Synthetic Peptides-Based Indirect ELISA for the Diagnosis of Bovine Anaplasmosis

Rosa E. Quiroz-Castañeda*[1]

Tania R. Tapia-Uriza[2]

Charlotte Valencia Mujica[2]

Sergio D. Rodríguez-Camarillo[1]

Jesús F. Preciado-de la Torre[1]

Itzel Amaro-Estrada[1]

Mayra Cobaxin-Cárdenas[1]

[1]Unidad de Anaplasmosis del Centro Nacional de Investigación Disciplinaria en Salud Animal e Inocuidad, INIFAP. Jiutepec, Morelos, México

[2]Facultad de Ciencias Biológicas, Universidad Autónoma del Estado de Morelos, México.

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ABSTRACT

This paper presents a first initial approach to diagnose bovine anaplasmosis using a synthetic peptides based indirect ELISA. The capacity of the positivity values obtained for a peptide mixture is higher than the capacity of the same peptides used individually, in other words, the combination of both peptides increases their antigenic potential. In some cases, the positivity values of sera show an almost six-fold increase when the peptide mixture is used as an antigen compared to the crude antigen of the in-house indirect ELISA (iELISA). The sensitivity of the assay using the peptide mixture is 100%, while the confidence for specificity is 95%. The design and use of synthetic peptides for anaplasmosis might be implemented as an alternative to the crude extract or recombinant proteins, which may result more expensive and experimentally complicated.

INTRODUCTION

Bovine anaplasmosis is an infectious, non-contagious disease caused by *Anaplasma marginale* (*A. marginale*), a rickettsial pathogen. The organism has a global distribution and infects erythrocytes, resulting in anemia, jaundice, fever, abortions, and even death. The disease is widely distributed in tropical and subtropical regions around the world, and is currently listed as a notifiable animal disease according to the OIE (http://www.oie.int/fileadmin/Home/eng/Health_standards/tahm/2.04.01_BOVINE_ANAPLASMOSIS) (Aubry and Geale, 2011; Howden et al., 2010; Kocan et al., 2010). Bovine anaplasmosis is highly important due to the productivity and economic losses involved, which have been estimated at more than $300 million per year in the United States and $800 million per year in Latin America (Aubry and Geale, 2011). In Mexico, according to the largest insurance company for livestock, bovine anaplasmosis has been held accountable for up to 26% of total losses in cattle movement (Rodriguez

et al., 1999). Detection of *A. marginale* can be carried out by direct microscope observation of stained blood-smear or using the Polymerase Chain Reaction (PCR) method (Aubry and Geale, 2011). Alternatively, indirect serologic assays, such as the card-agglutination test, the complement fixation test and the indirect enzyme-linked immunosorbent assay (iELISA) can be used to identify specific antibodies (Price et al., 1952; Welter, 1964).

The crude antigen used in the iELISA (obtained by the extruded bacteria from infected erythrocytes) was originally prepared for the card-agglutination test and later used for the complement fixation test (Barry et al., 1986; Gainer, 1961; Knowles et al., 1996). This, crude antigen however, renders variable results as an antigen for the iELISA (Barry et al., 1986) despite the fact that it is one of the recommended diagnostic methods by the OIE (OIE, 2015).

Recently, the use of synthetic peptides represents a major biotechnologic improvement resulting from the availability of complete pathogen genomes and computational approaches used in peptide science. Furthermore, their successful applications have been reported in several diagnostic methods (Carmona et al., 2012; Fleury et al., 2004; Link et al., 2017).

This work shows our preliminary results on the use of synthetic linear peptides designed based on the sequences of the Major Surface Proteins MSP1a and MSP5 of *A. marginale* and used as antigen in a iELISA.

MATERIALS AND METHODS

Materials

Synthetic peptides were commercially synthesized by Peptide 2.0 (Peptide 2.0 Inc., Chantilly, VA).

96-well microtiter plates were purchased from Corning Mexicana, Mexico. All other reagents used in antigenicity test were purchased from BioRad, Nestlé and Sigma-Aldrich.

Hyperimmunized and experimentally infected animals were purchased from *A. marginale*-free herds and tested negative to in house iELISA andMSP5-PCR prior to arrival at CENID-SAI and kept under conditions that precluded accidental infection with *A. marginale* or any other cattle pathogens.

All serum samples including positive and negative controls used in this study come from a serum bank kept at the premises and have been previously tested by the in-house iELISA with crude antigen. Hyperimmunized animals (135 and 6787) have received multiple (>20 and > 5 respectively) inoculations with both inactivated and live *A. marginale* derived from more than one *A. marginale* strain. Experimentally infected animals were infected according to Table 1.

