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Short communication. Sequence of *msp1α* gene of *Anaplasma marginale* Havana isolate and expression in eukaryotic cells

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Abstract

Bovine anaplasmosis is a disease of worldwide economic importance caused by the rickettsia *Anaplasma marginale.* Several major surface proteins with conserved gene sequences have been examined as potential candidates for vaccines. One of these proteins, MSP1a, is expressed from the single copy *msp1α* gene. The molecular weight of MSP1a varies among the geographic isolates of *A. marginale* because of a varying number of tandem repeated peptides of 28-29 amino acids. In the present study, the sequence of the *msp1α* gene of *Anaplasma marginale* Havana isolate was determined, with a size of 2,043 kb, demonstrating the presence of five tandem repeated regions. This gene was cloned into the eukaryotic expression vector pCIneo, transfected in COS-7 cells and expression verified by immunofluorescence. These data provide a basis for an immunization strategy using the *msp1α* gene in DNA immunization containing single or multiple genes encoding major surface proteins of *A. marginale.*

Additional key words: bovine anaplasmosis, major surface protein, MSP1a, rickettsia.

Resumen

Nota corta. Secuenciación del gen *msp1α* del aislado Habana de A*naplasma marginale* y expresión en células eucariotas

La anaplasmosis bovina es una enfermedad causada por la rickettsia *Anaplasma marginale* que afecta al ganado bovino. Las principales proteínas de la superficie codificadas por genes con secuencias conservadas constituyen candidatos potenciales para vacunas. Dentro de estas proteínas, MSP1a es expresada por un gen de copia simple *(msp1α*). El peso molecular de MSP1a varía entre aislados de *A. marginale* de diferentes regiones geográficas, debido a la presencia, en número variable, de una secuencia de 28-29 aminoácidos repetida en tándem. En el presente estudio se secuenció el gen *msp1α* del aislado Habana de *A. marginale,* de 2.043 kb, demostrando la presencia de cinco regiones repetidas en tándem. Se clonó este gen en un vector de expresión eucariota (pCIneo), se transfectó en células COS-7 y se verificó la expresión de la proteína MSP1a por inmunofluorescencia, demostrándose la expresión de este gen procariota en células eucariotas. Estos datos proporcionan las bases para la utilización de este gen en futuras estrategias de inmunizaciones de DNA conteniendo uno o varios genes que codifican para proteínas principales de superficie de *A. marginale.*

Palabras clave adicionales: anaplasmosis bovina, MSP1a, principales proteínas de superficie, rickettsia.

Anaplasma marginale, a member of the genogroup II of the Ehrlichias in the order Rickettsiales (Walker and Dumler, 1996), infects and replicates within the bovine erythrocyte. Like other pathogenic ehrlichias, *A. marginale* is biologically transmitted by ticks and in a mechanical way by mosquitoes and contaminated material. The disease is characterized by a noticeable anaemia, weight loss and, in 34% of clinical cases, death of the animal (Alderink and Dietrich, 1981). The external membrane fraction of *A. marginale* comprises at least six main polypeptides on the surface, which include the main proteins (MSPs), MSP1a, MSP1b, MSP2, MSP3, MSP4 and MSP5 (Tebele *et al.*, 1991; Visser *et al.*, 1992). Immunization with native proteins

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MSP1 or MSP2 induces protection in experimental infections, shown by a significant reduction in rickettsemia and the anaemia, when compared with the control (Palmer *et al.*, 1989). Specific antibodies against MSP1 block the union of *A. marginale* to the erythrocyte and opzonize the organism for phagocytosis by the macrophages (Cantor *et al.*, 1993). Nevertheless, in spite of the results obtained using the native complex MSP1a and b, immunization with recombinant MSP1a, MSP1b or a combination of these two proteins has not induced significant protection (Palmer and McElwain, 1995).

