

## Cloning, sequencing and antigenic characterization of rVirB9 of *Anaplasma marginale* isolated from Paraná State, Brazil

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**ABSTRACT.** *Anaplasma marginale*, a tick-borne bacterium, causes bovine anaplasmosis responsible for significant economic losses in tropical and subtropical regions worldwide. Various major outer membranes have been described, and VirB9, a type IV secretion system protein, has been recently indicated as a candidate in vaccine development against anaplasmosis. The *virB9* gene of an *A. marginale* strain isolated in Paraná, Brazil, was cloned by polymerase chain reaction and sequenced; its cloning into the pETSUMO vector produced a *virB9*-SUMO-6X His fusion gene construct. This recombinant clone was over-expressed in *Escherichia coli* BL21 (DE3), and the expressed fusion protein was solubilized with urea and purified with an Ni-NTA column. This method produced a relatively high yield of rVirB9. The deduced amino acid sequence encoded by VirB9 showed 99% homology to *A. marginale* isolates from St. Maries. rVirB9 was recognized by serum from cattle immunized with PR1 strain and by bovine sera infected with heterologous strains, showing that rVirB9 has conserved epitopes, which suggests that rVirB9 could be useful for the development of a vaccine against anaplasmosis.

**Key words:** *Anaplasma marginale*; Bovine anaplasmosis; Sequence of VirB9

## INTRODUCTION

*Anaplasma marginale*, a tick-borne bacterium, causes in cattle severe anemia, weight loss, abortion, and often death, resulting in significant economic losses in tropical and subtropical regions worldwide (Richey, 1981).

Immunization with purified outer membranes induces protection against acute *A. marginale* infection and disease (Tebele et al., 1991). Some well-characterized outer membrane proteins designated major surface proteins (MSPs), MSP1a, MSP1b, MSP2, MSP3, MSP4, and MSP5, were evaluated as potential candidates for antigens in vaccine production, diagnostic evaluations, and determination of intermolecular relationships (Vidotto et al., 1994; Palmer and McElwain, 1995; Alleman et al., 1997). Antibody responses to outer membrane vaccines are primarily directed against MSP2 and MSP3, but these proteins undergo antigenic variation and do not confer protection (Palmer et al., 2006). Twenty-one other proteins were identified within the outer membrane immunogen based on the proteomic and genomic approach (Lopez et al., 2005). Among the newly described proteins were type IV secretion system (TFSS) proteins VirB9, VirB10 and conjugal transfer protein (CTP) (Lopez et al., 2007).

In other Gram-negative bacteria, TFSS proteins form channels that facilitate the secretion of molecules, and are required for intracellular survival (Christie et al., 2005). The importance of the TFSS in intracellular survival and virulence has been widely documented in *Brucella suis*, *Legionella pneumophila* and *Helicobacter pylori* (Censini et al., 1996; Foulongne et al., 2000; Lamertyn and Anne, 2004). Also, these proteins have been described in rickettsial pathogens, but their functions are less well understood (Ohashi et al., 2002; Felek et al., 2003; Niu et al., 2006).

Because of their surface localization, highly conserved nature, and requirement for intracellular survival, Gram-negative bacterial TFSS proteins are logical targets for immunological intervention (Lopez et al., 2007). *A. marginale* VirB9, VirB10, and CTP induced B and T lymphocyte responses in outer membrane-immunized cattle. VirB9 induced the greatest proliferation in CD4<sup>+</sup> T cell lines, and VirB9-specific CD4<sup>+</sup> T cell clones responded to three *A. marginale* strains, confirming that the VirB9-specific T cell responses are directed against epitopes in the native protein (Lopez et al., 2007).

The objectives of the present study were the cloning, sequencing and antigenic characterization of VirB9 in an *A. marginale* strain from Paraná, Brazil.

## MATERIAL AND METHODS

### *Anaplasma marginale* strain and serum

DNA extraction from parasitized blood of splenectomized cattle and infected with the PR1 strain was performed according to the protocol of the Puregene Genta System®.

