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CHARACTERIZATION OF RECOMBINANT MSP5 *ANAPLASMA MARGINALE* HAVANA ISOLATE

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ABSTRACT

Anaplasma marginale is the causative agent of bovine anaplasmosis, a disease of worldwide economic importance. Major surface proteins (MSPs) are involved in host-pathogen and tick-pathogen interactions and they have been used as markers for the genetic characterization of *A. marginale* strains and phylogenetic studies. The major surface protein 5 (MSP5) is highly conserved in the genus *Anaplasma* and in all isolates of *A. marginale.* The aim of the present work was to carry out the cloning, sequencing and characterization of the recombinant MSP5 *Anaplasma marginale* Havana isolate. The sequence of the *msp5* gene of *Anaplasma marginale* Havana isolate with a size of 633 pb was determined (Acc. No. AY527217). This gene was cloned into pRSETB vector and expressed in *Escherichia coli.* The MSP5 protein was recognized by the monoclonal antibody ANAF16C1 and it showed a high similitude percent with the gene sequence described for other *Anaplasma marginale* isolates. These data are very important for the development of a diagnostic test for *A. marginale* using the MSP5 recombinant protein.

Key words: *Anaplasma marginale*, major surface proteins, characterization, Havana isolate, HMSP5

INTRODUCTION

Anaplasma marginale parasites mainly cattle mature erythrocytes causing severe economic losses in the tropical and subtropical regions (21). Six membrane surface proteins of the initial bodies of this organism, carriers of epytopes B and T have been characterized. They have been named major surface proteins (MSPs) and identified as 1a, 1b, 2, 3, 4 and 5. These proteins are recognized by neutralizing antibodies and they have a strong intermolecular relationship in the membrane of the initial bodies, performing important functions (20).

Genes encoding these proteins have been studied, showing their protein products to have a variable polymorphism. They can be represented in the genome by a single copy gene or forming part of multigenic families (4). The *msp5* gene is represented in the genome as a single copy, highly conserved among all *Anaplasma* species and all the *A. marginale* isolates studied. The MSP5 protein, for which it encodes, is of little structural complexity, equally conserved and it induces high antibody titters (10), thus, it is a strong candidate for bovine anaplasmosis diagnosis.

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In spite of the high economic losses produced world-wide every year, there is not an effective control method against the disease nowadays. Thus, it is important to develop a vaccine capable of preventing the infection with this pathogen and to rely on a more sensible diagnostic allowing the detection of carrier animals to be used in epyzootiological studies and for disease control (6).

Different techniques are used for anaplasmosis diagnostic: agent detection, Giemsa stain of blood smears, used for clinical cases; splenectomiced animals inoculation being the gold standard for the disease diagnostic, but it is not a practical system for routine diagnostic (31); and the molecular diagnostic, used nowadays for cattle international movement, due to it sensitivity and specificity. For antibody detection, complement fixation was widely used some decades ago; nowadays it is not used for the detection of asymptomatic animals due to its low sensitivity (33). At the present time, card agglutination, indirect immunofluorescence and ELISA test are used for antibody detection. Many of such techniques use crude antigens contaminated with erythrocytes, which give a high number of false negatives when persistently infected animals are tested (11). There are some ELISA reports developed from an *A. marginale* recombinant antigen using the MSP5 (25, 29) and using the MSP5 from *A. phagocytophilum* (1). Immunochromatographic tests of lateral flow constitute a very useful tool since they allow a quick obtainment of reliable data on diverse affections (23). The objective of the present study was to clone and sequence the *msp5* gene of *Anaplasma marginale* Havana isolate and to express and characterize the HMSP5 protein to be used for the diagnostic of bovine anaplasmosis.

MATERIALS AND METHODS

Blood from bovines experimentally infected with four Cuban isolates (two from western region: Havana and Valle del Peru, and two of the eastern region: East 1 and East 2) and bovine blood experimentally infected with *Babesia bigemina* and *B. bovis,* washed with PBS and preserved in 30% glycerol, were provided by the CENSA Parasitology and Immunology group, Cuba. Florida and Idaho isolates DNA, used as positive controls were gently provided by Dr. Guy H. Palmer (Veterinary Medicine and Parasitology Department, Washington State University).