MSP1a is encoded by a simple copy gene and is invariable within a strain. Although the gene is not part of a multigenic family there are differences between strains, by changes in the number of sequences repeated in tandem and other nucleotide substitutions, which result in differences in the primary structure from the polypeptide MSP1a (Allred *et al.*, 1990). In spite of the polymorphism of MSP1a between isolates, the epitope sensitive to neutralization is conserved (Palmer *et al.*, 1988), and, therefore, its importance in the design of a vaccine against the bovine anaplasmosis.

According to the results obtained by McGarey *et al.* (1994), MSP1a and MSP1b have functions of surface adhesins in charge of the adhesion of *A. marginale* to the bovine erythrocyte, as a part of the invasion process. This indicates the potential of *A. marginale* membrane proteins to be used as components of recombinant or nucleic acid vaccines for anaplasmosis. For these reasons, the use of MSP1a in a DNA-based vaccine against bovine anaplasmosis was investigated (Arulkanthan *et al.*, 1999).

DNA vaccines consist of a vector for expression in eukaryotic cells that contains the gene or genes of interest (Donelly *et al.*, 1997). A eukaryotic promoter directs the expression and the transcription is finished by a signal of polyadenylation in the eukaryotic cell. The intramuscular or intradermic inoculation of the DNA vaccine into the animal cell causes the protein to be expressed *in vivo* (Davis *et al.*, 1993). The antigens expressed in an endogenous form are processed and presented in the context of molecules of the histocompatibility complex (MHC) class I and class II, inducing specific cellular responses (lymphocytes CD4+ and CD8+) and antibody responses in the immunized host (Lekutis *et al.*, 1997). The objective of the present study was to obtain the sequence of the *msp1*α gene of *A. marginale,* Havana isolate, as well as to subclone this gene in a vector for expression in eukaryotic cells, to be used as an antigen model to evaluate the potential of DNA immunizations against bovine anaplasmosis.

The *msp1*α gene was amplified by PCR using the following primers: 5′GGAAGATCTTCCATGTCAGCAGAGTATG3′ and 5′GGAAGATCTTCCTTACGCCGCCGCCTG3′, corresponding to flanking regions of this gene, for Florida isolate (accession numbers M32871, M32872). These primers were used in a PCR assay to amplify DNA of *Anaplasma marginale* Havana isolate. Briefly, 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 2 min, followed by 30 cycles of 94°C for 1 min, 55°C for 1 min, 72°C for 2 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}. PCR product was purified by using the QIAquick Spin PCR purification kit (Qiagen, Inc., Chatsworth, Calif) as described by the manufacturer. The DNA was eluted in 30 μl of 10 mM Tris-HCl, pH 8.3.

The amplicon was cloned in a vector for expression in *E. coli,* pMAL (New England Biolabs) (data no presented) and the sequence was determined by the French Company Express Genome, using the same primers. Analysis and alignment of the sequence were done using the DNAsis program. In order to confirm the sequence identity, this was analyzed in the Blastn and Blastx, respectively (http: www.ncbi.nlm.nih.gov/).

The sequence obtained for the *msp1*α gene of *Anaplasma marginale* Havana isolate and for the MSP1a protein were deposited in the GeneBank with accession number AY489564, reporting a 2043 bp and 681 amino acids (aa), respectively.

The number of repeats (each 87 or 84 bp long) can be determined from the size of the resulting amplicon, because the 268 bp preceding and the 17 bp following the repeats are conserved in all strains (Palmer *et al.*, 2001, 2004). The presence of 5 regions repeated in tandem, in the Havana isolate, located at the beginning of the gene, with 84 to 87 bp (28 to 29 aa), immediately after the first 30 bp of the coding region, are observed showing a sequence similar to that of the

Table 1. Tandem repeat forms present in the Florida (FL), Virginia (VA), Washington (WA), Idaho (ID) (Allred *et al.*, 1990) and Havana (HA) variants of the MSP1a polypeptides

Forms	Sequences	No. tandem repeats				
		FL	VA	WA	ID	HA
A	DDSSSASGQQQESSVSSQS (EASTSS)QLG	1	1	0	0	1
B	ADSSSAGGQQQESSVSSQSD (QASTSS)QLG	7	1	3	0	4
C	ADSSSAGGQQQESSVSSQSG (QASTSS)QLG	0	0	1	0	0
D	ADSSSASGQQQESSVSSQS (EASTSS)QLGG	0	0	0	5	0
E	ADSSSASGQQQESSVSSQS (EASTSS)QLG	0	0	0	1	0

Florida isolate (Table 1). The repeated sequences vary minimally with remainders of 25 aa completely conserved.

