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Genetic variability of *Chlamydophila abortus* strains assessed by PCR-RFLP analysis of polymorphic membrane protein-encoding genes

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Abstract

This study used PCR-RFLP to investigate the genetic variability of *pmp*-encoding genes from fifty-two *Chlamydophila abortus* (*C. abortus*) strains originating from abortion cases from various geographical regions and host species. Six primer pairs were used to PCR-amplify DNA fragments encoding eighteen *pmps*. PCR products were digested using four restriction endonucleases and Bayesian methodologies were used to compare RFLP profiles and assign strains to a RFLP genotype. Strains could be assigned to 2 genotypes in the region encoding *pmp18D*, 3 genotypes in the regions encoding *pmp1A-pmp2B*, *pmp3E-pmp6H* and *pmp11G-pmp15G*, 4 genotypes in the region encoding *pmp7G-pmp10G* and 5 genotypes in the region encoding *pmp16G-pmp17G*. In all regions, the majority of strains (88.4%– 96.1%) had the same genotype as the reference strain S26/3. No correlation could be made between genotype, host species or geographical origin except for the two variant Greek strains, LLG and POS, which formed a discrete genotype in all *pmp*-encoding regions except *pmp18D*. Relative rates of evolution calculated for each *pmp*-encoding gene locus suggest that differing selective pressures and functional constraints may exist on *C. abortus* polymorphic membrane proteins. These findings suggest that although intraspecies heterogeneity of *pmp*-encoding genes in *C. abortus* is low, the sequence heterogeneity should be an important consideration when using *pmps* as the basis for novel diagnostics or vaccine development.

1. Introduction

Ovine Enzootic Abortion (OEA) is an economically important disease accounting for approximately 43% of diagnosed abortions in sheep in the UK. The aetiological agent, *Chlamydophila abortus* (formerly *Chlamydia psittaci* serotype 1), can also cause abortion in cattle and pigs and poses a zoonotic risk to pregnant women (Longbottom and Coulter, 2003).

The genetic heterogeneity of *C. abortus* has been reported to be low, with methods based on the cross-reactivity of monoclonal antibodies, Restriction Fragment Length Polymorphism (RFLP) and the phylogenetic analysis of 16S rRNA genes, failing to differentiate strains based on host, pathotype or geographical origin (Denamur et al., 1991; Everett et al., 1999; Salinas et al., 1995). More recently, methods such as Multiple Loci VNTR Analysis (MLVA) and Multi Locus Sequence Typing (MLST), have allowed the differentiation of *C. abortus* strains into novel genotypes (Laroucau et al., 2009; Pannekoek et al., 2010) that were partly related to geographical origin (Laroucau et al., 2009).

Genes encoding polymorphic membrane proteins (*pmps*) have been identified in all members of the family *Chlamydiaceae*. In *C. abortus* there are 18 *pmp*-encoding genes that are arranged as four loci composed of a single gene, two gene pairs and a large cluster of 13 genes (Thomson et al., 2005). Polymorphic membrane proteins have been implicated in chlamydial adhesion (Wehrl et al., 2004; Crane et al., 2006; Mölleken et al 2010), tissue tropism (Stothard et al., 2003; Gomes et al., 2006) and the induction of immune responses (Mygind et al., 2004; Wehrl et al., 2004; Livingstone et al., 2005) making them potential diagnostic and vaccine targets (Longbottom and Livingstone, 2006; Sachse et al., 2008). The purpose of this study was to analyse the genetic diversity of *pmp*-encoding loci from *C. abortus* isolates using PCR-RFLP and determine whether strains could be distinguished by geographical origin or host species.

2. Materials & Methods

2.1 Propagation of *C. abortus* and gDNA preparation

Fifty-two *C. abortus* strains isolated from various host species and geographical regions were used in this study (Table 1). Strains were propagated as previously described (Graham et al., 1995) and genomic DNA extracted using a Wizard Genomic DNA isolation kit (Promega).