Anaplasma marginale Cuban isolates DNA was purified according to the methodology described by Ambrosio and Potgieter (3). *B. bigemina* and *B. bovis* DNA purification was carried out according to the protocol described by Fahrimal *et al*. (7). Bovine DNA was purified according to the methodology described by Osta (17). *Tripanosoma* spp. DNA was provided by Genetics Laboratory, CINVESTAV, Mexico.

The *msp5* gene from *Anaplasma marginale* was amplified by PCR using the following primers: *5´CGGGATCCGATGAGAATTTTCAAG3´* and *5´CGGGATCCCTAAGAATTAAGCATG3´,* corresponding to the flanking regions of this gene for Florida isolate (Acc. No. M93392). Target DNA (50 ng) was amplified in a mixture of 0.4 mM deoxynucleoside triphosphates, 0.5 μM each primer, 20 mM $MgCl_2$, 100 mM KCl, 200 mM Tris-HCl (pH 8.2), 60 mM $(NH_4)_2SO_4$, 1% Triton X-100, 100 μg mL^{-1} of nuclease-free bovine serum albumin, and 2.5 U of native *Pfu* DNA polymerase (Promega). PCR was performed at 94°C for 1 min, followed by 30 cycles of 94°C for 1 min, 53°C for 1 min, 72°C for 1 min, with a final extension step at 72°C for 10 min. The amplicon was analyzed by gel electrophoresis on a 0.8% agarose gel in 1X TBE buffer (89 mM Tris, 89 mM boric acid, 2 mM disodium EDTA), containing ethidium bromide 0.5 μg mL^{-1}.

The amplified product was processed according to the methodology described by Sambrook *et al*. (27), digested with *BamH* I (Promega) and purified using the Wizard DNA CleanUp Resin kit protocol (Promega). The pRSET B plasmid (Invitrogen) was used to clone this gen. It was digested with *BamH* I (Promega), desphosphorilated with alkaline phosphatase (CIAP, Biolabs) and purified using the NucleinClean kit protocol (Sigma).

The fragment ligation was carried out using a DNA ligation kit (Amersham). The product was transformed into TOP 10F´ cells. The selection of recombinants was carried out using restriction enzyme digestion with *Bam*H I (Promega), and PCR using the primers described below. The direction of the cloning was confirmed by digestion with *Eco*R I (Promega). The results were analyzed on a 0.8% agarose gel. A clone containing a fragment of 633 bp in the correct direction was produced and purified by Plasmid Pure DNA Miniprep kit (Sigma) and the DNA purity was confirmed by measuring the optical density at 260 and 280 nm, followed by electrophoresis on agarose gel. It was denominated pHMSP5.

The sequence was determined by an automatic sequencer ALFexpress II (Amersham Pharmacia Biotech), using the same primers and reagents of Thermo Sequenase Cy5 Dye Terminator Kit (Amersham Pharmacia Biotech). The analysis and alignment of the sequence was carried out using Fasta 3, version 3.4t21 programs (24) and Vector NTI Suite 7. The sequence was deposited in the GenBank with the accession number AY527217, reporting a 633 bp and 210 aminoacids, respectively.

