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Molecular Cloning and Variability of a Subolesin Recombinant Peptide from a Mexican *Rhipicephalus (Boophilus) Microplus* Tick Strain

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Abstract - The cattle tick *Rhipicephalus* (*Boophilus*) *microplus* is the most important health problem for livestock at tropical and subtropical areas. Because of its hematofagous behavior, pathogen transmission, acaricide resistance, environmental impact and public health, new strategies for tick control are required. Vaccines, constitute a cost-effective and environment friendly alternative. The Bm86 tick antigen, originally identified in *R. microplus*, is the constituent of the only commercialized anti-tick vaccine. Nowadays, tick subolesin has been considered an important candidate. The aim of this study was to clone and characterize a recombinant peptide derived from the gen *Sub* in order to express it in *Escherichia coli*. The recombinant peptide was labeled with a polihistidine tag and identified by a monoclonal antibody against the tag after PAGE separation, purified by Ni affinity chromatography, and analyzed by the cluster analysis of variations with regards to ten different tick strains including Mexican ticks. Analysis of variations show, different clusters suggesting regional specific variations among Mexican strains, with probable implications on regional variability on protection efficiency. The tick protective antigen, subolesin, is a promising antigen and the use of immunogenic peptides predicted from in *silico* analysis approached in this study can be an alternative to improve the efficiency of tick vaccines in the future.

Keywords - Recombinant peptides, Subolesin, *Boophilus*, Bm86, Ticks

1. Introduction

The cattle tick *Rhipicephalus* (*Boophilus*) *microplus* is one of the most relevant ectoparasites affecting the cattle industry in tropical and sub-tropical areas [1]. Infestations with *R. microplus*, economically impact cattle production by reducing weight gain and milk production, and by transmitting pathogens that causes babesiosis and anaplasmosis [2, 3]. It is estimated that the worldwide economic impact of *R. microplus* reaches aproximately 10 billion US dollars per year [4].

Conventional tick control methods have relied mainly on the use of acaricides; however, the rapid appearance of resistant tick populations, and the presence of chemical residues in meat and milk are aspects that emphasize the need for novel control methods, such as vaccination [5, 6] and biological control [7]. In the animal production context, vaccine would be a tick control strategy widely accepted because of the reduction of environmental impact and food contamination.

Vaccines became commercially available in the early 1990s as a cost-effective alternative for cattle tick control that reduced the use of acaricides and the problems associated with them such as selection of acaricide-resistant ticks, environmental impact and milk and meat contamination with pesticide residues [8, 2, 9, 10, 11]. Vaccination programs with the commercial vaccines containing the recombinant *R. microplus* Bm86 gut antigen, Gavac® and Tick- GARD® have been established; However, different levels of efficacy to *R. microplus* strains have been experienced, and sequence variations in the target protein among different strains have been found to be associated with variable efficacy [12, 13, 14, 15].

The tick protective antigen, Subolesin, was discovered by cDNA expression library immunization (ELI) and analysis of expressed sequenced tags (EST) in a mouse model of tick infestations for identification of cDNAs protective against

Ixodes scapularis. [16, 17]. Subolesin was shown to be highly conserved at the nucleotide and protein levels among ixodid tick species [16, 18]. Furthermore, Subolesin was found to be protective against all tick developmental stages when used in recombinant protein immunization experiments, but the efficacy is around 50% [16, 19, 20, 21, 22]. These results proved that Subolesin is an important vaccine candidate for the control of multiple tick species, but higher efficacy is required. The advantages of characterizing a recombinant peptide vaccine include: high degree of antigenicity, large scale production, lack of contaminants and low cost production on industrial scales. Therefore, the aim of this research was to characterize a recombinant peptide derived from the *Sub* gene obtained from the *in silico* analysis of the immunogenic region, and compare the sequence with other Mexican tick strains in order to find out if the recombinant peptide could be used to develop an experimental vaccine against tick infestations that can be used all over the country or only for a region.

2. Materials and Methods

2.1. Sequence analysis of protective tick Subolesin

The inferred sequences of the native Subolesin protein reported in Mexico were analyzed to predict some protein properties, such as antigenic capacity, hydrophobic and hydrophilic properties. Nucleotide sequences were analyzed using the program Clustal W [23] to identify conserved regions within the gene, available at http://www.genome.jp/tools/clustalw/. Prediction of antigenic peptides was performed using the method of Kolaskar and Tongaonkar [24] by CLC Main Workbench 5.7 Software and ABCpred server available on the web http://www.imtech.res.in/raghava/abcpred/.