The pMAL plasmid, containing the *msp1*α gene of *A. marginale* Havana isolate was used to subclone this gene in a pCIneo plasmid (Promega). The inserted *msp1*α gene and the plasmid were purified using the QIAquick gel extraction kit protocol (QIAGEN, 28704). The ligation of fragments was made using a DNA ligation kit (Amersham, RPN 1507). The product was transformed into Xl-2 Blue cells (ultracompetent and supercompetent cells, Stratagene), using the protocol of transformation in 5 min described by Pope and Kent (1996). Selection of recombinants was done by PCR using primers T7 and T3 (Promega), in a final reaction volume of 25 µl containing PCR buffer 1X (Promega), 0.4 mM deoxynucleoside triphosphates, 0.5 µM each primer, 0.5 U of Taq polymerase (Promega). PCR was performed at 94°C for 2 min, followed by 30 cycles at 94°C for 1 min, 55°C for 1 min, 72°C for 2 min, with a final extension step at 72°C for 10 min. The results were visualized in a 0.8% agarose gel in TBE 1X, with ethidium bromide 0.5 µg ml^{-1}. The colonies positive by PCR were analyzed with *EcoR* I (Promega) and *Xba* I (Promega) restriction enzymes and the sense of direction of the cloned fragment was analyzed by digestion with *Nde* I (Promega). The resulting restriction enzyme digested DNA was analyzed in a 0.8% agarose gel.

A clone containing a fragment of 2043 bp, in the correct direction was produced and purified by Endofree Plasmid Maxi (QIAGEN). The purity of the DNA was confirmed by measuring the optical density at 260 and 280 nm, followed by electrophoresis in an agarose gel.

With the objective of verifying the expression of MSP1a in the eukaryotic cells, COS-7 cells were transfected with 5 µg of DNA of recombinant plasmid pCIneo/*msp1*α by plate of 65 cm^2, maintained until a confluence of 80% in the cell monolayer, using the method of transfections with DEAE-Dextran (Sambrook *et al.*, 1989). The transfected plates were left in DMEM medium (Sigma), supplemented with 5% bovine fetal serum (Sigma), for 72 h to 37°C, 5% CO_2. The cells were harvested, and the cellular debris was stored at –20°C for immunofluorescence, to make the immunodetection of the MSP1a protein.

After 72 h of transfection the coverslips with the cells transfected with the recombinant plasmid and the native plasmid were washed three times with PBS 1X and fixed with cold acetone for 10 min. The monoclonal antibody Ana22B1 was added (Vidotto *et al.*, 1994) and incubated in a humid chamber at 37°C for 1 h. This was followed by three washes with PBS 1X for 10 min and the addition of an antimouse antiserum conjugated with fluoresceine isothiocianate (Sigma), diluted 1/50 and incubated in the dark at 37°C for 1 h. After three washes with PBS 1X coverslips were left to dry and observed under the fluorescent microscope (Opton), following the methodology described in the manual of the International Office of Epizootics (OIE) (WHO, 1996).

The expression of pCIneo/msp1α in COS-7 cells (Fig. 1), was observed by the presence of fluorescent cells when these are transfected with the recombinant plasmid (panel A). No signal was observed in the cells transfected with the native plasmid (panel B), demonstrating the functionality of this gene in eukaryotic cells.

