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Cloning, sequencing, expression, and antigenic characterization of rMSP4 from *Anaplasma marginale* isolated from Paraná State, Brazil

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ABSTRACT. Anaplasmosis is a bovine intraerythrocytic disease caused by the bacterium *Anaplasma marginale*; it causes significant economic losses in tropical and subtropical regions, worldwide. The *msp*4 gene of an *A. marginale* strain isolated in Paraná, Brazil, was amplified by PCR and sequenced; its cloning into the pET102/D-TOPO® vector produced an *msp*4-6xHis-V5-HP thioredoxin fusion gene construct. This recombinant clone was over-expressed in *Escherichia coli* BL21(DE-3); the expressed fusion protein was found almost entirely in the insoluble form (inclusion bodies) in the cell lysate. The inclusion bodies were solubilized with urea and the recombinant protein was purified by Ni-NTA column and dialyzed. This method produced a relatively high yield of rMSP4, which was used to immunize rabbits. The deduced amino acid sequence encoded by MSP4 showed 99% homology to *A. marginale* isolates from Florida, USA, and from Minas Gerais, Brazil. Both rMSP4 and native MSP4 were recognized by post-immunization rabbit serum, showing that

rMSP4 has conserved epitopes. As antigenicity was preserved, rMSP4 might be useful for the development of vaccine against anaplasmosis.

Key words: *Anaplasma marginale*, Bovine anaplasmosis, MSP4, Sequence of *msp*4

INTRODUCTION

Anaplasmosis is a bovine intraerythrocytic disease caused by the tick-borne pathogen *Anaplasma marginale* (Anaplasmataceae) (Dumler et al., 2001). This disease occurs in tropical and subtropical regions worldwide and causes significant economic losses in unstable endemic regions. Following transmission, cattle develop rickettsemia, accompanied by severe anemia, weight loss, abortion, and often death (Richey, 1981).

The outer membrane of *A. marginale* includes six major surface proteins (MSPs), designated as MSP1a, MSP1b, MSP2, MSP3, MSP4, and MSP5 (Palmer and McGuire, 1984; Tebele et al., 1991; Visser et al., 1992; Oberle et al., 1993; Alleman and Barbet, 1996). Unlike MSP1a, MSP1b and MSP2, the initial body proteins, which vary widely in size among *A. marginale* isolates (Palmer and McElwain, 1995), significant variations in the size of a 31-kDa MSP4 protein have not yet been reported. MSP4 is encoded by a single gene and is highly conserved among different geographic isolates of *A. marginale* (Palmer and McGuire, 1984; Oberle et al., 1993). MSP4 was also found to be conserved in all Brazilian isolates analyzed by Western blotting with rabbit anti-MSP4 from the Florida strain (Kano et al., 2002; Oliveira et al., 2003). However, isolates from Minas Gerais, Brazil, have different *msp*4 sequences (de la Fuente et al., 2004). Four different *msp*4 genotypes have also been identified in *A. marginale* strains isolated from cattle farms in the province of Palermo, Sicily (de la Fuente et al., 2005).

The MSP4 outer membrane protein is a potentially useful antigen for the control of anaplasmosis; however, nothing is known about its structure or function. It is important to have information about the epitopes responsible for induction of protective immune responses by the MSP4 protein. We cloned, expressed and antigenically characterized MSP4 from an *A. marginale* isolate from Paraná, Brazil.

MATERIAL AND METHODS

Anaplasma marginale isolate

The isolate PR1 was purified following experimental infection of splenectomized cattle, which had previously tested negative for *A. marginale* by ELISA. Blood samples were collected by venipuncture into acid citrate, and washed four times in phosphate-buffered saline (PBS) to remove plasma and buffy-coat. Erythrocytes were suspended in PBS to a final concentration of 10^9 erythrocytes per mL; then they were frozen at -20°C, until used. DNA extraction from parasitized blood obtained from the splenectomized cattle was performed according to the protocol of the Puregene Genta System®.