2.2 PCR amplification of *pmp*-encoding loci

Oligonucleotide primers targeting the 18 *pmp*-encoding genes of *C. abortus* S26/3 [NC_004552] were designed using Lasergene 8 PrimerSelect software (DNASTAR). Three *pmp*-encoding loci encoding one or two genes in tandem were PCR-amplified as single fragments and are referred to in this study as BA (*pmp1B-pmp2A*), 16-17 (*pmp16G-pmp17G*) and 18D (*pmp18D*). The locus encoding 13 *pmps* was PCR-amplified as three fragments which are referred to in this study as EH (*pmp3E-pmp6H*), HG (*pmp6H-pmp10G*) and G (*pmp11G-pmp15G*).

PCR amplification mixes comprised 1 × Expand PCR buffer 3 (Roche Applied Sciences), 500 μM dNTPs, 300 nm each primer (see Table 2), 0.075 U Expand Long Template Polymerase (Roche Applied Sciences), and 20-100 ng *C. abortus* gDNA made up to a 25 μl final volume using nuclease free water. PCR consisted of 1 cycle at 94°C for 2 min followed by 10 cycles of 94°C for 10 s, 51.9 °C for 30 s and 68°C for 15 min. A second amplification stage consisted of 20 cycles of 94°C for 10 s, 51.9°C for 30 s and 68°C for 15 min with a 20 s increment per cycle followed by one cycle of 68°C for 7 min. Efficiency of amplification was assessed by agarose gel electrophoresis using 0.8% TAE-agarose gels containing 1 × GelRed (Biotium Inc).

2.3 Restriction Fragment Length Polymorphism (RFLP)

Restriction endonuclease (RE) digestions were performed using *Mbo*II (New England Biolabs), *Hae*III (New England Biolabs), *Rsa*I (Roche Applied Sciences) and *Alu*I (Roche Applied Sciences). Restriction endonuclease digestion reactions consisted of 1 × buffer, 5U of the appropriate RE and 5µl of PCR-amplified DNA. Restriction endonuclease digests were incubated at 37°C for 2 h. Five microlitres of each RE digest were electrophoresed on a 1.5% TAE-agarose gel containing 1× GelRed for 180 min or until the dye front had migrated to at least 15 cm from the sample wells. Gels were visualised under UV light and imaged using an AlphaImager 2200 (Alpha Innotech).

2.4 Data analysis

Restriction fragment length polymorphism gel images were imported into Bionumerics software (version 5.10, Applied Maths) and bands detected using default settings. The migration of each restriction fragment (RF) was expressed as a proportion of the total distance run on the gel, and strains were scored for the presence or absence of each RF generated with each of the four REs. RF positions were normalized to account for inter-gel variation using the RFLP profiles generated by the reference strain S26/3. The variation seen in technical replicates of S26/3 was also used as a general estimate of variation among technical replicates and guided the allocation of fragments to bins. The data were checked for errors and also evidence of horizontal gene transfer (HGT) using the NeighborNet method (Bryant and Moulton, 2004) available in the SplitsTree package (Huson and Bryant, 2006). Seventy of the 542 RFs that could not be unambiguously assigned were removed from the analysis. This resulted in a 0-1 data matrix of