The *msp1*α gene is genetically stable during the acute and persistent infection of *A. marginale.* It is a single copy gene which does not change when cultured *in vitro,* in ticks or animals (Brown *et al.*, 1998; Barbet *et al.*, 1999).

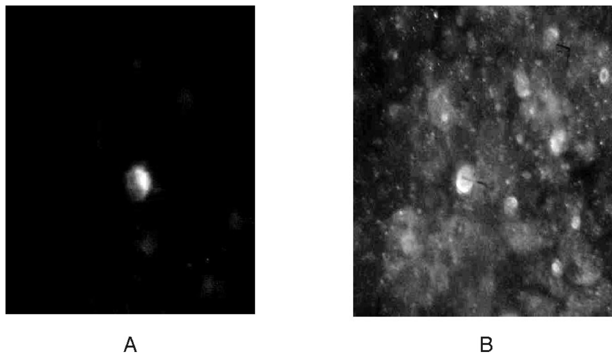

Figure 1. Immunofluorescence results. A: COS-7 cells transfected with pCIneo/*msp1*α; B: COS-7 cells transfected with pCIneo.

The MSP1a protein is an adhesin for the invasion of *A. marginale* to the bovine erythrocyte and the tick's cells. The molecular weight of this protein varies between geographic isolates of *A. marginale* by variation in the number of peptides repeated in 28-29 tandem amino acids. These repeated sequences can be present from 1 to 8 times (Palmer *et al.*, 2001) and constitute markers for the transmissibility by ticks, influencing the strategies of control and the vaccine design by subunits (de la Fuente *et al.*, 2001a).

It has been described (Allred *et al.*, 1990), that in the sequence of this gene in the different isolates, five forms of this sequence exist repeated in tandem. These vary minimally with 25 aa completely conserved in the five forms. This variation in the number of repeated sequences can explain the size polymorphism existing in this gene for the different isolates.

The different size of the coding sequence of this gene and the different length of the polypeptide for each studied isolate have been reported, with sizes of 2301 bp and 767 aa, 1779 bp and 593 aa, 1956 bp and 652 aa and 2124 bp and 708 aa in Florida, Virginia, Washington and Idaho isolates respectively (Allred *et al.*, 1990), but in all cases the presence of the epitope sensitive to neutralization is described. For the Havana isolate the size was 2043 bp and 680 amino acids, respectively. In this sense de la Fuente *et al.* (2003a,b) characterized 11 isolates of Oklahoma and found differences in the sequences of this gene for all the isolates. Moreover, Palmer *et al.* (2001) found an isolate that only had a single repeated region. In Table 1 it is possible to observe that the type of repeated sequences of the Havana isolate agrees with that described for the Florida isolate (Allred *et al.*, 1990), variation being evident only in the number of repetitions, unlike the rest of the isolates, that besides being different in relation to size, they also differed in the type of repeated sequence. If the geographical proximity of the two regions from where the isolates (Havana and Florida) came is taken into account, it is noteworthy that the Florida isolate, with relation to the *msp1*α gene has a greater similarity with the Havana isolate, compared with other isolates from different regions of the United States, according to Allred *et al.* (1990) and Palmer *et al.* (2001). In spite of the difference in the sequences repeated in tandem of this *msp1*α gene it has been found that a 6 aa epitope required for neutralization is present on all isolates and expresses immunity. This sequence was present in the repeats of the Havana isolate, studied in this work.

The study of these repeated sequences is very important, because they include markers for the transmissibility by ticks, but this gene cannot be considered as a marker to characterize isolates from different geographic regions (de la Fuente *et al.*, 2001a,b). It has been reported (de la Fuente *et al.*, 2001b) that MSP1a affects the infection and transmission of *A. marginale* by ticks of the genus *Dermacetor*. The mechanism of infection and transmission of *A. marginale* by ticks is important to formulate control strategies and to develop improved vaccine against the anaplasmosis. In addition, this is of extreme importance, because it is known that the sequence of MSP1a is conserved during all the life cycle of *A. marginale,* with no change in amino acids (Bowie *et al.*, 2002), indicating that this protein bound to MSP1b is a potential vaccine candidate against the anaplasmosis. Its important biological function within the pathogenesis of bovine anaplasmosis makes it of great interest as a future vaccine candidate. It may be possible to develop DNA vaccines where the genes can be cloned into plasmid combining several genes encoding for major surface proteins of the initial bodies of this hemoparasite. These proteins are targets for the immune response against this microorganism and DNA vaccines may induce a higher cellular and humoral response. This could elicit higher protection against the microorganism, judging from the results of Arulkanthan *et al.* (1999), which indicated the importance of this type of response in the immunity of the anaplasmosis.