### Cloning of the *virB9* gene and DNA sequence analysis

DNA from the PR1 *A. marginale* isolate was used to amplify the *virB9* gene by polymerase chain reaction (PCR). A pair of primers was constructed according to the sequence of GenBank VirB9 F (5'-ATGAATTTCTATAAACTTGCTTGCG-3') and VirB9 R (5'-CTAAAGCACCGTATTCACTACTTCGAC-3').

PCR was carried out in a total volume of 50  $\mu$ L containing 50 ng DNA template, 1  $\mu$ L each of the primers at 20 pmol, and 200  $\mu$ M of each deoxynucleoside triphosphate (Invitrogen Life Technologies). PCR conditions were as follows: 94°C for 5 min followed by 30 cycles of 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 on 1.0% agarose gels stained with ethidium bromide. The 100-bp ladder (Promega, Madison, WI, USA) was used as a standard for determining the molecular mass of PCR products. PCR products were quantified and 20 ng was used as insert in the pETSUMO<sup>®</sup> vector (5.5 kb) (Invitrogen, Carlsbad, CA, USA).

Chemically competent *E. coli* host strain TOP10 cells (Invitrogen) were then transformed with 3  $\mu$ L of the cloning reaction. Next, 200  $\mu$ L of transformation was spread on selective plates containing 100  $\mu$ g ampicillin and incubated at 37°C overnight. These positive clones were grown in LB containing ampicillin before extraction of the plasmid by alkaline lysis (Sambrook et al., 1989). The presence of *virB9* inserts was confirmed by restriction digestion of recombinant plasmids with *Apa*I, and by PCR using SUMO forward (5'-AGATTCTTGTACGACGGTATTAG-3') T7 reverse (5'-TAGTTATTGCTGAGCGGTGG-3') and VirB9 primers.

The *virB9* gene was sequenced using BigDye Terminator (Applied Biosystems, CA, USA) and the gene primers. The sequences were submitted to BLAST 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 computational programs "CAP3 Contig Assembly Program" and "Clustal W (1.81) Multiple Sequence Alignments" and "Six Frame Translation of Sequence".

### Expression of *virB9* gene in *E. coli* strain and purification of rVirB9

*E. coli* BL21 was transformed with the recombinant plasmid pETSUMO-*virB9*. The BL21/pETSUMO-*virB9* strain was grown to an OD<sub>600 nm</sub> of 0.8. Isopropyl-1- $\beta$ -D-thiogalactopyranoside (IPTG; Invitrogen Life Technologies) was then added to 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 determined in soluble and insoluble fractions on 12% SDS-PAGE gels.

For rVirB9 purification, the BL21/pETSUMO-*virB9* strain was grown for 4 h at 37°C. The cells were collected by centrifugation and incubated in buffer containing 6 M guanidine-HCl, 20 mM NaPO<sub>4</sub>, 10 mM Tris-HCl, pH 7.8 (100 mM NaH<sub>2</sub>PO<sub>4</sub>, 10 mM Tris-HCl, 8 M urea, pH 8.0) for 1 h at room temperature to assure thorough cell lysis, and the cell lysate was sonicated on ice with three 5-s pulses at high intensity. The lysate was then centrifuged at 10,000 g for 30 min, and the supernatant was transferred to Ni-NTA resin (Qiagen<sup>®</sup>) previously washed with denaturing binding buffer (8 M urea, 20 mM NaPO<sub>4</sub>, 10 mM Tris-HCl, pH 7.8). The supernatant and resin were incubated for 1 h with rotation. After centrifugation at 2000 rpm (microcentrifuge), the resin was washed twice with denaturing binding buffer, and twice with denaturing wash buffer (100 mM NaH<sub>2</sub>PO<sub>4</sub>, 10 mM Tris-HCl, 8 M urea, pH 6.3). The protein was eluted using elution buffer (100 mM NaH<sub>2</sub>PO<sub>4</sub>, 10 mM Tris-HCl, 8 M urea, pH 4.5). The protein content of the purified rMSP4 was measured using the Bradford 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. 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 bovine polyclonal sera (1:2000). The membranes were washed and the VirB9 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.