2.2. Tick strain

The Mexican Media Joya reference tick strain was kindly provided by Dr. Rodrigo Rosario-Cruz (CENID PAVET INIFAP, Jiutepec, Morelos, Mexico) and used as a source of genetic material, for experimental purposes. Originally, this tick strain was collected from infested cattle in Tapalpa, Jalisco, Mexico and reared at the CENID-PAVET, INIFAP tick research Unit.

2.3. Synthesis of cDNA and amplification by PCR

DNA and RNA were extracted from *R. microplus* (susceptible Media Joya). Approximately 100-150 unfed tick larvae were used for the experiment. Total RNA was extracted from homogenized tick samples using TRIZOL reagent (Life Technologies, Maryland, USA) according to manufacturer's instructions. Synthesis of cDNA was obtained from 5 μg of total RNA using RACE cDNA kit (Invitrogen, Carlsbad CA), which was used as template for PCR amplification reactions. Specific primers were designed using Primer 3 program, considering sequences flanking antigenic regions of Subolesin: FW 5'- CACCATGAAGCGACGCCGATGTATG-3' and RV 5'- CTCCTCCCGTATCTTGCTCTC-3'. PCR was performed in 25 μL reaction mixture for 40 cycles and amplification conditions 30 s at 95 ℃ and 1 min at 72 ℃ for annealing and extension steps, respectively. PCR products were electrophoresed on 1% agarose gels to check the size of amplified fragments by comparison with a DNA molecular weight marker (1 kb Plus DNA Ladder, Invitrogen).

2.4. Cloning and expression of subolesin recombinant peptide in E. coli.

Amplified fragments were cloned into the pET101/D-TOPO® vector (Invitrogen, Carlsbad, CA), subjected to a ligation reaction using 4 μL PCR product-amplified, 1 μL salt solution and 1 μL TOPO® vector and then transformed into competent *E. coli* One Shot® TOP10 cells (Invitrogen, Carlsbad, CA). Three μL of the TOPO® cloning reaction were used to transform the amplified fragments into a vial of chemically competent TOP10 cells. Positive clones were analyzed by PCR as described above. The fidelity and orientation of the constructs was verified by sequencing. For expression of recombinant peptide subolesin, plasmids were transformed into *E. coli* BL21 Star (DE3). Transformed *E. coli* were inoculated in LB containing 50 μg/ml ampicillin. Culture was grown at 37 ℃ to OD600 nm = 0.5. IPTG was then added to 1 mM final concentration, and incubation continued for 5 h to induce the recombinant peptide expression. Cells were collected by centrifugation and later analyzed by SDS-PAGE and Western blot.

2.5. Protein Gel Electrophoresis and Western Blot Analysis

Expression and purification of the recombinant peptide was confirmed by SDS-PAGE [25] and immunoblotting, by detection of the histidine tag by a monoclonal antibody anti-His (Invitrogen). Protein samples were loaded on 15% polyacrylamide gels that were stained with Coomassie Brillant Blue or were transferred to nitrocellulose membranes. Membranes were blocked with 5% skim milk for 60 min at room temperature and then over night at 4 ℃. Western blot analysis was performed using 1:2000 anti-His monoclonal antibodies (Invitrogen) alkaline phosphatase conjugate, for detection of recombinant fusion protein. After washing with TBS, the color was developed using BCIP/NBT alkaline phospatase substrate (Millipore).

2.6. Protein purification

E. coli cells expressing recombinant peptide were disrupted by sonication. Recombinant proteins were extracted with 10 mM imidazole in Phosphate-buffered saline (PBS) and purified by Ni affinity chromatography (Qiagen) following the manufacturer ́s instructions.

2.7. Sequences analysis

Sequencing of the recombinant peptide encoding sequence, was carried out by commercial sequencing at the Instituto de Biotecnologá from UNAM located at Cuernavaca Morelos,

México.

The nucleotide sequences of ten tick strains (DQ159965.1, DQ159964.1, DQ159963.1, JX193832.1, JX193834.1, DQ159966.1, JX193839.1, JX193838.1, JX193844.1, JX193842.1) were obtained from the GenBank database. The sequence corresponding to the region of interest was compared by the multiple sequence alignment algorithms and analyzed in order to deduce the genetic relationship among the tick strains based on the cluster analysis of variations on the nucleotide sequence of the predicted immunogenic region of the *Sub* gene. Based on this information, a dendogram was constructed in order to deduce the phylogenetic relationship of the cloned sequence from the Media Joya Strain with regards to the reported sequences obtained from de GenBank database (CLC Main Workbench 5.7 software).

