Analysis of membrane protein genes in a Brazilian isolate of *Anaplasma marginale*

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The sequencing of the complete genome of Anaplasma marginale *has enabled the identification of several genes that encode membrane proteins, thereby increasing the chances of identifying candidate immunogens. Little is known regarding the genetic variability of genes that encode membrane proteins in* A. marginale *isolates. The aim of the present study was to determine the degree of conservation of the predicted amino acid sequences of OMP1, OMP4, OMP5, OMP7, OMP8, OMP10, OMP14, OMP15, SODb, OPAG1, OPAG3, VirB3, VirB9-1, PepA, EF-Tu and AM854 proteins in a Brazilian isolate of* A. marginale *compared to other isolates. Hence, primers were used to amplify these genes:* omp1, omp4, omp5, omp7, omp8, omp10, omp14, omp15, sodb, opag1, opag3, virb3, VirB9-1, pepA, ef-tu *and* am854. *After polimerase chain reaction amplification, the products were cloned and sequenced using the Sanger method and the predicted amino acid sequence were multi-aligned using the CLUSTALW and MEGA 4 programs, comparing the predicted sequences between the Brazilian, Saint Maries, Florida and* A. marginale centrale *isolates. With the exception of outer membrane protein (OMP) 7, all proteins exhibited 92-100% homology to the other* A. marginale *isolates. However, only OMP1, OMP5, EF-Tu, VirB3, SODb and VirB9-1 were selected as potential immunogens capable of promoting cross-protection between isolates due to the high degree of homology (over 72%) also found with* A. (centrale) marginale.

Key words: membrane proteins - OMPs - *Anaplasma marginale* - Brazil

The outer membranes of tick-transmitted intracellular bacterial pathogens determine the functions necessary for survival, replication and transmission. Thus, proteins expressed on the surfaces of these pathogens are potential candidates for vaccine development, targeting the induction of protective immune responses in the vertebrate hosts and/or prevention of the colonization of the tick vector. The identification of surface proteomes is critical to the development of vaccines and this process has been accelerated by genome sequencing (Noh et al. 2008). A number of complete genome sequences of the family Anaplasmataceae have recently been reported, including *Anaplasma marginale* (Brayton et al. 2005), *Anaplasma marginale* ss. *centrale* (Herndon et al. 2010), *Anaplasma phagocytophilum*, *Ehrlichia chaffeensis*, *Neorickettsia sennetsu* (Hotopp et al. 2006), *Ehrlichia canis* (Mavromatis et al. 2006) and *Ehrlichia ruminantium* (Collins et al. 2005).

Genome sequencing and subsequent proteomic studies of *A. marginale* have enabled the identification of several membrane proteins and have expanded the range of vaccine candidates (Lopez et al. 2005, 2008, Brayton et al. 2006, Noh et al. 2008). However, some membrane proteins of this rickettsia vary between and even within isolates, depending on the rickettsemia cycle (Palmer et al. 2000). In the case of the major surface protein (MSP)2, genetic variability is due to the rearrangement of more than 10 whole functional pseudogenes with a single gene expression site (Brayton et al. 2001, 2005). The number of distinct configurations is exponentially increased by the conversion of gene segments, in which mosaics from the expression site are generated by the recombination of oligonucleotide segments from multiple allele donors (Barbet et al. 2000, Brayton et al. 2002, Palmer et al. 2009). This process involves rickettsia evasion from the immune response and ensures the maintenance of infections with low cyclical rickettsemia in the immune host (Palmer et al. 2000). Genetic variability is one of the factors to be considered in the evaluation of candidates for the development of vaccines, as it can result in significant antigen polymorphism and impair cross-protection between isolates (Palmer et al. 2000).

The aim of the present study was to assess the conservation of membrane proteins from a Brazilian isolate of *A. marginale* and compare the findings with other known isolates.