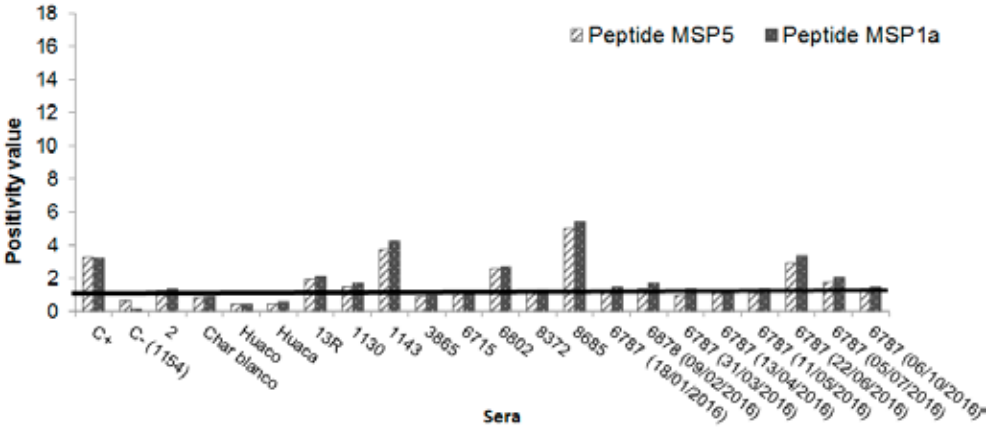
Peptide design

We designed the synthetic peptides based on the protein sequences of the MSP1a and MSP5 present in nine Mexican *A. marginale* strains that were aligned with PRALINE (http://www.ibi.vu.nl/programs/PRALINEwww). The sequences showing higher conservation scores were used as input to predict B cell epitopes from bioinformatic programs Bceprep (http://crdd.osdd.net/raghava/bcepred/index.html), Bepiprep 2.0 (http://www.cbs.dtu.dk/services/BepiPred/) and Epitopia (http://epitopia.tau.ac.il/). The sequences of the peptides are, MSP5: VGDKKPSDGDID and MSP1a: ERSRELSRARQEDQQ. The aminoacid sequence derived from MSP5 is common to both *A. marginale* and A. centrale, in contrast, the aminoacid sequence derived from MSP1A is not present in A. centrale, according to the bioinformatic analysis from published and our own *A. marginale* sequences.

iELISA protocol

Peptide antigenicity was tested by an in-house iELISA using the *A. marginale* crude antigen as a standard (Preciado de la Torre et al., 2006) and two synthetic peptides. Each synthetic peptide was resuspended in 1 ml of MQ sterile water and then diluted in 30 mM carbonate buffer (pH 9.6). 96-well microtiter plates (Corning Mexicana, Monterrey, NL, Mexico) were coated with 0.25 μg of each synthetic peptide or 5.0 μg of crude antigen

Figure 1. *Positivity values obtained from using individual A. marginale synthetic peptides MSP5 and MSP1a and individual sera from experimentally infected animals. C+, positive control; C- (1154), negative control. The line indicates the cutoff value, where values ≥1= positive and values <1= negative.*



(~200 µl). The plates were then incubated for 18 h at 4°C. Plates were then washed three times for 5 minutes using 200 µl of 1X phosphate buffered saline (PBS) supplemented with 0.5% Tween 20 (PBST20) (BioRad, Hercules, CA). Afterward, plates were blocked with 200 µl of 1X PBS, containing 0.05% Tween 20 (BioRad) and 5% Svelty® low-fat milk (PBST20 0.05%) (Nestlé, Mexico City, Mexico) for 1 h at 37°C being washed three times as mentioned above. After washing, plates were incubated with 200 µl containing different bovine sera diluted in PBST20 (1:100) and incubated for 1 h at 37°C. In all iELISA, the sera samples were used individually and never pooled. Then, plates were washed three times using 0.05% PBST20 and incubated with anti-bovine IgG alkaline phosphatase conjugated antibody in 0.05% PBST20 (1:5000) for 1 h at 37°C. After incubation, the plates were washed three times with 200 µl of 0.05% PBST20 and incubated for 1 h at room temperature with 200 µl of p-nitrophenyl phosphate substrate (SIGMAFAST p-Nitrophenyl phosphate tablets, Sigma-Aldrich, St. Louis, MO). All samples were run in triplicate. The plates were then read at 405 nm in a microtiter reader (BioRad) and absorbance values were recorded. Three wells containing all components, except for serum, were used as blanks. The mean absorbance of these three control wells was determined and subtracted from the absorbance of every other well in the plate. The mean absorbance of the triplicate samples of the positive and negative serum controls was also determined. The cutoff value was calculated as the mean absorbance value plus three times the standard deviation of the negative serum. The positivity index value for each serum was determined as the ratio of the mean absorbance divided by the cutoff index value (where, µ1= positive; <1= negative).