3. Results

The Mexican Media Joya Tick strain collected at Tapalpa Jalisco (Reference strain reared and maintained at the Tick Research Laboratory from the CENID-PAVET INIFAP), was used to obtain mRNA and to synthesize cDNA in order to be used as a template to set up the PCR reactions to amplify clone and get the nucleotide sequence of the Media Joya sequence encoding the *Sub* recombinant peptide.

Based on the Analysis of the Santa Luisa (DQ159965.1) subolesin gene sequence by the Kolaskar-Tongaonkar algorithm, the immunogenic region of the Subolesin gene was predicted (CLC Main Workbench 5.7), amplified by PCR (Fig 1), cloned, expressed (figure 2) and identified by PAGE and Western Blott (Fig 3). The aminoacid sequence was deduced by the algorithm contained within the software package CLC Main Workbench 5.7. The expected molecular weight of 15 kDa for the peptide subolesin expressed in this system was calculated using Protein Calculator v3.3 software.

The immunogenic predicted sequence of 303 bp, was cloned and the insert was sequenced and analyzed in order to confirm the size (Fig 1), sequence and putative function of the insert, before being expressed. Expression was induced by using the inducer IPTG and the molecular mass of the expressed peptide was aproximately 15 kDa as shown in figure 2. Putative function of the sequence was corroborated by the algorithm BLAST available at the NCBI web page showing a 100% homology and corresponded to the Santa Luisa subolesin gene sequence (Fig. 4), which is a member of the akirin family.

Expression levels of the recombinant peptide subolesin after induction reached approximately the 5-10% of total cellular proteins as shown by the analysis of the supernatants on 15% SDS-PAGE, protein expression profile revealed a band of approximately 15 kDa (Fig. 2). Western Blott analysis using a monoclonal antibody against the tag of histidines, identified the same 15 kDa recombinant peptide purified by Ni affinity chromatography showing the consistency of the expression and purification system (fig. 3).

The cluster analysis show three clusters of ticks, one constituted by strains from the European continent, a second group including the Media Joya (Jalisco State, Pacific Coast) sequence named "sub peptide" (figure 4) sharing a 100% of similitude with Santa Luisa (Brazil) strain and a lower similitude with Muñoz (unknown origin) and Cepich (Chiapas, México state, Pacific Coast), and a third group including a larger group of strains from the Mexican Gulf coast including Veracruz, Tamaulipas and Quintana Roo as well as a strain denoted as a susceptible strain sharing a 100% of homology with "Santa Luisa Strain" from Brazil.

4. Discussion and Conclusions

Tick control in México is becoming a major issue, since the appearance of multiple resistant tick strains is an increasing problem in most of the important livestock production regions all over Mexico. Currently, reinfestation of some pasture areas further the quarantine zone between Mexico and USA, have been reported [26]. Therefore, development of new strategies for tick control has been recently focused on anti tick vaccines. However, it is necessary to conduct and focus research on the integration of Bioinformatics, Immunogenomics, and systems biology [27] in order to find a better strategy in searching new targets or new antigens for a tick vaccine development.

Bioinformatics and data mining have become important tools for gene prediction of defined selection criteria, such as hidrofobicity, immunogenicity, cellular localization, and motifs to identify genes or predict regions of the gene sequence with immunogenic potential. There are lots of genes on public databases that can be used as a target, for this approach. Subolesin gene sequence was analyzed in this study in order to predict the immunogenic region, for amplification, cloning, expression and purification of a recombinant peptide for further immunological studies.

A TOPO-Subolesin recombinant plasmid attached with a tag of polihistidins was successfully constructed and proved capable to express a recombinant peptide derived from subolesin. Since the latter is soluble in the culture medium, it can easily be obtained from the supernatant by affinity chromatography which will facilitate the purification process and thus lead to a reduction in the production cost. In addition to being a simple purification system, the process preserves the antigenic and immunogenic properties of the expressed recombinant peptide, which is important for producing an effective immunogen [28]. However, it is necessary to evaluate the substitutions of the recombinant construct in order to find out if the expressed peptide will work as a tick vaccine (Fig. 4).