Cloning of the *msp*4 gene and DNA sequence analysis

DNA from the PR1 *A. marginale* isolate was used to amplify the *msp*4 gene by polymerase chain reaction (PCR). A pair of primers was constructed according to sequences in GenBank (L01987) F (5'-CACCATGAATTACAGAGAATTG-3') and R (5'-GCTGAACAGGA ATCTTGCTCC-3'). The CACC sequence from the 5' end of the forward primer anneals to the overhang sequence GTGG in pET102/D-TOPO® vector (6.3 kb) (Invitrogen, Carlsbad, CA, USA).

PCR was carried out in a total volume of 50 µL, containing 50 ng of DNA template, 1 µL each of the primers at 20 pmol, 200 µM of each deoxynucleoside triphosphate, 1X *Pfx* platinum buffer, 0.5 µL 50 mM of magnesium sulfate, 0.5X enhancer solution (Invitrogen), and 2.5 U *Pfx* platinum DNA polymerase (Invitrogen Life Technologies). PCR conditions were as follows: 94°C for 5 min, followed by 30 cycles at 94°C for 1 min, annealing at 55°C for 1 min, and 68°C for 1 min, followed by a final extension at 68°C for 7 min in a thermal cycler (Gene Amp PCR System 9700/Perkin Elmer). The amplified DNA was visualized in 1.0% agarose gels stained with ethidium bromide. A 100-bp ladder (Promega, Madison, WI, USA) was used as a standard for determining the molecular mass of PCR products. PCR products were quantified; 20 ng was used as an insert in the pET102/D-TOPO® vector (Invitrogen Life Technologies).

Chemically competent *Escherichia coli* host strain TOP10 cells (Invitrogen) were transformed with 3 µL of the cloning reaction. Then, 200 µL of transformed bacteria culture was spread on selective plates containing 100 µg ampicillin and incubated at 37°C, overnight. These positive clones were grown in Luria Bertani broth containing ampicillin, before extraction of the plasmid by alkaline lysis (Sambrook et al., 1989). The presence of *msp*4 inserts was confirmed by restriction digestion of recombinant plasmids with *Bam*HI, and by PCR, using *msp*4 and pET102/D-TOPO® (*Trx Fus* forward: 5'-TTCCTCGACGCTAACCTG-3' and *T7* reverse: 5'-TAGTTATTGCTGAGCGGTGG-3') primers.

PCR products of the *msp*4 gene were cloned with the pCR4-TOPO TA cloning kit for sequencing (Invitrogen™) and sequenced using Big Dye Terminator (Applied Biosystems, CA, USA) and the gene primers. The sequences were submitted to BLASTn and BLASTx (Altschul et al., 1990, 1997) through the NCBI website (http://www.ncbi.nlm.nih.gov/) to check sequence identity. DNA and amino acid sequence analysis were carried out with the computer programs "CAP3 Contig Assembly Program", "ClustalW (1.81) Multiple Sequence Alignments" and "Six Frame Translation of Sequence".

Expression of the *msp*4 gene in the *Escherichia coli* strain and purification of rMSP4

E. coli BL21 was transformed with the recombinant plasmid pET10-*msp*4. The BL21/pET10-*msp*4 strain was grown to an optical density of 600 nm 0.8; IPTG (isopropyl-1-β-D-thiogalactopyranoside) (Invitrogen Life Technologies) was added to a final concentration of 1 mM and aliquots were removed at different times to choose the best time for expression. The cells were collected by centrifugation and expression was analyzed from soluble and insoluble fractions on 12% SDS-PAGE gels.