128 52 unique strains by 472 RFs. The number of RFs for each of the six regions was 71 (16-17
 129 region), 51 RFs (BA), 54 RFs (18D), 105 RFs (EH), 93 RFs (G), and 98 RFs (HG). As there was
 130 no evidence of HGT in the NeighborNet diagram, the application of a phylogenetic tree model
 131 was judged to be valid. Bayesian phylogenetic methods have become increasingly important for
 132 the analysis of molecular sequence data but can also be applied to RFLP data using the MrBayes
 133 package (Huelsenbeck and Ronquist, 2001). The evolutionary relationships among strains at
 134 individual *pmp*-encoding loci were therefore analysed in MrBayes using the restriction datatype
 135 model. Relative evolutionary rates of the six regions were modelled using a gamma distribution.
 136 The Bayesian analysis produced an estimate of an overall tree topology based on all six regions
 137 plus a relative evolutionary rate for each region. The settings for the Markov chain Monte Carlo
 138 runs were as follows: two simultaneous runs of 625,000 generations with a burn-in period of
 139 125,000 generations with trees sampled every 100 generations. The analyses were assessed for
 140 convergence of the two runs using the MrBayes potential scale reduction factor (PSRF) values
 141 and the standard deviation of split frequencies between simultaneous runs. After an initial run,
 142 the value of the rate parameter specified for the exponential prior on branch lengths was altered
 143 to be 100 rather than the default value of 10 due to the observation of very high PSRF values for
 144 the relative rate parameters for each region. This setting reflects a prior belief that branch lengths
 145 are short (the rate parameter is the inverse of its mean). All analyses were then carried out with
 146 this altered branch length prior distribution. The PSRF values of 93% (211 out of the 226) of the
 147 taxon bipartition parameters were less than 1.20 (the maximum value was 2.04 and 14 other
 148 values were between 1.21 and 1.58). In addition, all 25 model parameters (namely, total tree
 149 length, the six pairs of parameters for the presence and absence of RFs on each of the six regions,
 150 the alpha shape parameter for each of the six regions and the relative rates for each of the six

regions) had PSRF values of less than 1.14. These PSRF values suggest good convergence of the two runs (i.e. less than the PSRF threshold of 1.2 as suggested by Gelman et al., 1995). As noted in the MrBayes output, the MrBayes PSRF values should be treated as a rough guide to convergence as not all assumptions are met for a PSRF convergence diagnostic in the phylogenetic context. Another convergence diagnostic, the standard deviation of split frequencies between simultaneous runs, was close to zero (<0.08), confirming convergence. Six additional Bayesian analyses were carried out for each of the six regions individually to produce phylogenetic trees for each region, with similar convergence diagnostics. The statistical support for groups in a Bayesian phylogenetic tree is expressed as a posterior probability ranging from 0.0 to 1.0, rather than the zero to 100 scale used in other phylogenetic approaches. RFLP groupings were assigned based on posterior support being present for each grouping. Phylogenetic tree diagrams were produced using the Dendroscope program (Huson et al., 2007).

3. Results and discussion

The genetic diversity of *pmp*-encoding genes from fifty two *C. abortus* strains were investigated by PCR-RFLP. Six primer pairs were used to PCR-amplify approximately 55kb spanning the 18 *pmp*-encoding genes and corresponding intergenic regions. PCR products of the expected size were obtained from 48 unique strains using primer set BA and 39, 43, 32, 47 and 52 unique strains using primer sets EH, HG, G, 16-17 and 18D respectively. PCR amplification was more variable when primer pairs generating larger PCR fragments (>10 kb) were used, probably due to the shearing of genomic DNA during DNA extraction. Where PCR amplification was poor or absent, the presence of *pmp*-encoding loci was confirmed by PCR amplification of the region as smaller PCR fragments (data not shown).

Restriction fragment length polymorphism was performed by digesting PCR amplicons with the four REs *Hae*III, *Alu*I, *Rsa*I and *Mbo*II and resolving RFs within the size range of 50 bp to 10 kb on an agarose gel. RFLP patterns for individual strains were scored based on the presence or absence of each RF and phylogenetic trees were generated to graphically represent strain similarity (Figure 1, Supplementary figures 1-6). Strains were assigned to RFLP groups based on the posterior probability assigned to each clade (Table 3). The number of genotypes assigned at each *pmp*-encoding locus varied from two genotypes observed at the 18D locus to 5 genotypes observed at the *pmp16-17* locus. At each of the six loci, the majority of strains belonged to a single genotype, typified by the reference strain S26/3. Based on the assumption that different RFLP patterns indicate that nucleotide changes have occurred in the gene locus, either at the RE recognition site or by nucleotide insertions or deletions in the intervening gene regions, the majority of strains belonging to a single genotype similar to S26/3 suggests a high degree of conservation both of the DNA sequence and the arrangement of the *pmp*-encoding genes.

