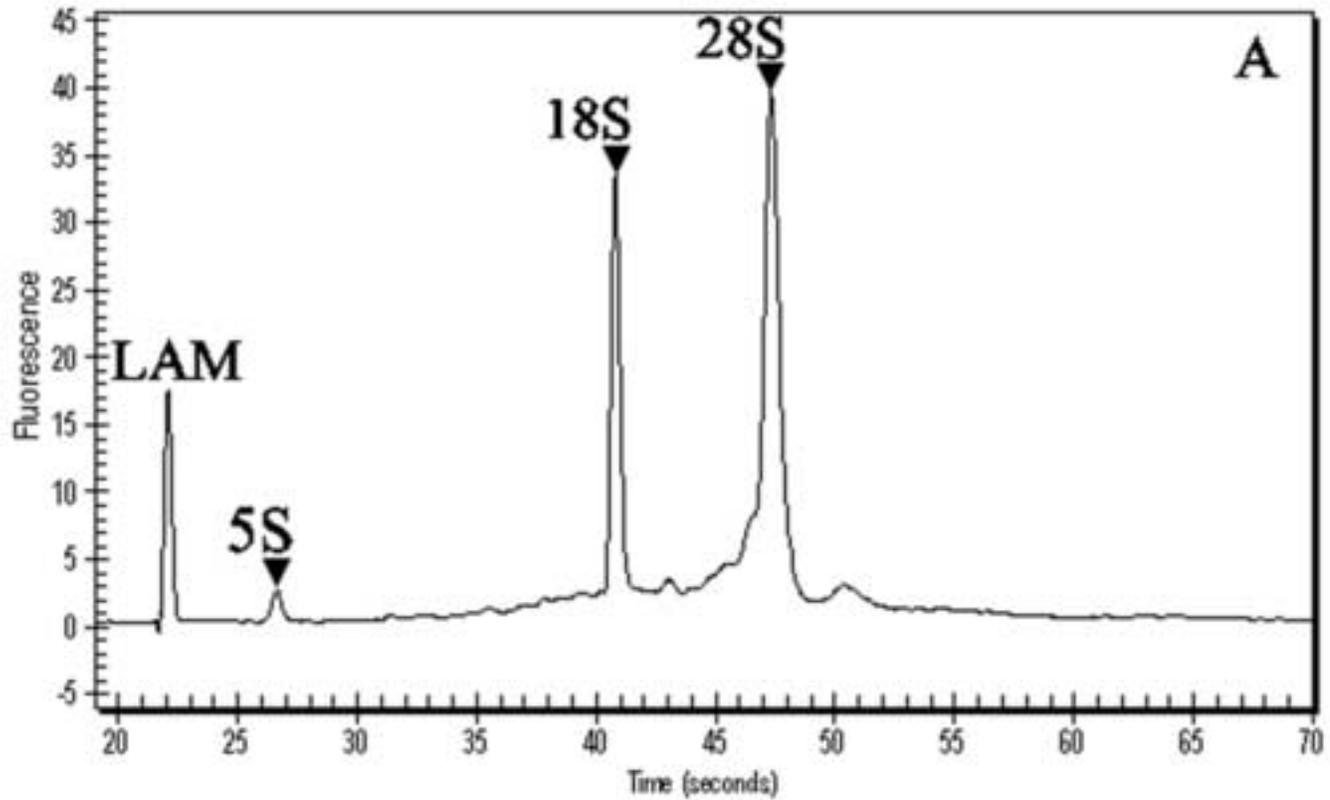
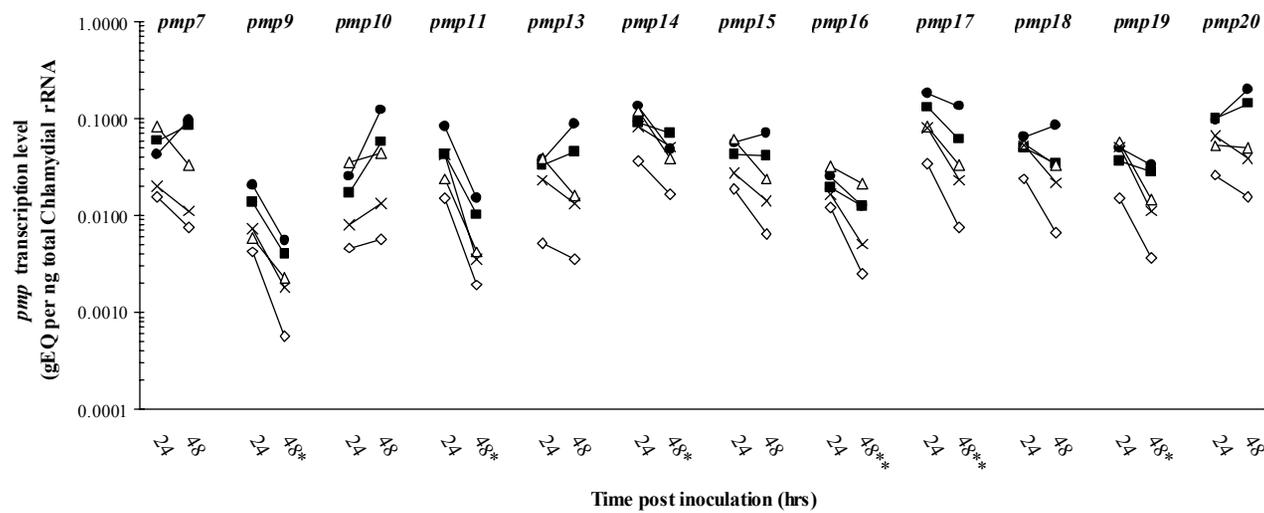