Results obtained from cluster analysis of subolesin gene alignments show that variations of the gene have to deal with some geographical segregation, there is a cluster for gulf coast strains while the sequence reported (sub peptide) stay at a different cluster together with some pacific coast strains such

as the sub peptide sequence derived from a strain isolated from Jalisco state (pacific coast) and Cepich from Chiapas (Pacific coast) (Fig. 4).

The first tick vaccine based on the glycoprotein Bm86, was commercialized and proved to be a cost effective alternative in the early 1990 ś and still is the only commercially available vaccine [10]. However, because of the limited efficiency of this vaccine when it has been used in different geographic latitudes, new antigens have been identified and proposed as new protective antigens for tick vaccine development [29, 30].

Recently, Subolesin used in cattle vaccination experiments, resulted in a reduction of tick infestations and a reduced infection rate of two different pathogens: *Anaplasma marginale* and *Babesia bigemina* [29]. However the use of the recombinant subolesin has not been totally succesfull, since only a 60% efficiency has been obtained in controlled infestations experiments [22].

The approach used in this study is based on previous immunization of cattle using synthetic peptides (SBm4912 and SBm7462) derived from the *R. microplus* gut protein (Bm86). Use of synthetic peptides resulted in significant reduction of the reproductive indices of ticks, represented by the number and weight of adult females, and their egg laying capacity, providing an improvement of the overall efficacy over 80% [31, 32]. A similar modified approach using a single recombinant peptide has been proposed in this study, focused on the immunogenic region of the subolesin gene in order to improve and propose a new formulation for a subolesin derived tick antigen to be used in future experiments.

The use of a prokaryotic expression system to produce a specific recombinant peptide with a potential for use in vaccines was thus achieved in the present investigation, showing advantages for efficient production of the recombinant peptide, low cost and lack of contaminants. However, analysis of variations on the gene sequence, is necessary in order to find out possible substitutions that could affect the efficacy of the vaccine in future infestations trials. Results presented from cluster analysis, suggest some geographical segregation of gene variants that could cause variations on the efficiency of the immunogen. Therefore, analysis of substitutions on single genes or peptides derived from them, is necessary in order to predict the region of the country in which vaccines are going to be used, since substitutions can also affect the immunogenicity of tick vaccines from a geographical area to another, if conformational epitopes are determinant for a protective immune response. The tick protective antigen, subolesin, is a promising antigen and the use of immunogenic peptides predicted from in *silico* analysis approached in this study can be an alternative to improve the efficiency of tick vaccines in the future.

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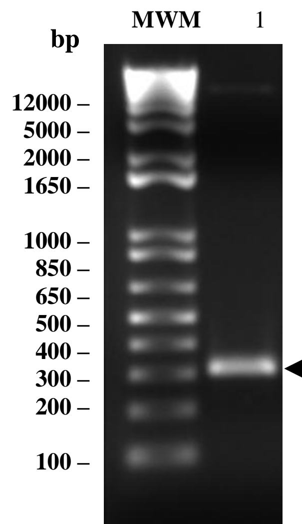
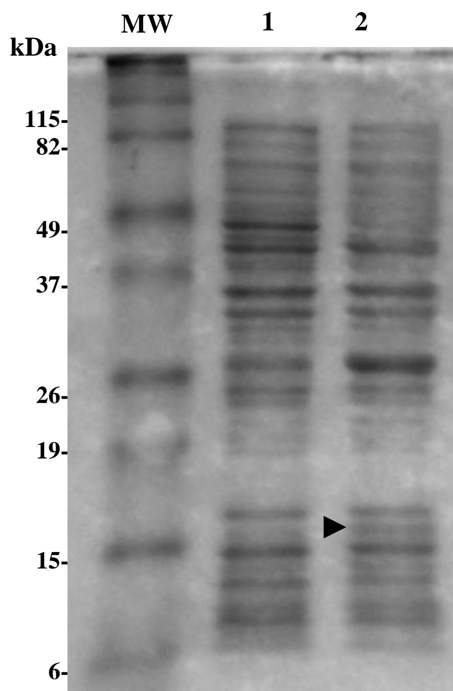
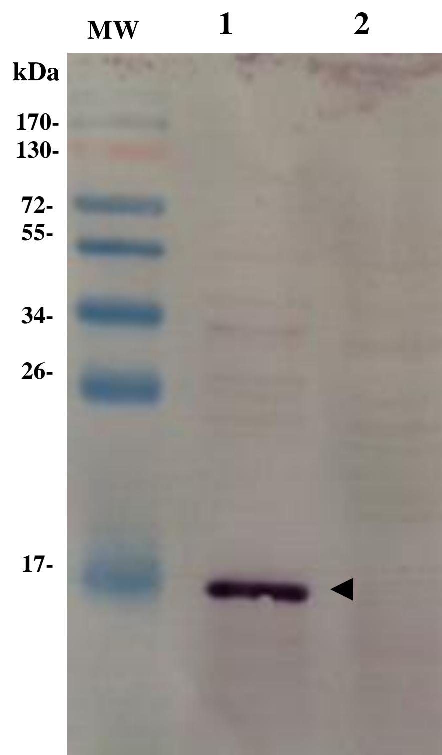