MATERIALS AND METHODS

A. marginale isolate - The Pernambuco-Zona da Mata isolate of *A. marginale* (AMBR) was maintained in liquid nitrogen, in the stabilized form of infected blood containing 10% glycerol at Embrapa Gado de Corte (Brazilian

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TABLE I
Primer sequences used to amplify Brazilian *Anaplasma marginale isolate*

Gene	Primer	Sequence (5'-3')	Amplicon size (bp)	Region
Omp1	F	AAGGTCTACGGCTTGGTTTACGCTGCGTTGTCTTT	977	1027576-1025552
	R	AGTCTGATACCGCACTCAAACCCAAAATAGTCCAG		
Omp4	F	CAGCATATCATTGAATACAGGAGGAAGTCGCTTAT	1,128	1040447-1041574
	R	GTGGCTAGCCGCTTTGGAGATGTTACCAG		
Omp5	F	CTGGTGGTGGAGAGTTTGCCTATG	1,112	1041985-1043096
	R	AACTCGAGCTTCAGCCCCAGATTG		
Omp7	F	TCTTTTCTGTTGGGTGCGGTTGTA	1,121	1088542-1089662
	R	CGCGCCTTGACATCTTCCTC		
Omp8	F	TTTCTGTTGAGCGCGGTTGTAGTT	1,127	1089797-1090923
	R	CGCGCTCTGATATTTTCCCTTTCA		
Omp10	F	TTGCTGCGTTGCGACGGTTTC	1,202	1092307-1093508
	R	GCGAGATTCAACACCCCAAAAGAG		
Omp14	F	GCGTTTAGCCTCCTGTT	1,207	63058-64247
	R	ATCCGAACCTGATTCCTA		
Omp15	F	GAATTCACGCATATACCTTGGCTCACCGTT	422	900095-900516
	R	GAATTCCTATATTGCGTAGCCCGAAGGATGCTGAGA		
Am097	F	ATGAAAAAGGCTTTCATGGTTT	813	77775-78587
	R	CTACCCACGTCCCCTTCTG		
Am197	F	ATGAAAAAGCTTGAGCTTGCTAG	510	166838-167347
	R	CTACTTGGGCTTTCTAGGAGCC		
Am254	F	ATGACAGAAGGGAGAAAGCC	1,182	832189-833370
	R	CTACTCCAAAATCTCAGTTATGATAC		
Am854	F	ATGCTGCATCGTTGGTTAGC	711	789000-789710
	R	CTATTCAGGCGCGACCAC		
Am956	F	ATGTGCTATGGTACTCGCATC	1,605	876183-877787
	R	TCACTTTTCGTAATACTTCGACACA		
Opag1	F	AGTTGCAGAGCATTTTCCTTG	390	1030618-1031007
	R	CTAAAAAACCAAAAAAACCGT		
Opag3	F	TGTGGGTTGCACACACTACC	936	1028730-1029665
	R	CTAAAAACCATCACCAAATGC		
Virb3	F	TCGTCCGGTAGCGTAAAGAC	294	752143-752436
	R	CTACATCACATCGTAAGAATT		
SodB	F	TCACACACCCGCGGTTTCAAGCCT	664	752533-753196
	R	TCCGCTGCGCAGTTGGTATACAT		

Cattle Research Institute), Campo Grande, Mato Grosso do Sul, Brazil. Rickettsemia in the sample at the time of the preparation of the stabilized solution was 87%.

DNA extraction was performed with the Easy-DNA™ kit (Invitrogen) according to the manufacturer's instructions. The concentration of extracted DNA and the A_{260}/A_{280} ratio were determined with a GeneQuant spectrophotometer (Amersham Pharmacia Biotech, USA).

Gene amplification by polimerase chain reaction (PCR) for sequencing - The membrane proteins prioritized in this work were those for which less information was available and for which amplification was successful. Primers were designed as described in Ramos et al. (2007) for the amplification of the *omp1, omp4, omp5, omp7, omp8, omp10, omp14, omp15, sodb* (*am197*)*, opag1, opag3, virb3, virb 9-1* (conjugal transfer protein/*am097*), pepA (*am956*), ef-tu (*am254*) and *am854* genes (Table I) using the PCR. Amplification reaction mixtures were prepared in a volume of 25 µL containing 10 mM Tris-HCl (pH 8.3), 50 mM KCl, 1.5 mM $MgCl_2$, 0.2 mM of each deoxynucleoside triphosphate, 12 pmol of each primer, 100 ng of genomic DNA and 1.25 U of *Taq* DNA polymerase (Invitrogen). Amplifications were carried out in a thermocycler (MyCycler, Biorad) as follows: 94ºC for 1 min (denaturation), 30 cycles at 94ºC for 1 min (denaturation), 55ºC for 30 s (annealing) and 72ºC for 1 min (extension). The PCR products were analyzed by electrophoresis in 1% agarose gels stained with SybrGold (Invitrogen).