With the objective of verifying the expression of MSP5 in *Escherichia coli*, the recombinant plasmid was transformed into BL21DE3pLysS cells. The cells transformed with the native and recombinant plasmids were grown in SOB medium, and induced with 1mM IPTG during six hours. The results of the MSP5 recombinant protein expression, named HMSP5, were corroborated by electrophoresis on 12% polyacrilamide-SDS gel. The proteins were transferred to nitrocellulose membranes and the immunological detections were done by immunoblot from the semidry transference of proteins to nitrocellulose membranes (Bio-Rad), according to the methodology described by Sachse *et al*. (26). For the immunological detection, the monospecific polyclonal antiserum X-MSP5, obtained in bovine with the native MSP5 protein (positive sera), the monoclonal antibodies ANAF16C1 (32) (positive sera) and Tryp1E1, anti-variable surface glycoprotein (VSG) of *Trypanosoma brucei* (negative sera) (provided by Washington State University) and the monospecific polyclonal antiserum anti MSP5 obtained in rabbit using the native MSP5 protein (provided by the CENSA Parasitology and Immunology group), were used. The molecular weight of HMSP5 protein was determined using the electrophoretic mobility of the proteins with respect to the molecular weight marker (Molecular Wide Weight Range, Sigma) and by densitometry using the Molecular Program Analyst Software (Bio-Rad Laboratories, Version 1.4.1, 1992-1996).

RESULTS AND DISCUSSION

Using *A. marginale* DNA from four different geographic regions of Cuba, the amplification of a 633 bp fragment was obtained (data not showed). This was in accordance with Visser *et al*. (32) that describing the *msp5* gene as a highly preserved single copy gene.

Similar to the *msp1α* and *msp4* genes, *msp5* is present in the genome as a single copy gene. This makes easy the use of this gene in the diagnostic of bovine anaplasmosis by PCR (29), being different from *msp1β*, *msp2* and *msp3* genes, which are present in multiple copies in *A. marginale* genome (19). MSP3 has been found to be particularly useful for detecting cattle that are long-term carriers of *A. marginale* (13). However, as MSP3 has been reported to be encoded by a polymorphic, multigene family (2), it remains to be determined which *msp3* alleles could be useful for producing recombinant protein which should contain immunodominat conserved epytopes useful for diagnostic purposes.

The *msp5* gene is conserved in all *Anaplasma* species: *A. marginale*, *A. centrale*, *A. ovis* and *A. phagocytophilum* and in all *A. marginale* studied isolates. It is present in the erythrocytes, as well as in all the *A. marginale* stages, including the in vitro organism culture (4). The protein expressed has an epytope, recognized by the monoclonal antibody ANAF16C1, conserved among the widely divergent strains (15), in *A. ovis* and in *A. centrale* salivary gland stage (4). That is why both constitute important candidates for *A. marginale* diagnostic.

The DNA from Havana Cuban isolate was selected as a template for amplification, cloning, sequencing and expression of this gene.

After the digestion of possible recombinant plasmids with *Bam*H I, the band of 2.9 kb, corresponding to the vector, and the band of 633 bp, corresponding to the cloned *msp5* gene appeared in the plasmids analyzed (Fig. 1). Amplification by PCR of the *msp5* gene was observed (Fig. 2). This corroborated the presence of the cloned gene in the plasmid. From the restriction enzyme analysis with *Eco*R I, the plasmids containing the gene cloned in the correct sense could be determined by the presence of two bands of 3.3 kb and 233 bp (Fig. 3).

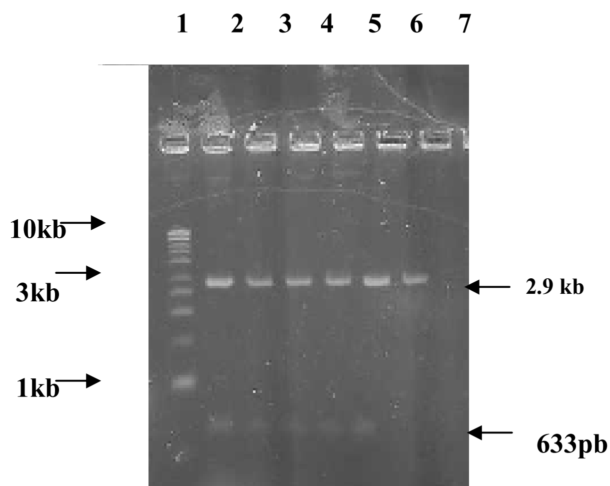

Figure 1. Restriction analysis with *Bam*H I of the possible recombinant plasmids. Line 1: Molecular Weight Marked 1 kb (Promega); 2-6: possible recombinant plasmids; 7: native pRSET B plasmid.