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References

ALDERINK F.J., DIETRICH R., 1981. Anaplasmosis in Texas: epidemiologic and economic data from a questionnaire survey. Proc Seventh Ntnl Anaplasmosis Conf (R.J. Hidalgo and E.W. Jones, eds). Mississippi State University Press, USA. pp. 27-44.

ALLRED R., McGUIRE T.C., PALMER G.H., LEIB S.R, HARKINS T.M, MCELWAIN T.F., BARBET A.F., 1990. Molecular basis for surface antigen size polymorphims and conservation of neutralization-sensitive epitope in *Anaplasma marginale.* Proc Natl Acad Sci USA 87, 3220-3224.

ARULKANTHAN A., BROWN W.C., McGUIRE T.C., KNOWLES D.P., 1999. Biased immunoglobulin G1 isotype responses induced in cattle with DNA expressing msp1α of *Anaplasma marginale.* Infect Immun 67(7), 3481-3487.

BARBET A.F., BLENTLINGER R., YI J., LUNDGREN A.M., BLOUIN E.F., KOCAN K.M., 1999. Comparisons of surface proteins of *Anaplasma marginale* grow in tick cell culture, tick salivary glands, and catlle. Infect Immun 67(1), 102-107.

BOWIE M.V., DE LA FUENTE J., KOCAN K.M., BLOUIN E.F., BARBET A.F., 2002. Conservation of major surface protein 1 genes of *Anaplasma marginale* during cyclic transmission between ticks and cattle. Gene 282(1), 95-102.

BROWN W.C., ZHU D., SHKAP V., McGUIRE, E.F., KOCAN K.M., PALMER G.H., 1998. The repertoire of *Anaplasma marginale* antigens recognized by CD4+ T-lymphocyte clones from protectively immunized cattle is diverse and includes major surface protein 2 MSP2 and MSP3. Infect Immun 66, 5414-5422.

CANTOR G.H., PONTZER C.H., PALMER G.H., 1993. Opzonization of *Anaplasma marginale* mediated by bovine antibody against surface protein MSP1. Vet Immunol Immunopathol 37, 343-350.

DAVIS H.L., DEMENEIX B.A., QUANTIN B., COULOMBE J., WHALEN R.G., 1993. Plasmid DNA is superior to viral vectors for direct gene transfer into adult mouse skeletal muscle. Hum Gene Ther 4, 733-740.

DE LA FUENTE J., GARCÍA-GARCÍA J.C., BLOUIN E.F., RODRÍGUEZ S.D., GARCÍA M.A., KOCAN K.M., 2001a. Evolution and function of tandem repeats in the major surface protein 1a of the ehrlichial pathogen *Anaplasma marginale.* Anim Health 2(2), 163-173.

DE LA FUENTE J., GARCÍA-GARCÍA J.C., BLOUIN E.F., McEWEN B.R., CLAWSON D., KOCAN K.M., 2001b. Major surface protein 1a effects tick infection and transmission of *Anaplasma marginale.* Int J Parasitol 31(14), 1705-1714.

DE LA FUENTE J., VAN DEN BUSSCHE R.A., PRADO T.M., KOCAN K.M., 2003a. *Anaplasma marginale msp1* alpha genotypes evolved under positive selection pressure but are not markers for geographic isolates. J Clin Microbiol 41(4), 1609-1616.