## RESULTS AND DISCUSSION

### Cloning and sequencing of *virB9* gene

The genetic and antigenic conservation of a protective protein is important for its efficacy as a vaccine. The TFSS proteins VirB9, VirB10 and CTP are components of the protective *A. marginale* outer membrane fraction and because they are highly conserved due to functional constraints, they may be more appropriate candidates for vaccines than the antigenically variant MSP2 and MSP3 (Lopez et al., 2005, 2007).

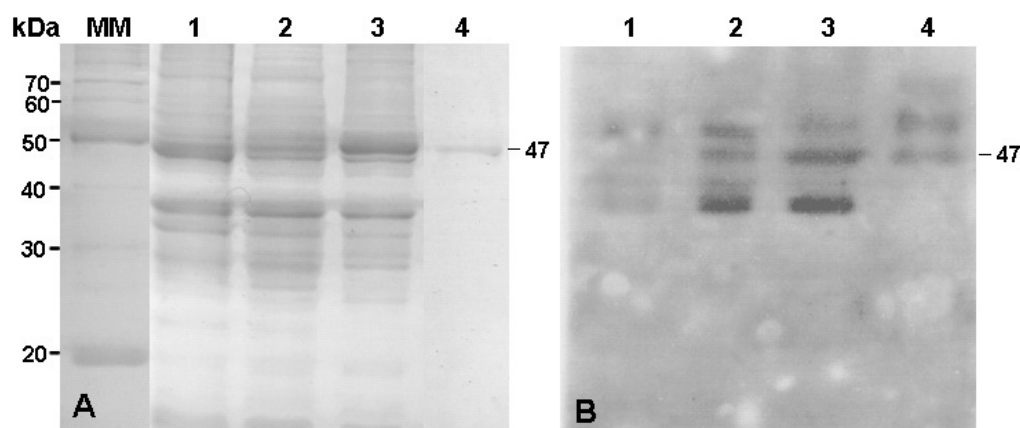
In this study, the *virB9* (840 bp) from the PR1 *A. marginale* strain was cloned and sequenced. The colonies obtained from cloning into pETSUMO were screened first by PCR with specific primers for the *virB9* gene and the vector. All clones VirB9<sup>+</sup> showed the amplicon, and after cleavage of the recombinant plasmid pETSUMO-*virB9* from a positive clone with the restriction enzyme *Hind*III, a 6.4-kb fragment was released corresponding to 5.6 kb of the vector plus 0.84 kb of the insert. The cleavage of the pETSUMO-*virB9* with *Apa*I showed two fragments of 4.4 and 2.0 kb, demonstrating the correct position of *virB9* gene.

The complete sequence of *virB9* gene amplified from the PR1 *A. marginale* was deposited in GenBank (accession number 1064466). The analysis of the *virB9*-coding region from the PR1 strain showed high identity in nucleotides (98%) with the sequence of *virB9* from the St. Maries strain of *A. marginale* (accession number YP\_154362). When the amino acid sequence of VirB9 from the PR1 strain was compared with the St. Maries strain, there was 99% similarity. Additionally, the St. Maries and Florida strains have complete amino acid identity in VirB9, and the topology prediction algorithm calculated VirB9 to be either an outer membrane protein or surface associated protein (Lopez et al., 2007). These predictions are consistent with surface localization of similar TFSS proteins in other bacteria (Christie et al., 2005).