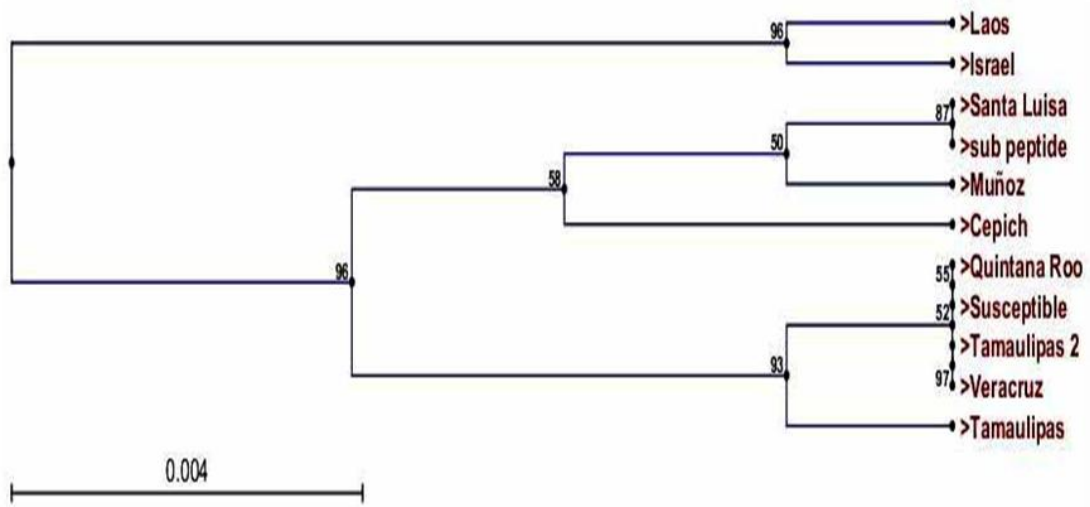
Rosario-Cruz Fig 1. Subolesin immunogenic sequence was amplified by PCR reaction by using specific primers from the *R. microplus* subolesin cDNA sequence. The length of the amplified PCR product was 303 bp. Arrow indicate the size of the peptide subolesin (303 bp). MWM, molecular weight markers (1 Kb DNA Ladder, Promega).



Rosario-Cruz Fig 2. Total protein of both induced and non induced *E. coli* colonies with and without the insert were grown in LB Broth and analyzed by PAGE. Protein samples were loaded on 15% polyacrylamide gels stained with Coomassie Brilliant Blue. Lane 1, induced *E. coli* cells (control); lane 2, induced *E. coli* expressing the recombinant peptide subolesin. Arrow indicate the size of the peptide subolesin (15 kDa). MW, molecular weight markers (Bio-Rad, SDS-PAGE molecular weight standards, Broad Range).



Rosario-Cruz Fig 3. Western blot analysis of the recombinant peptide of subolesin (lane 1) and induced *E. coli* cells (control), (lane 2) Samples of induced *E. coli* proteins and the recombinant peptide of subolesin were separated by SDS-PAGE and reacted with anti-His tag monoclonal antibody. Arrow indicates the size of the peptide subolesin (15 kDa.) MW, molecular weight markers (Fermentas, PageReguler Prestained Protein Ladder).



Rosario-Cruz Fig. 4. Cluster Analysis including a partial sequence of *Sub* gene from the cattle tick *Rhipicephalus (Boophilus) spp*, in order to evaluate the variability of a recombinant peptide compared to sequences at the GenBank database and its possible effects on its immunogenic efficiency in different regions of Mexico.