DE LA FUENTE J., VAN DEN BUSSCHE R.A., GARCÍA-GARCÍA J.C., RODRÍGUEZ S.D., GARCÍA M.A., GUGLIELMONE A.A., MANGOLD A.J., PASSOS L.M., FRICHE, M.F., BARBOSA R., BLOUIN E.F., KOCAN K.M., 2003b. Phylogeography of New World isolates of *Anaplasma marginale* based on major surface protein sequences. Vet Microbiol 88(3), 275-285.

DONELLY J.J., ULMER J.F., SHIVER J.W., LIU M.A., 1997. DNA vaccines. Annu Rev Immunol 15, 617-648.

LEKUTIS C., SHIVER J.W., LIU M.A., LETVIN N.L., 1997. HIV-1 env DNA vaccine administered to rhusus monkeys elicits MHC class 11-restricted CD4+ helper cells that IFN-γ and TNF-α. J Immunol 158, 4471-4477.

McGAREY D.J., BARBET A.F., PALMER G.H., ALLRED D.R., 1994. Putative adhesins of *Anaplasma marginale* major surface polypeptides (MSP)1a and 1b. Infect Immun 62, 4594-4601.

PALMER G.H., BARBET A.F., MUSOKE A.J., KATANDE J.M., RURANGIRWA F.R., SHKAP V., 1988. Recognition of conserved surface protein epitopes on *Anaplasma centrale* and *Anaplasma marginale* isolates from Israel, Kenya and the United State. Int J Parasitol 18, 33-38.

PALMER G.H., BARBET A.F., CANTOR G.H., McGUIRE T.C., 1989. Immunization of cattle with the MSP1 surface protein complex induces protection against structurally variant *Anaplasma marginale* isolate. Infect Immun 57, 3666-3669.

PALMER G.H., McELWAIN T.F., 1995. Molecular basis for vaccine development against anaplasmosis and babesiosis.Vet Parasitol 57, 233-253.

PALMER G.H., KNOWLES D.P., RODRÍGUEZ J.L., GNAD D.P., HOLLIS, L.C. MARSTON T., BRAYTON K.A., 2004. Stochastic transmission of multiple genotipically distinct *Anaplasma marginale* strains in a herd with high prevalence of *Anaplasma* infection. J Clin Microbiol 42, 5381-5384.

PALMER G.H., RURANGIRWA F.R., McELWAIN T.F., 2001. Strain composition of the ehrlichia *Anaplasma marginale* within persistently infected cattle, a mammalian reservoir for tick transmission. J Clin Microbiol 39, 631-635.

POPE B., KENT H.M., 1996. High efficiency 5 minutes transformation of *Escherichia coli.* Nucleic Acids Res 24(3), 536-537.

SAMBROOK J., FRITSCH E.F., MANIATIS T., 1989. Molecular cloning: A Laboratory Manual. Cold Spring Harbor Laboratory, N. Y.S

TEBELE N., McGUIRE T.C., PALMER G.H., 1991. Induction of protective immunity using *Anaplasma marginale* initial body membrane. Infect Immun 59, 3199-3204.

VIDOTTO M.C., McGUIRE T.C., McELWAIN T.F., PALMER G.H., KNOWLES D.P., 1994. Intermolecular relationships of major surface proteins of *Anaplasma marginale*. Infect Immun 62, 2940-2946.

VISSER E.S., McGUIRE T.C., PALMER G.H., DAVIS W.C., SHKAP V., PIPANO E., KNOWLES D.P., 1992. The *Anaplasma marginale* msp5 gene encodes a 19 kDa protein conserved in all recognized *Anaplasma* species. Infect Immun 60, 5139-5144.

WALKER D.H., DUMLER J.S., 1996. Emergence of the ehrlichiosis as human health problems. Emerg Infect Dis 2, 18-29.

WHO, 1996. Manual of standard for diagnosis test and vaccines. World Organization for Animal Health, Paris, France. pp. 295-300.