For rMSP4 purification, the BL21/pET10-*msp*4 strain was grown for 5 h at 37°C. The cells were collected by centrifugation and incubated in buffer containing 6 M guanidine-HCl, 20 mM $NaPO_4$, and 10 mM Tris-HCl, pH 7.8 (100 mM NaH_2PO_4, 10 mM Tris-HCl, 8 M urea, pH

8.0) for 1 h at room temperature to assure thorough cell lysis; the cell lysate was sonicated on ice with three 5-s high-intensity pulses. The lysate was then centrifuged at 10,000 *g* for 30 min and the supernatant was transferred to Ni-NTA resin (Qiagen®), previously washed with denaturing-binding buffer (8 M urea, 20 mM $NaPO_4$, 10 mM Tris-HCl, pH 7.8). The supernatant and resin were incubated for 1 h with rotation. After centrifugation at 2,000 rpm (in a microcentrifuge), the resin was washed twice with denaturing-binding buffer, and twice with denaturing wash buffer (100 mM NaH_2PO_4, 10 mM Tris-HCl, 8 M urea, pH 6.3). The protein was eluted using elution buffer (100 mM NaH_2PO_4, 10 mM Tris-HCl, 8 M urea, pH 4.5). The protein content of the purified rMSP4 was measured using the Bradford (1976) method and analyzed on 12% SDS-PAGE gels.

SDS-PAGE and Western blotting

Lysates and purified proteins were suspended in electrophoresis sample buffer (0.025 M Tris-HCl, 2% SDS, 15% glycerol, 2.5% 2-mercaptoethanol, pH 6.8), boiled for 5 min, and electrophoresed on 8% SDS-PAGE gels. The gels were either stained with Coomassie blue or were used for Western blotting. For Western blotting, proteins were transferred onto nitrocellulose membranes (Pharmacia Biotech) (Towbin and Gordon, 1984), and the membranes were blocked in blocking buffer (PBS + 0.1% Tween 20 + 5% nonfat dry milk) for 1 h at room temperature, with agitation. Membranes were washed in PBS-T (PBS + 0.1% Tween 20) and incubated for 1 h with a 1:5000 dilution of anti-His (C-term)-HRP (Invitrogen) directed against the hexamer histidine tag. Monospecific polyclonal anti-MSP4 serum (1:2000) and HRP anti-rabbit IgG 1:2000 (Sigma Immuno Chemicals) were also used. The membranes were washed and the MSP4 was detected by means of the enhanced chemiluminescence Western Blotting System (Amersham International, Amersham, UK). Protein molecular mass markers (Rainbow™ colored, Amersham Life Science) were used as standards.

Immunizations with rMSP4

Two rabbits were maintained according to the National Institute of Health rules for use of laboratory animals. The animals were immunized with rMSP4 to produce hyperimmune serum. Each rabbit was immunized subcutaneously with 100 µg of protein, together with complete Freund's adjuvant, followed by three inoculations with incomplete Freund's adjuvant after 14 days. Five days after the last boost, the rabbits were bled, and the serum was separated, inactivated, and adsorbed on *E. coli* BL21 Star™ (DE-3). Control serum was obtained from a non-immunized animal.

The immune response against rMSP4 was analyzed by Western blotting using a pool of preimmune sera (1:2000 dilution) and a pool of immune serum produced against rMSP4 (1:5000).

RESULTS AND DISCUSSION

Cloning and sequencing of *msp*4

The genetic and antigenic conservation of a protective protein is important for its efficacy as a vaccine. Serum from *A. marginale* outer membrane-immunized cattle, which were

protected against challenge by a virulent isolate, recognized MSP4 (Oberle et al., 1993), and the immunization of cattle with native MSP4 induced protection against challenge with a homologous isolate of *A. marginale* (Palmer ad McElwain, 1995). We sequenced the *msp*4 gene from the PR1 *A. marginale* isolate, producing an 849-bp fragment (GenBank accession number AY714546).

Analysis of sequence variation in the PR1 *A. marginale msp*4-coding region showed some heterogeneity in nucleotides (97.9 identity) and predicted amino acid sequences (99% similarity), when compared to the Florida strain. The *msp*4 sequence of the PR1 isolate differed from the sequence of Minas Gerais isolates only at nucleotide position 798. Some heterogeneity of *msp*4 was observed among *A. marginale* Brazilian isolates from endemic areas, showing that this gene can be used to obtain phylogeographic information (de la Fuente et al., 2004).

