SEROLOGICAL EVIDENCE OF *Anaplasma* spp. IN SMALL RUMINANTS FROM VENEZUELA USING RECOMBINANT MSP5 IN IMMUNOENZYMATIC ASSAY

Evidencia serológica de *Anaplasma* spp. en pequeños rumiantes de Venezuela utilizando MSP5 recombinante en ensayos inmunoenzimáticos

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ABSTRACT

Anaplasma marginale causes a disease in cattle characterized by fever, anemia and decrease in milk and meat production. Small ruminants do not show signs of disease when infected, but it has been suggested they could act as reservoirs. Goat and sheep breeding is socially and economically important in arid and semi-arid areas in Venezuela, and these species often share space and food with cattle. The aim of this work was to detect antibodies against *Anaplasma* spp. in Venezuelan goat and sheep flocks. To accomplish this goal, an indirect ELISA using recombinant MSP5 as antigen of *A. marginale* was performed. Sera obtained from experimental infection in goat and a hyperimmune sheep serum were used as positive controls. Blood sera were obtained from 45 sheep and 48 goats located in Guárico State, an endemic area to bovine anaplasmosis. After standardization of assay for each species, 80.46% of the sheep and 59.25% of the goat sera showed to have antibodies against MSP5. No signs of clinical disease were detected in sampled animals. These results suggest that small ruminants could harbour *A. marginale* and consequently may be reservoirs for neighbouring cattle if appropriate vectors are present. The development of clinical diseases caused by *A. marginale* under stress situations and the existence of other *Anaplasma* species (e.g. A. *ovis*) in small ruminants should also be investigated.

Key words: *Anaplasma marginale,* ELISA, goat, MSP5, sheep.

RESUMEN

Anaplasma marginale ocasiona una enfermedad en los bovinos caracterizada por fiebre, anemia y disminución de la producción de leche y carne. Los pequeños rumiantes generalmente no muestran signos clínicos, por lo que pudieran actuar como reservorio. En Venezuela, los ovinos y caprinos tienen gran importancia económica y socialmente en zonas áridas y semi- áridas e incluso, en muchas ocasiones comparten su espacio y alimento con los bovinos. El objetivo de este trabajo fue detectar anticuerpos contra *Anaplasma* spp. en rebaños de ovinos y caprinos. Para ello, se estandarizó un ELISA indirecto con la MSP5 recombinante de *A. marginale*, empleando sueros provenientes de infecciones experimentales en caprinos y un suero hiperinmune ovino como controles positivos. Posteriormente, fueron obtenidos sueros sanguíneos de 45 ovinos y 48 caprinos localizados en una zona endémica a anaplasmosis bovina del estado Guárico. De estos, 80,46% de los ovinos y 59,25% de los caprinos presentaron anticuerpos que reconocieron la MSP5, sin embargo, ninguno de estos animales positivos presentaron signos clínicos de la enfermedad. Estos resultados sugieren que los pequeños rumiantes son portadores de *A. marginale* y por ende, pueden estar actuando como reservorio de la enfermedad para los bovinos en el caso que se encuentren los vectores apropiados. Por lo tanto, se debe profundizar en los estudios sobre el desarrollo de sintomatología clínica en condiciones de estrés y la existencia de otras especies de *Anaplasma* (como *A. ovis*) en los ovinos y caprinos de Venezuela.

Palabras clave: Anaplasma marginale, ELISA, caprinos, MSP5, ovinos.