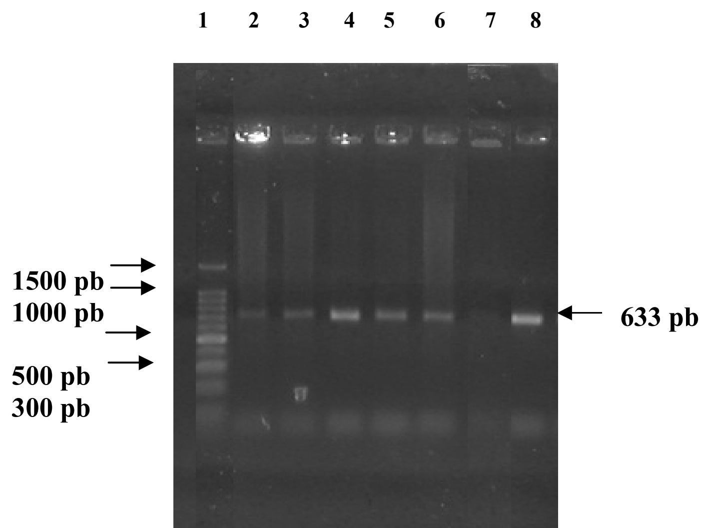

Figure 2. PCR amplification of *msp5* gene in the possible recombinant plasmids. Line 1: Molecular Weight Market 100 pb (Promega); 2-6: DNA of possible recombinant plasmids; 7: pRSET B; 8: DNA of *A. marginale* Havana isolate.

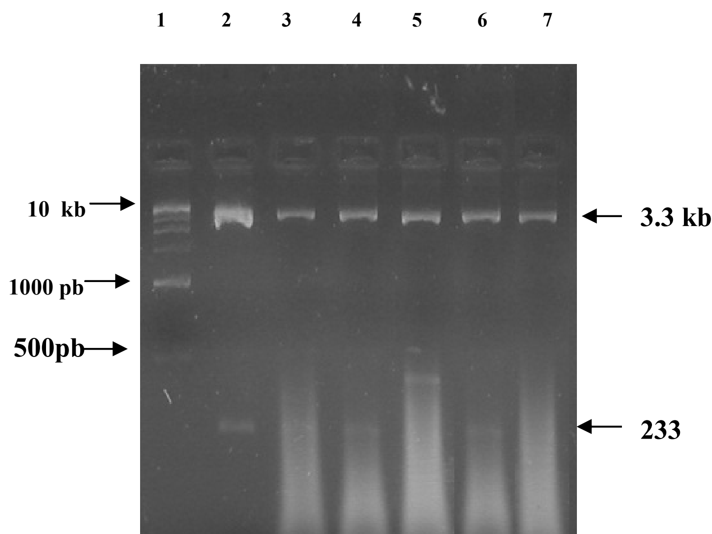

Figure 3. Restriction analysis with *Eco*R I to recombinant plasmids. Line 1: Molecular Weight Market 1 kb (Promega); 2, 3, 4, 6: plasmids with the *msp5* gene cloned in the correct sense; 5: plasmid with the *msp5* gene cloned in the incorrect sense; 7: pRSET B.

From these results, a plasmid named pHMSP5 was selected as recombinant and the sequence of the gene cloned was determined (AY527217). The analysis and comparison of the gene sequence allowed knowing that it had a 98.42% similarity with the sequence described for the Florida isolate (accession number M93392). This difference is given by precise changes of bases that cause no changes in the deduced sequence of amino acids, showing a 99.04% similarity. In the same way, *A. centrale* (accession number AY054384) showing 96.68% and 87.46% similarity in the nucleotide sequence was compared with the sequence of this gene described for a Brazilian isolate (Acc. No. AY245428) and a 97.14% and 92.85% with respect to the protein sequence.