The colonies obtained from cloning *msp*4 into pET102-TOPO were screened first by PCR with specific primers for the *msp*4 gene and the vector; all clones showed the amplicon, as expected. After cleavage of the recombinant plasmid pET102-*msp*4 from a positive clone with the restriction enzyme *Bam*HI, a 7.2-kb fragment was released, corresponding to 6.3 kb of the vector plus 0.85 kb of the insert.

Expression in *Escherichia coli* BL21 and purification under denaturing conditions

The recombinant plasmid pET102-*msp*4 was transformed into the *E. coli* BL21 (DE-3) expression host, a bacterial strain designed for gene expression regulated by the T7 promoter. This expression vector was chosen based on the presence of sequences encoding six histidine residues, V5 epitope tag and His-Patch thioredoxin. The MSP4 fusion protein should have an estimated size of about 47 kDa on SDS-PAGE. The expected band around 47 kDa was more evident after 5 h of induction with IPTG (Figure 1, line 2). This band was observed in the insoluble fraction and in inclusion bodies (Figure 1, line 3). Although His-Patch thioredoxin increases the solubility of recombinant proteins, rMSP4 was insoluble. The 47-kDa band was absent in the negative control.

rMSP4 was solubilized and purified with the Ni-NTA purification system under denaturing conditions, using urea. The targeted 6xHis-tag MSP4 was eluted in fractions, among which the third and fourth gave the highest concentrations (Figure 1, lines 5 and 6). Based on SDS-PAGE, rMSP4 was successfully expressed in BL21 (DE-3) and purified by means of Ni-NTA.

The monospecific polyclonal serum produced against rMSP4 of Florida strain and anti-His antibody reacted with a 47-kDa protein from BL21/pET102-*msp*4 induced by IPTG and also reacted with rMSP4 in Western blotting (Figure 2), confirming the fusion protein expression. This approximately 55-kDa protein is nonspecific since it reacted with rabbit serum and because this protein was also present in untransformed BL21.

The rMSP4 used to immunize rabbits induced an immune response that produced specific anti-MSP4 reactive with both recombinant (47 kDa) and native MSP4 (Figure 3), showing that rMSP4 has conserved epitopes and maintained antigenicity. This result agrees with those obtained by Oberle et al. (1993), who found a single conserved gene coding MSP4, in contrast to MSP1b, MSP2 and MSP3, which are encoded by a multigene family (Palmer et al., 1994; Alleman et al., 1997; Barbet et al., 2000; Brayton et al., 2002) and can change by intragenic recombination. Previous study demonstrated conservation of MSP4 in Brazilian isolates by means of Western blotting with monoclonal antibodies (Kano et al., 2002; Oliveira et al., 2003).

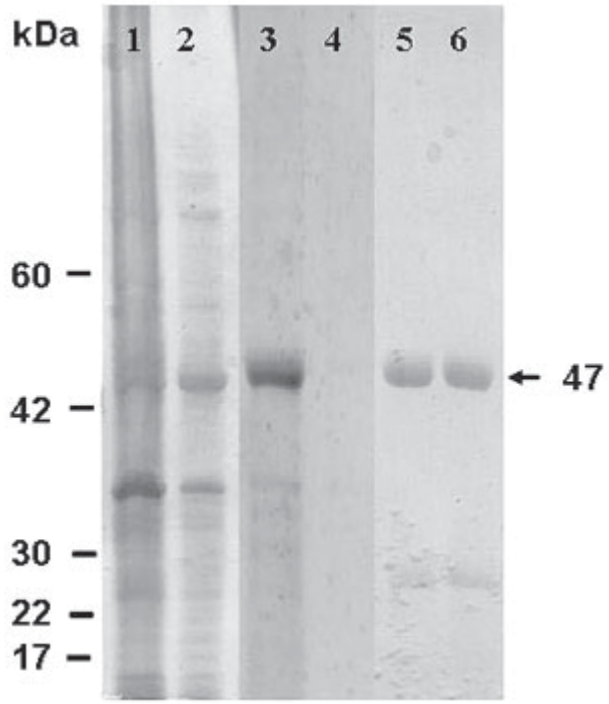

Figure 1. SDS-PAGE (12%) gel stained with Coomassie brilliant blue shows expression and purification of rMSP4 protein from clone BL21/pET102-*msp*4. *Lane 1*, BL21/pET12/*msp*4 not induced; *lane 2*, BL21/pET102/*msp*4 induced with 1 mM IPTG; *lane 3*, insoluble fraction; *lane 4*, soluble fraction; *lanes 5* and *6*, 3rd and 4th elution fractions.