RFLP profiles of the 18D locus were identical in all strains except AB7 and FAG which formed a discrete cluster with a posterior probability of 0.98 based on a single unique restriction fragment which was observed when 18D PCR products were digested with the RE *Rsa*I (data not shown). The relative rate of evolution, calculated by modelling RFLP data using a gamma distribution while constraining each region to estimate the same tree topology was 0.135, suggesting that this gene locus was evolving more slowly compared to other *pmp*-encoding gene loci (Table 4). Pmp 18D exhibits high levels of DNA and amino acid sequence conservation across the *Chlamydiaceae*. Putative roles based on the timing of expression *in vitro* and the inhibition of *in vitro* infection using neutralizing antibodies against Pmp18D suggest a role in the

adhesion of *C. pneumoniae* and *C. trachomatis* to host cells (Crane et al., 2006; Wehrl et al., 2004; Mölleken et al., 2010). However the expression of the gene early in the replication cycle suggests that this protein may have an additional function during the late replicative/early differentiation phase (Wheelhouse et al., 2009).

Other *pmp*-encoding loci showed a greater degree of gene heterogeneity with discrete genotypes observed in each region. Strains S404/5, S507 and S82/3 (posterior probability = 0.70) formed a discrete grouping in region EH (Table 3). Strains Zar-2B and AO-4 (posterior probability = 0.72) and strains AB7 and C94/2 (posterior probability = 0.56) formed a discrete grouping in region HG (Table 3). In *C. trachomatis* PmpH has been implicated in the mechanisms of pathogenesis due to the observation that *pmpH* gene sequences diverged into 3 clades that were consistent with the three different disease pathotypes, urogenital, ocular and lymphogranuloma venereum (Stothard et al., 2003; Gomes et al., 2006). It is still unknown if the grouping in the region HG in *C. abortus* has a similar pathophysiological basis.

Strains AO-4 and Zar-5 formed a discrete grouping at regions G (posterior probability = 0.77) (Table 3) and strains C94/1 and C94/2 (posterior probability = 0.83), strains C1/98, C17/98 and C9/98 (posterior probability = 0.55) and strains 15 and 532 (posterior probability = 0.51) formed a discrete grouping in region 16-17 (Figure 1, Table 3). The relative rate of evolution in *pmpG* and *pmp16-17* regions was predicted to be 1.488 and 1.468 respectively. The higher relative rate of evolution of the *pmp* G family of proteins, a family that has a number of paralogues present in *C. abortus*, suggests that the *pmpG* and *pmp16-17* may be under different selective pressures to other *pmp*-encoding genes. It is unclear whether these genes have a high relative rate of evolution because they contribute to antigenic variance or whether they are redundant in the infection cycle.

Two strains, LLG and POS, showed a markedly different genotype to other strains with posterior support observed for the discrete grouping of strains LLG and POS at *pmp*-encoding regions BA (posterior probability = 0.59), EH (posterior probability = 0.87), HG (posterior probability = 0.79), G (posterior probability = 0.62) and 16-17 (posterior probability = 0.91). This was the only RFLP group that could be correlated with geographical location, with both strains originating from Greece. However LLG and POS are not typical Greek strains having been identified as variant strains by MLVA (Laroucau et al., 2009) and the DNA sequencing of the 23S domain I rRNA (Siarkou et al., 2002). Phenotypic differences have been observed in the inclusion morphology and polypeptide profiles of these two strains, in mouse cross-protection experiments and in antibody cross-reactivity to variable segments of MOMP (Vretou et al., 1996; Vretou et al., 2001). Studies in pregnant mice have demonstrated these strains to be different from other *C. abortus* strains in terms of their ability to colonise the placenta and fetus (Bouakane et al., 2003). Furthermore, mouse protection studies have shown a decreased effectiveness of the live 1B vaccine in terms of organism recovery (Bouakane et al., 2005). However, the differences observed did not prevent inactivated AB7-based vaccines from providing protection against heterologous challenge with LLG (Caro et al., 2003).