### Expression of *virB9* gene in *E. coli* BL21 and characterization of rVirB9

The recombinant plasmid pETSUMO-*virB9* was used to transform *E. coli* BL21

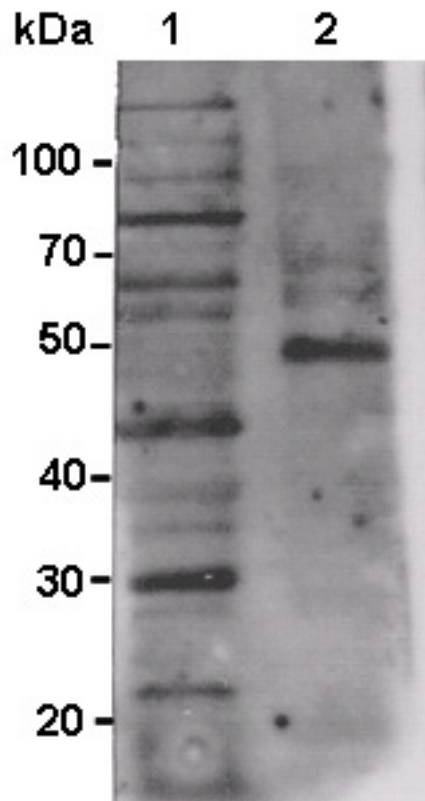
(DE3), a bacterial strain designed for gene expression regulated by the T7 promoter. This expression vector was chosen based on the presence of sequences encoding six histidine residues, V5 epitope tag and His-Patch thioredoxin. The VirB9 fusion protein should have an estimated size of about 47 kDa on SDS-PAGE. The expected band around 47 kDa was more evident after 4 h of induction with IPTG (Figure 1A, lane 3). This band was observed in the insoluble fraction or inclusion bodies. Although His-Patch thioredoxin increases the solubility of recombinant proteins, the results showed that rVirB9 is insoluble. The 47-kDa band was absent in the negative control.



**Figure 1.** Expression and purification of rVirB9 protein from clone BL21/pETSUMO-*virB9*. **A.** 12% SDS-PAGE stained with Coomassie brilliant blue. **B.** Western blotting of rVirB9 with homologous serum, polyclonal serum produced against PR1 strain. *Lane 1*, BL21; *lane 2*, BL21/pETSUMO-*virB9* not induced; *lane 3*, BL21/pETSUMO-*virB9* induced with 1 mM IPTG; *lane 4*, elution protein. MM = molecular mass.

rVirB9 was solubilized and purified with the Ni-NTA purification system under denaturing conditions using urea. The targeted 6X His-tag rVirB9 was eluted in fractions (Figure 1A, lane 4). The results obtained from SDS-PAGE showed that rVirB9 was successfully expressed in BL21 (DE3) and purified by means of Ni-NTA.

The polyclonal serum from cattle immunized with PR1 strain of *A. marginale* reacted with a 47-kDa protein from BL21/pETSUMO-*virB9* induced by IPTG and with rVirB9 in Western blotting (Figure 1B). Pre-immune serum did not react with rVirB9. Additionally, rVirB9 reacted with bovine polyclonal heterologous sera from cattle of other regions of Brazil by Western blotting (Figure 2), showing conserved epitopes and antigenicity. These results agree with those obtained from Lopez et al. (2007), which showed the presence of a conserved gene coding for rVirB9 and antibody from outer membrane vaccines recognizing recombinant forms of the proteins studied.



**Figure 2.** Western blotting of rVirB9 with heterologous serum. *Lane 1*, BL21 strain; *lane 2*, eluted VirB9.

In outer membrane-immunized cattle, three *A. marginale* TFSS proteins, VirB9, VirB10, and CTP, elicit significant CD4<sup>+</sup> T lymphocyte proliferation, IFN- $\gamma$  secretion, and IgG2 production, immune responses associated with protective immunity (Lopez et al., 2007). However, VirB9 was selected since this antigen was consistently recognized by memory T cells and induced strong proliferative responses from all cattle tested (Lopez et al., 2007). Transcriptional analyses of TFSS genes in *E. chaffeensis* and *A. phagocytophilum* have shown that VirB8, VirB9, VirB10, VirB11, and VirD4 are transcribed polycistronically (Ohashi et al., 2002). However, in *E. canis*, VirB9 is expressed throughout various stages of infection, and is antigenic in *E. canis*-infected dogs (Felek et al., 2003). Additionally, elevated levels of VirB9 were expressed during replication of *A. phagocytophilum* within neutrophils, whereas basal levels of VirB9 were expressed upon neutrophil lysis (Niu et al., 2006), demonstrating differential levels of expression of TFSS proteins during the mammalian infection cycle.

This study shows that rVirB9 is highly conserved and that the rVirB9 possesses conserved epitopes and maintained antigenicity, suggesting its use for subunit vaccine development.

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