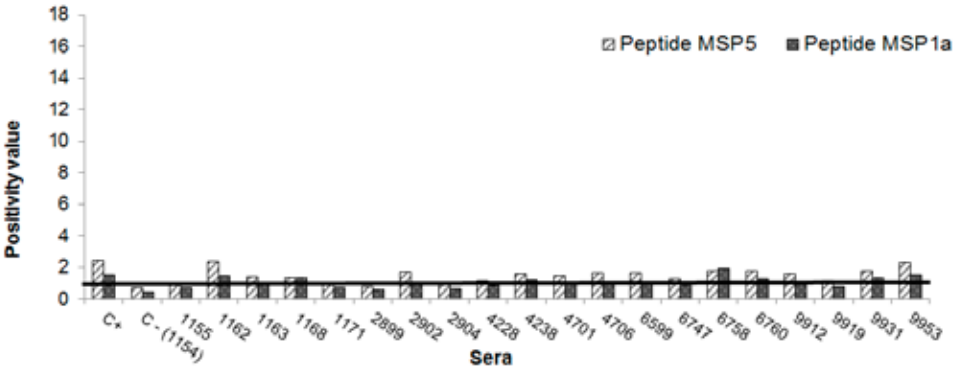
Statistical analyses

Specificity, sensitivity and Cohen´s kappa coefficient were calculated according to (Kundel and Polansky, 2003), using on-line available MedCalc utility for diagnostic test evaluation https://www.medcalc.org/calc/diagnostic_test.php).

RESULTS

The iELISA using 0.25 µg of MSP1a and MSP5 synthetic peptides showed that peptides have an antigenic potential when used separately. Even at different concentrations (starting at 0.25, 0.5, 1 and 2 µg of each peptide per well), the antibodies of the sera reacted with the antigens. In fact, antibodies

Figure 2. *Positivity values obtained using individual A. marginale synthetic peptides MSP5 and MSP1a and individual sera from naturally infected animals.*
C+, positive control; C- (1154), negative control. The line indicates the cutoff value, where values ≥1= positive and values <1= negative.



in the sera from animals experimentally and naturally infected with *A. marginale* reacted against both peptides when tested individually with positivity indexes exceeding 1.

In naturally infected animals, the highest positivity values are 5.07 and 5.45, corresponding to serum 8685, which reacted with MSP5 and MSP1a peptides, respectively (Figure 1).

In experimentally infected animals, the highest positivity values are those of sera 1162 and 9953, with 2.39 and 2.31 for synthetic peptide MSP5 and 1.49 and 1.52 for synthetic peptide MSP1a, respectively (Figure 2).

This shows that peptides were recognized by the specific antibodies against *A. marginale*, this is, those antibodies that recognize linear or conformational epitopes in *A. marginale* proteins.