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INTRODUCTION

Bovine anaplasmosis is an infectious disease of cattle (*Bos indicus - taurus*), caused by rickettsial bacteria *Anaplasma marginale.* This pathology is characterized by the presence of typical intraerythrocitic bodies associated with a progressive anaemia, due to a loss of 60-65% of red blood cells [10, 23]. From the time of Theiler discovery in 1910 [28], who initially described *A. marginale*, anaplasmosis has been recognized in tropical and subtropical areas worldwide and identified as an endless menace to cattle industry. In ovines (*Ovis aries*), the disease caused by *Anaplasma ovis* produces a slight to severe decrease in packed cell volume and death in affected animals. The disease in that specie has been mainly reported in Africa, but some outbreaks have occurred in Southeast Europe countries as well in USA, Russia and China in the middle of the past century [23]. Curiously, it has been reported that small ruminants are not severely affected by *A. marginale* and cattle show an innate resistance to *A. ovis* [15, 23].

The presence of *Anaplasma* species in goats (*Capra hircus*) was first reported in 1912 in Africa [8]. Later, *A. ovis* was described in North America, China, Turkey and Iran [9] as the etiological agent of a non-pathogenic disease showing minor clinical complications with low prevalence in goat flocks [9, 25]. The number of affected animals and clinical manifestations could become more severe under nutritional stress situations [8]. Until now, there are no reported cases of goat or sheep anaplasmosis by *A. ovis* in Venezuela.

Of the immunodominant major surface proteins of *A. marginale,* a protein of 19 KDa named MSP5 [27] was cloned, sequenced and proposed as a diagnostic tool [30]. Now, MSP5 has shown to be an excellent protein in diagnosis of bovine anaplasmosis worldwide [14, 20, 29, 30]. The recognition of MSP5 by sera of *A. marginale* infected animals demonstrated that this protein is conserved [12, 29 30]. It has been also recognized that MSP5 shows cross-reaction with *A. ovis* and *A. centrale* [16, 30]. Molecular works have shown that the protein is codificated by a single gene conserved between all the isolates and species of the genus *Anaplasma* tested [1, 19, 30]. In the present work, recombinant MSP5 *A. marginale* was used to detect antibodies against *Anaplasma* spp. in small ruminant flocks from Venezuela.

MATERIALS AND METHODS

Sheep and goat sera. Blood sera were obtained from 45 sheep and 48 goats maintained in field conditions in Estación Experimental La Iguana, located at Guárico State, Venezuela, regardless of breed and age.

Recombinant *Anaplasma marginale* major surface protein 5 (rMSP5). Recombinant protein was obtained as described [19]. Protein concentration was estimated by a Lowry assay.

Hyperimmune sheep sera to rMSP5. 50 µg of recombinant MSP5 in Freund complete adyuvant (Grand Island Biological Company, cat. No. 6605721, USA) was once inoculated to a one year-old lamb plus three equal doses in Freund incomplete adyuvant (Grand Island Biological Company, cat. No. 6605720, USA) in a fortnight basis. Fifteen day after the last inoculation, sera was collected and frozen (Whirpool, Model ARC 4110 IX, USA) at -20°C until use.

Negative sheep and goat control sera. Negative sheep sera were obtained from an anaplasmosis-free area (kindly given by Dr. Blasco, Centro de Investigación y Tecnología Agroalimentaria, Aragón, Spain) and from Estación Experimental La Iguana, Guárico State, Venezuela. All sera were previously tested by Western blot against rMSP5 and after used in ELISA assays using a protocol previously described [5].

Experimental infection of goat. To obtain positive sera to *A. marginale*, a five month-old goat was inoculated with 5 x 10^8 *A. marginale* infected erythrocytes of a Zulia State isolate [22] and re-inoculated on day 58. Parasitemia and packed cell volumen was measured and recorded daily until day 105 post-inoculation. Blood thin smears were stained with Hemacolor® (Merck). Sera were collected every other day from the day of inoculation with A. marginale in order to find an adequate serum to use it as positive control.