The amplicons were cloned in the pGEM-T Easy plasmid (Promega, USA) according to the manufacturer's instructions. The recombinant plasmids were then used to transform *Escherichia coli* (TOP-10) cells, which were seeded in Luria-Bertani agar (LB) containing 100 µg/mL ampicillin. The plates were incubated at 37ºC for approximately 12 h and the colonies were analyzed by PCR with M13 primers to confirm the presence of the inserts. Colonies that were PCR-confirmed to contain the inserts (1 per gene) were grown in LB broth with ampicillin and at 37ºC for 12 h with agitation. The cells were then recovered by centrifugation and plasmid DNA was extracted using the Wizard Plus Miniprep kit (Promega).

Sequencing and analysis - Sequencing reactions were performed using the BigDye Terminator (Applied Biosystems), following the manufacturer's instructions. Gene sequences were obtained with an automatic sequencer (model ABI 3130, Applied Biosystems). Four sequencing reactions were performed for each gene, two with the M13 forward primer and two with the M13 reverse primer.

The gene sequences were assembled and the consensus sequences were generated with the Sequencher program v.4.1.4 (Gene Codes). The search for homologues was performed using the BLASTn program (ncbi.nlm.nih.gov) with comparisons to the sequences obtained from the following *A. marginale* isolates: Saint Maries (AMSM) (Genbank CP000030), Florida (AMFL) (Genbank CP001079) and *A. marginale centrale* (AMCE) (Genbank CP001759).

TABLE II

Percentage of identity between the predicted amino acid (AA) sequences in the membrane proteins of the Pernambuco-Zona da Mata isolate of *Anaplasma marginale* (AMBR) compared to the American isolates Saint Maries (AMSM), Florida (AMFL) and *A. (centrale) marginale* (AMCE)

AMBR protein	AAs n	AMBR GenBank n	AMSM %	AMFL %	AMCE %
AM854	236	GU991617	100	100	-
OMP1	329	GU991619	99	99	72
OMP4	404	GU991620	92	92	66
OMP5	380	GU991621	97	94	75
OMP7	381	GU991622	65	72	40
OMP8	399	GU991623	97	92	66
OMP10	420	GU991624	100	98	65
OMP14	404	GU991625	95	94	70
OMP15	147	GU991626	94	§	#
OPAG1	130	GU991627	100	100	#
OPAG3	312	GU991628	95	95	66
EF-Tu	393	GU991618	100	100	97
PepA	518	GU991629	97	97	65
VirB3	98	GU991632	100	100	96
ViB9-1	270	GU991631	97	100	97
SODb	223	GU991630	99	100	89

§: the OMP15 predicted amino acid sequence of the AMFL (available on GenBank) is incomplete; #: *A. marginale centrale* does not have the complete genes that codify the proteins AM854, OMP15 and OPAG1 in its genome.

Predicted amino acid sequences for the genes of AMBR were obtained using the MEGA 4 program (Tamura et al. 2007) and multiple alignments between these sequences and those from the AMSM, AMFL and AMCE isolates was performed using the CLUSTALW program (ebi.ac.uk/Tools/clustalw2/index.html).

RESULTS

Most of the alignments of predicted amino acid sequences of membrane proteins from the Brazilian (Table II) and American *A. marginale* isolates were more than 92% homologous (Table II), thereby demonstrating conservation.

A. marginale membrane protein genes are grouped into two superfamilies, MSP1 and MSP2 (Brayton et al. 2005). Most members of the MSP1 superfamily in the American *sensu stricto* isolates (AMSM and AMFL), such as MSP1a, MSP1b 1-2 and MLP 2-4, have low degrees of conservation, with only 13-48% homology between amino acid sequences (Herndon et al. 2010). The MSP2 superfamily has a number of conserved genes, some of which were studied in the present investigation.