This study presents the first assessment of the genetic diversity that exists within the 18 *pmp*-encoding genes of *C. abortus*. The *pmp*-encoding regions of *C. abortus* were largely homogeneous with the majority of strains exhibiting genotypes similar to reference strain S26/3. However, novel genotypes were identified in all *pmp*-encoding loci. Although the biological significance of this variation in terms of pathogenesis is currently unknown, this sequence heterogeneity should be an important consideration when using *pmps* as the basis for diagnostic test or vaccine development.

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Conflict of interest statement

The authors declare no conflict of interest.

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Figure Legends

Figure 1.

(A) *C. abortus* RFLP profiles generated following RE digestion of polymorphic membrane protein 16-17 locus PCR products from twenty representative strains; 1, DC1. 2, POS. 3, LLG. 4, 8385. 5, S152/3. 6, FAG. 7, S26/3. 8, S26/3. 9, 53. 10, C94/1. 11, Z339. 12, AO-2. 13, T28. 14, Syva-1. 15, S26/3. 16, AO-7. 17, T19. 18, AO-4. 19, 532. 20, 15. The RFs used for analysis have been highlighted. The size of the upper and lower RF is indicated at the side of the gel. (B) Evolutionary relationships of *C. abortus* strains at the 16-17 locus. The posterior probability of discrete groupings is indicated by the number at the node.

Table 1. *C. abortus* strains used for PCR-RFLP analysis

Country of origin	Host species	Strain
France	Sheep	AB7
Germany	Cow	03DC34, 03DC36, C11/98, Z339
	Goat	532
	Pig	DC12
	Sheep	53, C1/98, C2/98, C7/98, C9/98, C10/98, C17/98, DC1, DC4, DC7
Greece	Goat	FAG, LLG
	Sheep	POS
Ireland	Sheep	C94/1, C94/2, C95/23, C95/27, VS88/576
Italy	Cow	LV350/93
Namibia	Goat	C21/98
Spain	Goat	Syva-1
	Sheep	AO-2, AO-3, AO-4, AO-7, Zar-2B, Zar-5
Tunisia	Goat	15
UK	Sheep	83/12, 86/30, 90/345, A22, H574, S3/3, S26/3, S82/3, S95/3,
		S124/3A, S152/3, S404/5, S507, T17, T19, T20, T28

Table 2. Primers used for the PCR-amplification of *pmp*-encoding genes.

Region ^a	Forward primer (5'-3')	Reverse primer (5'-3')	Amplicon size (bp) ^a
BA [<i>pmp1B</i> , <i>pmp2A</i>]	AGAATATCATACGTGCACCGCAGTTAG	TTCACTCGCACCACTGACTC	8329
EH [<i>pmp3E</i> , <i>pmp4E</i> , <i>pmp5E</i> , <i>pmp6H</i>]	AAGATTAACGAGCTTCCTGC	CTTGCTTATGCAGCTCGGGG	10547
HG [<i>pmp7G</i> , <i>pmp8G</i> , <i>pmp9G</i> , <i>pmp10G</i>]	TTAGAATGTTCTTGCAAGCCTGT	TGACGGTTCTACAGGGACC	14514
G [<i>pmp11G</i> , <i>pmp12G</i> , <i>pmp13G</i> , <i>pmp14G</i> , <i>pmp15G</i>]	TTAAAAATGCACCCTGCTG	CCTCTGCACCTCAAAACACA	15145
16-17 ^b [<i>pmp16G</i> , <i>pmp17G</i>]	TGAGCCAAATCCTTCTACAGCAAT	AGTCTTAGCGTCACAGGCCGAG	5613
18D [<i>pmp18D</i>]	TTAGAAGATCAAACGCATACCACAATTC	GTCTACATTTTCTCACTCCGTA	4569

^a Based on *C. abortus* S26/3 genome sequence [NC_004552]^b Primers have been described previously as S5765 and S2429 (Longbottom et al., 1998)

Table 3. *C. abortus* strains organized by RFLP grouping at each *pmp*-encoding locus.