Immunoenzimatic assays. Polystyrene plates (Polysorp, Nunc, Denmark) were coated with increasing concentrations of rMSP5 (0.5, 1, 2 µg/mL) diluted in 50 mM bicarbonate-carbonate buffer pH 9.6 and incubated overnight at 4°C. Wells were washed five times with 200 µL of 150 mM NaCl-Tween 0.1% in ELISA washer (Columbus, model No.30008658, Tecan, Austria). Non-specific binding sites were blocked with 5% non-fat dry milk diluted in phosphate buffer saline solution at 20 mM, 150 mM NaCl, pH 7.2 (PBS) for 1 hour at 37°C (Thelco, model No. 6DG, Thelco, USA). After five additional washes, positive, negative and sampled sera diluted 1:100 and 1:200 in PBS-1% Tween were added by duplicate. Following incubation 1 hour at 37°C, and washed again, wells were filled with 100 µL of horseradish peroxidase anti-ovine immunoglobulin conjugate (InmunoPure® cat. Nº.31480, Pierce, USA) or horseradish peroxidase anti-goat immunoglobulin conjugate (InmunoPure® cat. No. 31402, Pierce, USA) diluted 1:20000 in PBS-Tween buffer.

After incubation of 1 hour at 37°C, conjugate solution was discarded and 50 µL de TMB (Tetramethylbenzidine, Research Organics, cat. No. 3020T, USA) in appropriate buffer (Citrate phosphate buffer 50mM, pH 4, 0,01% TMB in dimetil sulfoxide and 1% H_2O_2) were added into each well. The plates were left at room temperature for 30 minutes. Colour development was stop by adding 50 µL 1M H_2SO_4. Absorbance values were recorded within ten minutes in an ELISA plate reader (BioRad Model 3550, USA) at 450 and 630nm.

Cut-off. In order to obtain cut-off values, sheep and goat negative sera were tested in standardized ELISA conditions as described above. Result of mean values of optical density plus

3 x standard deviation value was used as cut-off point (X + 3SD) in each specie [3].

RESULTS AND DISCUSSION

In Venezuela, bovine anaplasmosis has been extensively studied using immunological or epizootiological approaches [5, 12, 19, 21, 22]. However, the impact of this disease in small ruminant flocks is still unknown, despite their importance in economical activities of several localities of the country. In this work, It was successfully standardized a sensitive indirect ELISA to be used in small ruminants, using a recombinant MSP5 of *A. marginale* as antigen.

Standardization results of ELISA/rMSP5 for sheep sera are shown in FIG. 1. The largest positive/negative sera ratio was obtained with 1 µg/mL of antigen, sera dilution of 1:100 and secondary antibody dilution of 1.20,000. Under these conditions, hyperimmune serum had an absorbance of 1.4 and OD and mean of negative control sera was 0.135. In this condition the ratio between positive OD/ negative OD was 10.37.

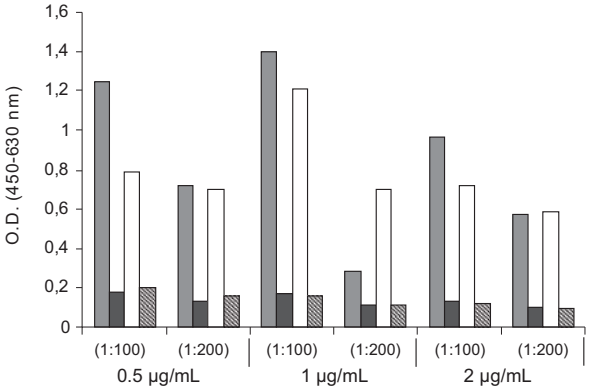

FIGURE 1. STANDARIZATION OF ELISA WITH rMSP5 USING SHEEP SERA. ANTIGEN DILUTION: 0.5 µg/mL, 1µg/mL y 2µg/mL. SERA DILUTIONS 1:100, 1:200. HYPERINMUNE SHEEP SERUM ■, POSITIVE SHEEP SERUM ▨, NEGATIVE SHEEP SERUM ■, SPANISH NEGATIVE SHEEP SERUM ▨. CONJUGATE DILUTION: 1:20,000/ ESTANDARIZACIÓN DEL ELISA CON LA MSP5r EMPLEANDO SUERO OVINO. DILUCIÓN DEL ANTÍGENO 0.5 µg/mL, 1µg/mL Y 2µg/mL. DILUCIÓN DEL SUERO 1:100, 1:200. SUERO HIPERINMUNE DE OVINO ■, SUERO OVINO POSITIVO ▨, SUERO NEGATIVO OVINO ■, SUERO OVINO NEGATIVO ESPAÑOL ▨. DILUCIÓN DEL CONJUGADO: 1:20.000.