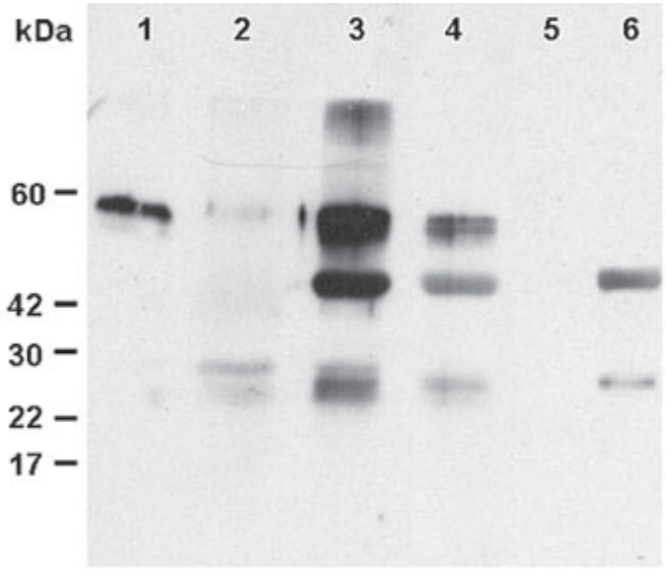

Figure 2. Western blotting of recombinant MSP4. *Lanes 1* to *4* were reacted with polyclonal anti-MSP4 rabbit serum produced against Florida strain and *lanes 5* and *6* were reacted with anti-HIS-TAG. *Lane 1*, BL21 strain; *lane 2*, BL21/pET102-*msp*4 not IPTG induced; *lane 3*, BL21/pET102-*msp*4 induced with IPTG; *lane 4*, eluted rMSP4; *lane 5*, BL21 strain; *lane 6*, eluted rMSP4.

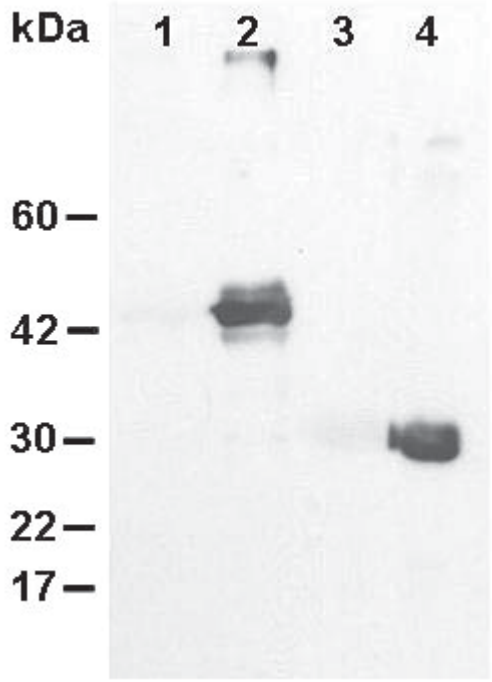

Figure 3. Western blotting of recombinant and native MSP4. *Lanes 1* and *3*, pre-immune rabbit serum and *lanes 2* and *4*, anti-rMSP4 polyclonal rabbit serum produced against PR1 recombinant MSP4. *Lanes 1* and *2*, eluted rMSP4; *lanes 3* and *4*, *Anaplasma marginale* initial bodies PR1 strain.

We conclude that *msp*4 is highly conserved and that the 47-kDa rMSP4 possesses conserved epitopes and maintains antigenicity, suggesting its use for subunit vaccine development.

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