Locus	RFLP group	Strains	PSRF
BA	1	03DC34, 03DC36, 83/12, 86/30, 90/345, 532, A22, AB7, AO-2, AO-4, AO-7, C1/98, C2/98, C7/98, C9/98, C10/98, C17/98, C21/98, C94/1, C94/2, C95/23, C95/27, DC1, DC4, DC7, FAG, H574, 15, LV350/93, Syva-1, S3/3, S26/3, S82/3, S95/3, S124/3A, S152/3, S404/5, S507, T17, T19, T20, T28, Zar-2B, Z339	unresolved
	2	LLG, POS	0.59
	3	C11/98, 53	0.75
EH	1	03DC34, 53, 532, 83/12, 86/30, 90/345, A22, AB7, AO-2, AO-4, AO-7, C2/98, C7/98, C9/98, C10/98, C17/98, C21/98, C94/1, C94/2, C95/23, C95/27, DC1, DC4, DC7, DC12, FAG, LV350/93, S3/3, S26/3, S95/3, S124/3A, S152/3, T17, T19, T20, T28, Zar-2B, Z339	unresolved
	2	LLG, POS	0.87
	3	S404/5, S507, S82/3	0.70
HG	1	03DC34, 53, 532, 83/12, 86/30, 90/345, A22, AO-2, AO-7, C2/98, C7/98, C9/98, C10/98, C17/98, C21/98, C94/1, C95/23, C95/27, DC1, DC4, DC7, FAG, H574, LV350/93, S3/3, S26/3, S82/3, S95/3, S124/3A, S152/3, S404/5, S507, T17, T19, T20, T28, Z339	unresolved
	2	LLG, POS	0.79
	3	AO-4, Zar-2B	0.72
	4	AB7, C94/2	0.56
G	1	53, 532, 83/12, 86/30, 90/345, A22, AB7, AO-7, C7/98, C9/98, C21/98, DC1, DC7, FAG, LV350/93, S3/3, S26/3, S82/3,	unresolved

Locus	RFLP group	Strains	PSRF
		S95/3, S124/3A, S152/3, S404/5, S507, T17, T19, T20, T28, Z339	
	2	LLG, POS	0.62
	3	AO-4, Zar-5	0.77
16-17	1	03DC34, 03DC36, 53, 83/12, 86/30, 90/345, A22, AB7, AO-2, AO-4, AO-7, C2/98, C7/98, C10/98, C21/98, C95/23, C95/27, DC1, DC4, DC7, FAG, H574, LV350/93, Syva-1, S3/3, S26/3, S82/3, S95/3, S124/3A, S152/3, S404/5, S507, T17, T19, T20, T28, Zar-2B, Z339	unresolved
	2	LLG, POS	0.91
	3	C94/1, C94/2	0.83
	4	15, 532	0.51
	5	C1/98, C17/98, C9/98	0.55
18D	1	03DC34, 03DC36, 53, 532, 83/12, 86/30, 90/345, A22, AO-2, AO-3, AO-4, AO-7, C1/98, C2/98, C7/98, C9/98, C10/98, C11/98, C17/98, C21/98, C94/1, C94/2, C95/23, C95/27, DC1, DC4, DC7, H574, 15, LLG, LV350/93, POS, Syva-1, S3/3, S26/3, S82/3, S95/3, S124/3A, S152/3, S404/5, S507, T17, T19, T20, T28, VS88/576, Zar-2B, Zar-5, Z339	unresolved
	2	AB7, FAG	0.98

Table 4. Relative evolutionary rates for *pmp*-encoding gene loci

Region	Mean	95% confidence	
		interval, lower	
		limit	limit
BA	0.425	0.142	0.770
EH	0.928	0.569	1.406
HG	0.874	0.648	1.114
G	1.488	1.105	1.885
16-17	1.468	1.005	1.948
18D	0.135	0.015	0.368

Figure 1