Torioni *et al*. (29) analyzed the sequence of 30 products of a nested PCR with a segment of 345 bp of the *msp5* gene from animals of a herd. There was a 95% identity in the analyzed sequence, compared with the sequence described for the Florida isolate. Two of these sequences were identical to that of Florida isolate and the rest 28 only showed precise changes of bases. This favors our results regarding the conservation of this gene sequence in the *A. marginale* isolates studied. A similar *msp5* sequence conservation level was demonstrated in an animal (two peaks separated from rickettsemia cycle) (8). Kano *et al*. (9) demonstrated the conservation of MSP5 epytopes among all Brazilian isolates. This coincides with previous results indicating the conservation of this protein in America (10), Africa and Israel isolates (14). Strik *et al*., (28) demonstrated a 100% sequence identities in the *msp5* genes among all the *A. phagocytophilum* isolates from the United State and a horse isolated from Sweden.

To study the HMSP5 protein expression, BL21DE3pLysS *E. coli* cells were transformed with recombinant plasmid pHMSP5. In Figure 4, a band of an apparent molecular weight of 22 kDa can be observed in the protein pattern of this clone induced with IPTG. This band was neither observed in the cells transformed with the native plasmid and that were induced and not-induced with IPTG.

The molecular weight of the protein HMSP5, with respect to the weight pattern used and estimated by densitometry, was 22 kDa (Fig. 4), being higher than that described for this protein in the Florida isolate (19 kDa) (32) and other studied isolates, where this gene is expressed as a 19 kDa polypeptide in the surface membrane of the initial bodies (16). This size difference (3 kDa) is due to the expression system used, where the protein is expressed fused to a histidine hexapeptide in the N-terminal region. It is

known that the histidine residues fused to proteins in the regions N-terminal and C-terminal allow the small proteins to increase their size for being synthesized and fused to such residues, becoming more stable and allowing to be easily purified by IMAC (The QIA Expressionist, 1997).

MSP5 native protein maintains size conserved in all *A. marginale* isolates studied (16). Other *A. marginale* proteins, MSP1 and MSP2 show variation in their size; MSP1a is characterized by a size polymorphism up to 50% (70-105 kDa) (22). Size variation is more limited for MSP1b, which is up to 3% (97-100 kDa) (6), MSP2 up to 9% (33-36 kDa) among *A. marginale* strains (5; 12).

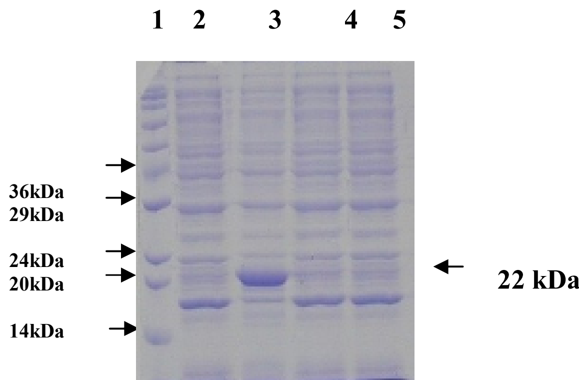

Figure 4. Expression of the *msp5* gene of *A. marginale* Havana isolate. Line 1: Molecular Wide Weight Range (Sigma); 2: Non induced recombinant strain BL21DE3pLysS/pHMSP5, 3: Recombinant strain BL21DE3pLysS/pHMSP5 induced with IPTG 1mM during six hours, 4: Non induced strain BL21DE3pLysS/pRSET B, 5: Strain BL21DE3pLysS/pRSET B induced with IPTG 1mM during 6 hours.