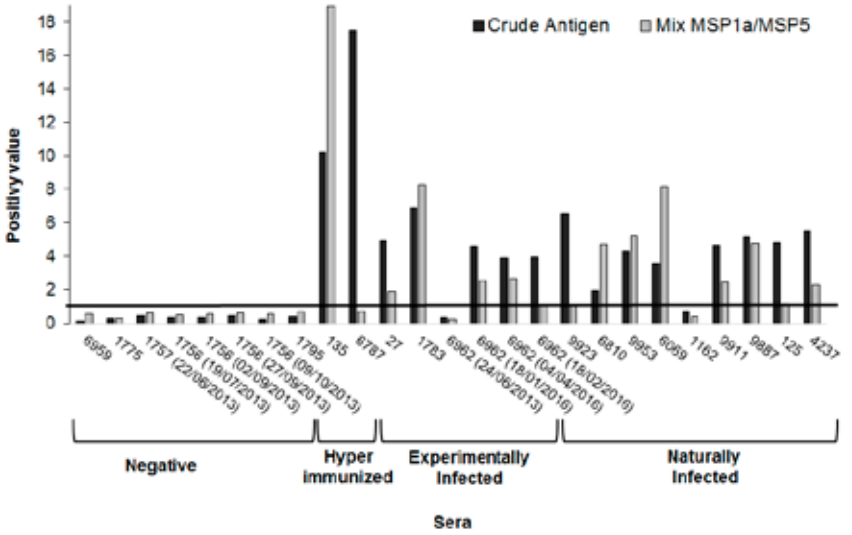
In an attempt to improve the capacity of individual peptide-antigens to be detect by a specific antibody, 96-well ELISA plates were coated with a mixture containing 0.25 µg of each synthetic peptide. Results were compared with those obtained with the standard crude extract antigen (5 µg/well) for the in-house iELISA. In this case, the sera used in the iELISA includes: positive and negative controls, sera from experimentally or naturally infected animals and sera from hyper-immunized animals. The highest values were obtained from sera of hyper-immunized animal 135 (10.21 and 18.90 to both, crude antigen and MSP5-MSP1a mixture, respectively). Sera from experimentally infected animal 27 against crude antigen shows higher positivity values compared to the MSP5-MSP1a peptide mixture. Serum 1783 yielded higher positivity values against the MSP5-MSP1a mixture. Animal serum 6962 was sampled on different dates. Sera from naturally infected animals 9923, 991, 9887, 125 and 4237 yielded higher values against crude antigen than for the peptide mixture, while sera from animals 6810, 9953, and 6069 gave higher values against the peptide mixture than to the crude antigen (Figure 3). The results of this assay show an excellent level of agreement between the crude antigen and the peptide mixture with regards to positive and negative sera. In a number of cases, the magnitude of response was better for the peptide mixture.

The calculated sensitivity for the peptide mixture was 100% and the Cohen´s kappa coefficient was 84.62 at 0.95 confidence for specificity.

DISCUSSION

The development of diagnostic methods based on synthetic peptides derived from antigenic proteins have been reported previously (Vordermeier et al., 2001). In this work we used synthetic peptides to diagnose

Figure 3. *Positivity values obtained using a crude antigen of A. marginale and a MSP5-MSP1a mixture against individual selected sera. The line indicates the cutoff value, where values ≥1= positive and values <1= negative.*



bovine anaplasmosis.

As observed in results, individual peptides have a poor capacity to be detected by *A. marginale* specific antibodies, nonetheless, the mixture of these peptides has a much better performance when detecting by a specific antibody. Even when compared to the standard crude antigen, the positivity index is usually higher in those reactions containing peptide mixture, with a few exceptions. Synthetic peptides have been successfully used as antigens to diagnose bovine tuberculosis in cattle, anaplasmosis in domestic sheep, Aleutian mink disease and Foot-and-Mouth disease virus in different animals, among other veterinary relevant diseases (Faria et al., 2011; Ma et al., 2016; Mason et al., 2017; Vordermeier et al., 2001). Synthetic peptides can be synthesized commercially, their purity exceeds 75% and their cost is affordable. For MSP1a and MSP5 peptides, the sequences were derived from bioinformatic analyses that included a large number of B cell epitopes that are present in many proteins conserved in at least nine Mexican *A. marginale* strains. These sequences were required to be on outer membrane proteins and, according to available bioinformatic programs, the epitope sequences themselves were exposed in the proteins.

Our study confirms that a synthetic peptide mixture may have the potential to act as antigens in bovine anaplasmosis diagnosis in a simpler and more accurate manner as compared to the previously available test, which is based on the use of a crude antigen. If it were to be implemented as a diagnostic tool in Mexican certified laboratories, most cattle producers would benefit from accurate evaluation of their herd for bovine anaplasmosis infection, particularly those animals mobilized within genetic enhancement and stock replacement programs. We believe that these peptides may have potential in the differential diagnostics of A. centrale from *A. marginale*, yet we did not test for A. centrale as this organism is not present and it is classified as exotic in Mexico.

CONCLUSION

Synthetic peptides may be used as an alternative to crude antigen or recombinant proteins usually used in bovine anaplasmosis diagnosis. According to our results, the mixture of synthetic peptides is well recognized by antibodies of sera from infected

animals, with 100% of sensitivity and 95% of specificity.

ANIMAL RIGHTS

This study was approved by the Animal Experimentation and Ethics Committee of the Centro Nacional de Investigación Disciplinaria en Salud Animal e Inocuidad (CENIDSAI) branch of the Instituto Nacional de Investigaciones Forestales Agrícolas y Pecuarias (INIFAP). The study took ethic and methodological aspects into considerations in accordance with the Mexican regulations on use, housing and transportation of experimental animals (NOM-062-ZOO-1999 and NOM-051-ZOO-1995).

CONFLICT OF INTEREST

The authors declare no potential conflicts of interest with respect to the research, authorship, and/or publication of this article.

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