FIG 3



ACCEPTED

**Table 1: Primer pairs used for conventional PCR and sequence analysis of *pmp* genes**

Gene	Sense primer (5' to 3')	Antisense primer (5' to 3')	Amplicon size (base pairs)
<i>Pmp7</i>	CGCACAGACACCAACAGAAG	AAGCACGAACGACTAAAGCA	698
<i>Pmp9</i>	GCGATTCATGTAGCAGCAAA	GTCCAGCTTCCTTGATACCC	632
<i>Pmp10</i>	CACCGGCAGGAAATAAATCA	TATAGCGTTGGAGCCATCTT	695
<i>Pmp11</i>	CCTTTGATAAACTGGTCTTTAGGG	TTGCGAGGATTTGCGTAGTT	615
<i>Pmp13</i>	GCGATTCACACAGCAACAA	CATAGGACCGTTGGCATTAC	525
<i>Pmp14</i>	CGGCTTTCATAACCTAAACCA	TTCCACCTGCCAATATGTAA	534
<i>Pmp15</i>	TGCTATTCAACACGGCACTC	TTCCAGTAAGCCGCTATGT	694
<i>Pmp16</i>	CAGCCCGTGTACTTTGTGAA	GCACATTGGCGTTATTTCCCT	619
<i>Pmp17</i>	CAGCAAATACCTCGCATCGT	GGAGCATAACAGCAAAGCTCA	552
<i>Pmp18</i>	AGATGGGGAGGAGCCTTACT	AGGCCAGAAAGAGTGATTG	642
<i>Pmp19</i>	CTGGTCTGGGGAAGAATCAA	ACGCGAAGAAGCAAAATACC	691
<i>Pmp20</i>	ACGATGCCGTAAACATCACA	GATTGGAGTTCCCAAGTTCC	688

**Table 2: Primer pairs and probes used for real-time PCR analysis of *pmp* genes and mouse G3PDH**

Gene	Sense primer (5' to 3')	Antisense primer (5' to 3')	TaqMan Probe (5' to 3') (* <i>Antisense</i> )	Amplicon size (base pairs)
<i>Pmp7</i>	GCTTTCGGACTCTTATGTCTTG	TCCTTCACTTGTGGCTCTTG	TGTTCCCTTTCCTGAAGGCACAGATGA	100
<i>Pmp9</i>	ACCAACCCTACAGCCACAAA	TCAGCAAAGATTCCCAAAGG	TCTACAGCCGGCAATGCCCA	83
<i>Pmp10</i>	GCAATACCGCAGATACAAA	TTAGCGAAGATCCCTGACAAA	TGCTCCCTTACCAGTTGCAACGG*	123
<i>Pmp11</i>	GCCTTTATTGCCGACGTATTT	TGCCGATAGAGCTTGTCTTG	CGATCCCTTCGCCCTATGGACA	117
<i>Pmp13</i>	TCCGCTACCATTTCTTCTC	ATCCATTTGCTGCGGTATTT	ATTTTGCGGCATGCGGTGGT	127
<i>Pmp14</i>	TCAAGCCTTTGCCTCTAAACA	GGCACGCTAATATCCACAAGA	AAAAGGAGCCCGTGTGCGCT	96
<i>Pmp15</i>	AAAGTGCCTCTGCTATATCTTTCTG	GACCAGGTCCTTGATGTCC	CCCGCATTGATGGATGCGAT*	113
<i>Pmp16</i>	CTCAGGAAATCTGGGCAACA	GATCGTTGGGGAGTGCATAG	TCCACTACGACGCAGGGCTTTG*	82
<i>Pmp17</i>	GGGGCCTATGTCTCACAAA	GGACTTGATGCTTGAGTAGAGTAACC	AACTCGCAACTTAAAACCAGGGTTTGA	86
<i>Pmp18</i>	GGATATTCGTTCCCGACTCC	AGCTAATCCCGCTCGATCTT	CAATAGCAAGCACGCCGTATTTGA*	113
<i>Pmp19</i>	GGTGTCGTTTCTAGCGTTATGG	GTGTTGATGAGAGGGGAAG	TGACCCGCCTGTGTGGAAGA	107
<i>Pmp20</i>	CTGGGGCTCATAGAAGCAAA	TGGTCTACGTCTGGAGCTTGT	CAAAGTGCCTTCCACAAGCAA*	67
<i>G3PDH</i>	GCTGCCAAGAACATCATCC	GTCAGATCCACGACGACAC	TCACTGGCATGGCCTTCCGT	134

**Table 3: Number of GGA(I/L/V) and FXXN tetrapeptide motifs within the N- and C-terminal halves of the putative pmp proteins**

Pmp protein <sup>1</sup>	GGA(I/L/V) <sup>2</sup>		FXXN <sup>3</sup>	
	N	(C)	N	(C)
Pmp7	6	(0)	10	(1)
Pmp9	3	(0)	4	(2)
Pmp10	4	(0)	6	(1)
Pmp11	3	(0)	7	(2)
Pmp13	5	(0)	5	(1)
Pmp14	1	(0)	1	(1)
Pmp15	7	(0)	9	(3)
Pmp16	4	(0)	11	(1)
Pmp17	5	(0)	10	(0)
Pmp18	6	(0)	8	(2)
Pmp19	6	(0)	12	(2)
Pmp20	7	(4)	10	(12)

<sup>1</sup> *C. felis* pmp name from the *C. felis* genome (3).

<sup>2</sup> Number of GGA(I/L/V) or <sup>3</sup> FXXN motifs within the (N) N-terminal and (C) C-terminal halves of each protein.