Operon-associated genes (OPAG) 1 and 3 are components of the MSP2 superfamily and their function remains unknown (Löhr et al. 2002). The observation that bacterial structural genes are often organized into groups that encode proteins with related functions (Lewin 2000, Löhr et al. 2002) suggests that OPAG proteins take part in interactions in the membrane of *A. marginale*. It is unknown whether the *OPAG1* gene is expressed in small amounts or not expressed at all. *OPAG3* is expressed only within erythrocytes during acute rickettsemia. The differential expression of outer membrane proteins (OMP) within the same operon is a new finding in tick-transmitted bacteria and expression regulation should be broadly applicable in order to understand how the pathogen adapts to transitions in the host (Löhr et al. 2002).

The American isolates exhibit identical predicted OPAG1 and OPAG3 amino acid, with 100% and 95% homology with AMBR sequences, respectively. AMCE does not have the *OPAG1* gene and its predicted amino acid sequence for *OPAG3* demonstrated only 66% homology with AMBR isolate sequence. Therefore, although the degree of conservation in the *OPAG1* gene is greater than that found in *OPAG3* for the AMSM and AMFL isolates, *OPAG3* is probably a better candidate vaccine component. However, while AMCE lacks the *OPAG1* gene, the immunity achieved by vaccination with this isolate is sufficient to prevent the development of clinical disease in cattle (Pipano 1995). How this protection is achieved with *OPAG3* remains unknown.

Fifteen OMP with sequences similar to those of MSP2 and/or MSP4 have been identified in the MSP2 superfamily. These genes can be separate or grouped in structures similar to an operon (Brayton et al. 2006). Within this group, OMP2, OMP3 and OMP6 are probably pseudogenes, as their transcripts have not been detected in erythrocyte stages of different *A. marginale* isolates (Noh et al. 2006, Ramos et al. 2007).

The functions of OMPs 4, 7, 10 and 14 remain undefined in *A. marginale*. These proteins are designated as members of the MSP2 superfamily due to sequence identity with surface antigens of the PFAM01617 family (Brayton et al. 2005). However, these OMPs are the targets of antibodies induced by the immunization of cattle with purified outer membranes (Lopez et al. 2005) and specifically by IgG2, which is associated with protective immunity (Brown et al. 1998).

The vaccine isolate AMCE has *OMP1*, *OMP4*, *OMP5*, *OMP7*, *OMP9*, *OMP10*, *OMP11*, *OMP12*, *OMP13* and *OMP14* genes in its genome (Genbank CP001759). According to Herndon et al. (2010), the family of OMPs 1-15 present in the *sensu stricto* American isolates *of A. marginale* is reduced in AMCE. The latter has no distinct *omp7*, *omp8* or *omp9* genes; they are assembled into a single coding region, constituting an operon and OMP 2, 3, 6 and 15 homologues are missing. In the present study, not all the genes in AMCE were studied; however, the predicted amino acid sequences of OMPs 1, 4, 5, 7, 8, 10 and 14 exhibited 72%, 66%, 75%, 38% 65%, 65% and 70% homology, respectively, between the AMBR isolate sequences (Table II).

DISCUSSION

Considering the OMPs in the *A. marginale* studied, OMP1, OMP5 and OMP10 take on greater importance among antigens derived from the membrane of rickettsia due to their greater than 94% homology with predicted amino acid sequences of the other isolates studied (Table II). As the inoculation of AMCE can protect against isolates of *A. marginale* (Pipano 1995), these proteins that have over 65% sequence homology with AMCE proteins are probably the best candidate vaccine antigens among the OMPs studied.

Most of the AMBR isolate OMP amino acid sequences analyzed in the present study exhibited greater than 92% homology with the American isolates, indicating that the genes in this family of proteins are also conserved in the Brazilian isolate. However, the predicted OMP15 amino acid sequence in the AMFL isolate (available on Genbank) is not complete and has only 105 amino acids (Fig. 1).

OMP7 was the only protein with predicted amino acid sequence homology below 90% (Fig. 2). According to Noh et al. (2006), this variation is probably associated with nucleotide substitutions in the central region of this gene, which maintain the reading frame but introduce variations in amino acids. The genes that encode OMPs

Fig. 1: multiple alignment of the predicted amino acid sequence in OMP15. The sequence is smaller in *Anaplasma marginale* Florida (AMFL) isolate in comparison to *A. marginale* Saint Maries (AMSM) and *A. marginale* Brazilian (AMBR) isolates.