To optimize ELISA conditions in goat assays, day 84 post-inoculation serum was used as positive control and a preimmune serum (day 0) as negative control. Optimal conditions were obtained with a sera concentration of 1:400 and secondary antibody concentration of 1:20000. Under these conditions, positive OD (0.789) / negative OD (0.210) ratio was 3.7 (FIG. 2).

Cut-off values obtained in standardization assays for both species were 0.476 and 0.473 in sheep and goat, respectively.

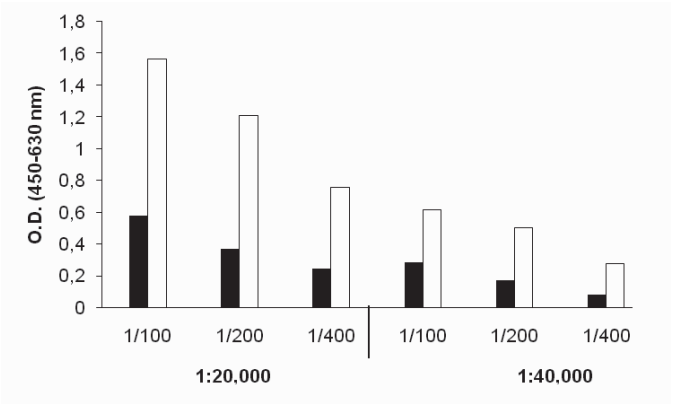

FIGURE 2. STANDARDIZATION OF ELISA WITH MSP5r USING GOAT SERA. ANTIGEN DILUTION: 1 µg/ml. SERA DILUTIONS 1:100, 1:200 AND 1:400. GOAT POSITIVE CONTROL SERA □, GOAT NEGATIVE CONTROL SERA ■. CONJUGATE DILUTION: 1:20,000 AND 1:40,000/ ESTANDARIZACIÓN DEL ELISA CON MSP5r EMPLEANDO SUERO CAPRINOS. DILUCIÓN DEL ANTÍGENO 1 µg/ml. DILUCIÓN DEL SUERO 1:100, 1:200 AND 1:400. SUERO DE CONTROL POSITIVO CAPRINO □. SUERO DE CONTROL NEGATIVO CAPRINO■. DILUCIÓN DEL CONJUGADO: 1:20.000 Y 1:40.000.

Kinetics of antibody response against rMSP5A in an experimentally infected goat is observed in FIG 3. Two peaks of antibody response on days 21 and 78 (7 days after reinoculation) were observed (FIG. 3), but started to decrease a few days later. Parasitemia level was up to 0.01% in blood thin films along three days, from day 21 after inoculation. After reinoculation, 0.01% of parasitemia persisted for two days (data not shown). No evidence of fever or decrease in packed cell volume values were observed.

Previous reports of undetected rickettsemia in experimentally infected goats with *A. marginale* [15] may indicate that these animals could act as dead-end hosts because they do not express enough levels of the microorganism [4]. However, splenectomized goats were shown to be latent carriers of *A. marginale* with positive serological reaction to complement fixation tests [13], so it seems that they could act as reservoirs.