The HMSP5 protein expressed was recognized by a monospecific polyclonal antiserum X-MSP5, obtained in bovine, by the monoclonal antibody ANAF16C1 and by a monospecific polyclonal antiserum anti MSP5, obtained in rabbits. It was not recognized by the monoclonal antibody Tryp1E1, used as negative control (Fig. 5). In Figure 3A, unspecific bands were observed since the antiserum used was not previously absorbed against *E. coli*, and this bacterium is part of the normal flora of the digestive tract in bovine. That is why antibodies against *E. coli* proteins are present in the serum. The conservation of MSP1 and MSP2 epytopes among *A. marginale* isolates of Kenya, Israel and the United States, as well as *A. centrale* was previously demonstrated (18). Nevertheless, Ndung'u *et al*. (16) demonstrated for the first time that a common epytope defined by the monoclonal antibody ANAF16C1 was in *A. marginale* and *A. ovis*, despite the latter was a primary pathogen of ewes. The results obtained by Molad *et al*., (14) show that the protein MSP5 of *Anaplasma marginale* and *Anapalsma ovis* has a common epytope defined by this monoclonal antibody. However, the results obtained by Alleman *et al*. (1) indicate that that recombinant MSP5 has potential for use as a diagnostic test antigen to detect infection with *A. phagocytophilum* in both dogs and humans, but sequence similarities among orthologs of MSP5 in related species of anaplasma and ehrlichia suggest that cross-reactivity among these pathogens is likely if the entire peptide is used as a test antigen.

Munodzana *et al*. (15) reported that in region 5', nucleotide 390 (aa 91), as well as all nucleotides of the region 492-600 (aa 125-161) were required for binding of the monoclonal antibody ANAF16C1, and that the presence of cysteines in the N-terminal region and the intermolecular bond of disulfides in the conformation of MSPs are essential for the binding (30). The fact that the monoclonal antibody recognizes the recombinant protein HMSP5 indicates that the presence of the histidine hexapeptide does not alter the recognition of the epytope by the monoclonal antibody, and corroborates the results obtained by Munodzana *et al*. (15). They stated that the epytope, defined by the binding to this monoclonal antibody, is conserved in all *A. marginale* isolates.

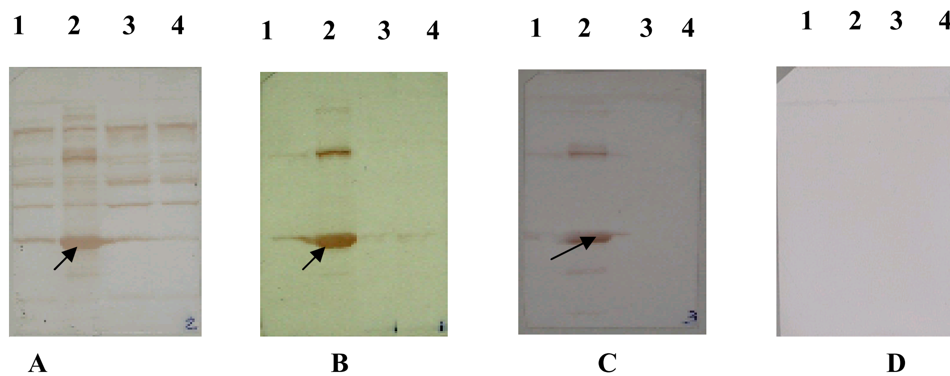

Figure 5. Recognition of HMSP5 protein by different antisera. A: Monospecific polyclonal antiserum X-MSP5 obtained in bovine, B: Monoclonal antibody ANAF16C1, C: Monospecific polyclonal antiserum antiMSP5 obtained in rabbit, D: Monoclonal antibody Tryp1E1. Line 1: Non induced recombinant strain BL21DE3pLysS/pHMSP5, 2: Recombinant strain BL21DE3pLysS/pHMSP5 induced with IPTG 1mM during six hours, 3: Non induced strain BL21DE3pLysS/pRSET B, 4: Strain BL21DE3pLysS/pRSET B induced with IPTG 1 mM during six hours.

With these results we have an *E. coli* recombinant strain expressing the MSP5 recombinant protein from Havana isolate that can be used for the development of a diagnostic test for bovine anaplasmosis.

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