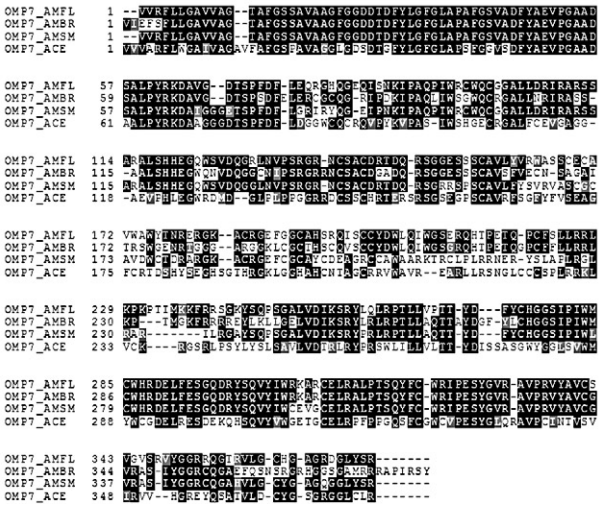

Fig. 2: multiple alignment of the predicted amino acid sequence in OMP7 with low identity between isolates due to the variability of this gene. AMBR: Pernambuco-Zona da Mata isolate of *Anaplasma marginale*; AMCE: *A. (centrale) marginale* isolate; AMFL: Florida isolate of *A. marginale*; MSM: Saint Marie isolate of *A. marginale*.

7-9 share large areas of identity at the 5' and 3' termini, where recombination can occur, while C and N terminal areas exhibit relative conservation.

The PepA and EF-Tu were previously classified as cytoplasmic proteins, but an *in silico* analysis by the TMHMM program suggested them to be proteins anchored to the outer membrane and exposed to the extracellular environment (Lopez et al. 2005). Leucyl aminopeptidase is an enzyme that promotes the breaking of peptide bonds by hydrolysis in the metabolic pathways of amino acids. After alignment, the predicted amino acid sequence of the PepA protein in AMBR isolate exhibited 97% homology with the sequences of the same protein in AMSM isolate and AMFL isolate and 65% homology with the gene in AMCE (Table II). The percentage of homology found for this protein sequence is relatively high considering its size (521 amino acids) and the fact that the protein sequence in the *sensu stricto* American isolate differs from the AMBR isolate sequence by only four amino acids. As PepA, after analysis, was suggested to be a membrane-associated protein and its protein provides a conserved predicted amino acid sequence, more studies should be devoted to the evaluation of its immunogenicity and potential as a vaccine antigen.

EF-Tu is an enzyme belonging to the family of hydrolases involved in protein synthesis, promoting chain elongation during polypeptide synthesis in the ribosome. Ramos et al. (2007) assessed the transcription pattern of several membrane proteins' genes in *A. marginale*, including EF-Tu, which, after analysis, was suggested to be a membrane-associated protein (Lopez et al. 2005). Araújo et al. (2008) and Lopez et al. (2008) expressed EF-Tu in *E. coli* and the recombinant protein was able to induce a proliferative response in T lymphocytes and titers of IgG2 greater than those of IgG1, thereby demonstrating the desired capacity for immunostimulation.

The predicted amino acid sequence of EF-Tu in AMBR isolate exhibited 100% homology with the sequence in the American isolates and 97% homology with the sequence in *A. (centrale) marginale*. In comparison to the genes of the membrane proteins investigated in the present study, EF-Tu takes on significance as a possible candidate vaccine antigen, as it exhibits the highest percent homology among the predicted amino acid sequences in the isolates analyzed and is considered a highly conserved gene. This finding corroborates a study carried out by Araújo et al. (2008), who report the conservation of this gene. Moreover, it is capable of stimulating the proliferative response in T lymphocytes, as reported by Lopez et al. (2008).