Even supposing small ruminants could be hosts of *Anaplasma marginale,* rickettsemia must reach optimal levels under stress situations to be acquired by adequate arthropods. But, although the cattle tick *Boophilus microplus* has been traditionally incriminated in transmission of *A. marginale* in tropical areas and prevalences ranged 8-18% in goats which inhabit in cattle farms in Venezuela [24], its role in transmission of *A. marginale* is still a matter of discussion [2]. Haematophagus diptera (Tabanids) and even mosquitoes are implicated by immediate and delayed transfer feeding from acutely infected bovines to susceptible animals [6,7]. Hence, it would be an appropriate spreading route from cattle to sheep and goats in shared environments.

After standardization of working conditions of the ELISA, 45 sheeps sera and 48 goats sera from Venezuelan

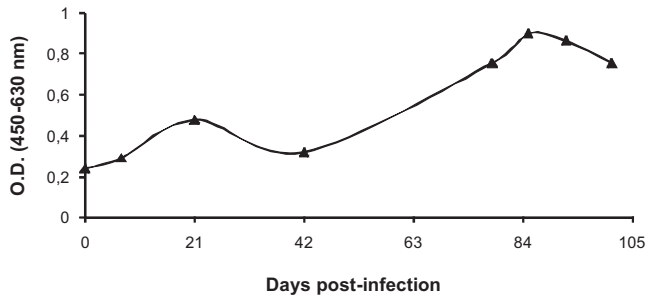

FIGURE 3. KINETIC OF ANTIBODY RESPONSE AGAINST rMSP5 IN AN EXPERIMENTALLY INFECTED GOAT WITH CRYOPRESERVED *A. marginale* BODIES. FIRST INOCULATION WAS MADE ON DAY 0 AND REINOCULATION ON DAY 78. CONJUGATE DILUTION 1:20,000. GOAT SERA DILUTION 1:400 AND ANTIGEN CONCENTRATION 1 µg/mL/ CINÉTICA DE ANTICUERPOS FRENTE A MSP5r EN UN CAPRINO EXPERIMENTALMENTE INFECTADO CON UN CRIOPRESERVADO DE CUERPOS DE *A. marginale*. LA PRIMERA INOCULACIÓN FUE HECHA EN EL DÍA 0 Y REINOCULADO EL DÍA 78. DILUCIÓN DE CONJUGADO 1:20.000. DILUCIÓN DEL SUERO CAPRINO 1:400 Y DE LA CONCENTRACIÓN DE ANTÍGENO 1 µg/mL.

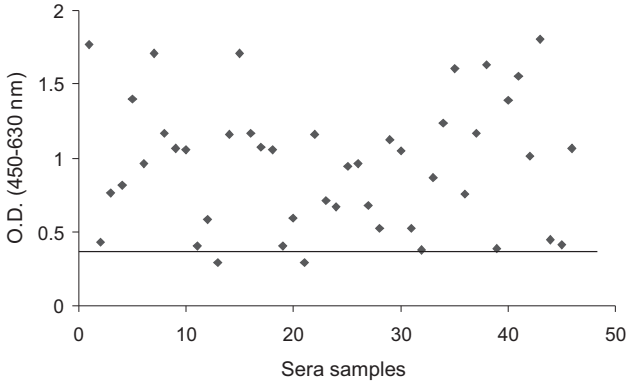

FIGURE 4. ELISA/rMSP5 IN STANDARDIZED CONDITIONS FOR 45 FIELD SHEEP SERA FROM VENEZUELAN SAVANNA, GUÁRICO STATE. CUTOFF: 0.476. SEROPREVALENCE 80.46% / ELISA/MSP5r EN CONDICIONES ESTANDARIZADAS PARA 45 OVINOS DE CAMPO PROVENIENTES DE LOS LLANOS VENEZOLANOS, ESTADO GUÁRICO. PUNTO DE CORTE: 0,476. SEROPREVALENCIA DE 80,46%.

were tested by ELISA/rMSP5. Of all sera tested, 80.46% of sheep and 59.25% of goat sera recognized rMSP5 (FIGS. 4 and 5). OD ranged between 0.3 to 1.75 in sheep sera and 0.15 to 1.56 in goat sera. Some of the positive sheep sera showed a higher OD than those from goats.