The type IV secretion system (TFSS) transports macromolecules through the membrane in an ATP-dependent manner and is related to the conjugation system in Gram-negative bacteria (Niu et al. 2006). TFSS is one of the few groups of syntenic genes among all sequenced Rickettsiales, suggesting that the coordination of the expression of these genes is critical (Hotopp et al. 2006). It is possible that TFSS expression is regulated and associated with survival in intracellular bacteria (Niu et al. 2006). Due to its location on the cell surface, its highly conserved nature and intracellular survival requirements, TFSS proteins in Gram-negative bacteria are consistent targets of immunological investigations (Lopez et al. 2007). In the present study, the predicted amino acid sequences of two TFSS proteins, VirB3 and VirB9-1 (Sutten et al. 2010) were analyzed. The function of VirB3 is not well defined and VirB9-1 is responsible for the transfer of protein-DNA complexes and macromolecules between cells (Lopez et al. 2007).

After alignment with sequences from the previously mentioned isolates, the predicted amino acid sequences of VirB3 and VirB9-1 in AMBR isolate exhibited nearly 100% homology with the American *sensu stricto* isolates (Table II) and approximately 96% homology with AMCE (Table II), demonstrating a high degree of conservation. In studies with other TFSS group members (VirB9-2 and VirB10), Araújo et al. (2008) and Vidotto et al. (2008) suggested conservation among different isolates of *A. marginale* and demonstrated that VirB9-2 and VirB10 proteins are recognized by antibodies from naturally and experimentally infected cattle. The VirB3 protein in AMBR isolate has the same conservation profile as other components of the TFSS group analyzed by Araújo et al. (2008); thus, it is expected to exhibit the same antigen profile, as the conservation of B cell epitopes between and within isolates is likely important for controlling infection. Lopez et al. (2007) also found that VirB9-2, VirB10 and VirB9-1 are conserved among isolates. Furthermore, these proteins are recognized by effector cells of the immune system in cattle inoculated with the outer membrane of *A. marginale*.

Superoxide dismutases (SOD) are metalloenzymes that catalyze the dismutation reaction $O_2 \rightarrow H_2O_2 \rightarrow O_2$. There are other types of SODs that are distinguished by the metal present in the active site (for example: MnSOD, ZnSOD). According to Ohashi et al. (2002), the *SODb* gene in *Anaplasma* and *Ehrlichia* spp probably

encodes a FeSOD. Iron SODs and manganese SODs are known to defend bacterial cells from reactive oxygen generated from the bacterial metabolism and to protect DNA from oxidation damage.

The alignment of the predicted amino acid sequence of SOD of AMBR isolate with the others analyzed in this paper exhibited nearly 100% homology with SODs from the American *sensu stricto* isolates (Table II) and 89% homology with AMCE (Table II). Thus, as SOD is also highly conserved among *A. marginale* strains, the immunogenic potential of this protein should also be examined for vaccine development.

Lopez et al. (2005) annotated AM854 in the *A. marginale* genome as a putative protein without defined function and a BLAST search showed identity with an OMP and peptidoglycan-associated protein in *E. canis*. Noh et al. (2008) reported that AM854, along with other proteins, was identified on the surface of *A. marginale* from erythrocytes and tick cells. The predicted amino acid sequence for the *am854* gene in AMBR isolate exhibited 100% homology with the sequences in the American isolates (Table II). According to Lopez et al. (2005), AM854, along with other proteins, was recognized by IgG in at least one animal, thereby proving to be antigenic. Although the gene that encodes the AM854 protein is not complete, compared to the American isolates, the AMCE genome has a protein annotated as a putative OMP. This protein has 211 amino acids and has 79% identity (167/211) to the sequence of the American isolates (236 amino acids) when compared using BLAST, showing considerable conservation. This fact must be taken into account, since immunization with this subspecies of *Anaplasma* is capable of generating a protective immune response against *A. marginale* in cattle (Pipano 1995).

In conclusion, among the predicted amino acid sequences of the proteins evaluated, OMP1, OMP5, EF-Tu, VirB3, SODb and VirB9-1 had the highest degrees of homology to the isolates used for comparison purposes (including AMCE) and demonstrated a high degree of conservation. This suggests that these proteins are important to the development of immunogens capable of promoting cross-protection between isolates. Further studies should be carried out to gain a better understanding of the real importance of the EF-Tu, VirB3, SODb and VirB9-1.

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