Previously, Tavares-Marques and Reyna-Bello [26] showed *Anaplasma* spp. in sheep from Venezuela by molecular methods. Recently, it has been reported in Brazil [18] a serological evidence of *Anaplasma* spp. in small ruminants. In this work, it was found a considerable number of animals with antibodies against rMSP5 if compared with findings in Brazil of 12 and 16% (sheep and goats, respectively) [18].

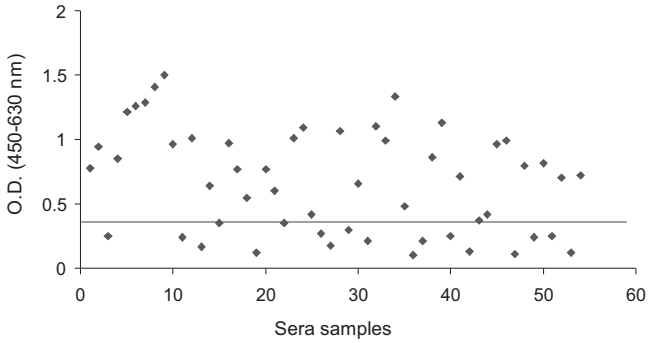

FIGURE 5. ELISA/rMSP5 IN STANDARDIZED CONDITIONS, FOR 48 FIELD GOAT SERA FROM VENEZUELAN SAVANNA, GUÁRICO STATE. CUT-OFF: 0.473. SEROPREVALENCE 59.25% / ELISA/MSP5r EN CONDICIONES ESTANDARIZADAS PARA 48 SUEROS CAPRINOS DE CAMPO PROVENIENTES DE LOS LLANOS VENEZOLANOS, ESTADO GUÁRICO. PUNTO DE CORTE 0,473. SEROPREVALENCIA 59,25%.

The experimental infection in a goat suggests this specie can harbour *A. marginale* with no anemia, fever or other signs of anaplasmosis. Similar results were obtained when an experimental infection in sheep was done (data not show). As MSP5 is found in *A. marginale* as well in *A. ovis*, a question arises: were sampled field animals infected by *A. ovis* instead of *A. marginale*? In Africa, anaplasmosis in goats due to *A. ovis* seemed to be of economic importance, because 25% of the whole goat world population inhabit in this continent [11]. Anaplasmosis in small ruminants could become a severe disease under some conditions as stress [17]. In arid and semiarid locations of Venezuela, where goat breeding is the main way of life, there is no previous report of *A. ovis* or the disease caused by the rickettsia.

CONCLUSIONS AND RECOMENDATIONS

In this work, an indirect ELISA using recombinant MSP5 from *Anaplasma marginale* to be used in detection of antibodies in small ruminants was standardized. These assays allowed demonstrating the existence of the antibody anti-MSP5 antibodies in goat and sheep from Venezuela. Using ELISA/rMSP5, a seroprevalence of 80.46% and 59.25% was found in sheep and goat sampled population, respectively.

As *Anaplasma marginale* infection does not cause clinical signs in small ruminants, the absence of disease in the sampled animals (as well as the experimentally infected) suggests that they were not infected by any other rickettsia than *A. marginale.* If *Anaplasma ovis* were the agent responsible for the ELISA results obtained in this study, classical clinical signs of *A. ovis* infection (anemia, fever and rickettsemia) would have been detected.

Since small ruminants may be carriers of *Anaplasma marginale*, it is important to detect harboring animals in places

where bovines, sheep and goats are raised together. Moreover, the frequent introduction of goats and sheep from other countries to Venezuela means *A. ovis* infections should be under epizootiological surveillance.

In order to conclude about the presence and impact of *A. ovis* or A. marginale in small ruminants from Venezuela, epizootiological studies (including sequencing the 16S ribosomal gen of *Anaplasma* species) should be widely performed.

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