Global Advanced Research Journal of Medicine and Medical Sciences (ISSN: 2315-5159) Vol. 5(6) pp. 188-193, June, 2016
Available online http://garj.org/garjmms

Full Length Research Paper

Evaluation of the hemolymph extraction technique in *Rhipicephalus sanguineus* sensu lato for detecting *Anaplasma platys*

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Accepted 06 June, 2016

Diagnosis of *Anaplasma platys*, can be performed in the laboratory by using hemolymph test vector organs or tissues using serological colorants and molecular methods. The purpose of the research was to evaluate the hemolymph extraction technique in the brown tick of the dog (*Rhipicephalus sanguineus sl*) for the detection of the rickettsia, identify the canine anatomical region of most collection of specimens, the site for greater fluidity of hemolymph, rickettsial infection prevalence in the tick and compare the reference method (Test hemolymph) with indirect immunofluorescence test (IIF). The vector was placed in a petriplate in ventral position; an incision in the coxal suture (intersegmental membrane), between the front and rear coxotrocanteral joints, was performed to visualize the spontaneous release of hemolymph; this was collected in a calibrated capillary glass (10µL). Hemolymph samples were performed with the following procedures: they were stained with rapid staining Hemacolor®. Antigens were prepared to practice test (IIF) and dilutions were performed at the hemolymph in PBS phosphate buffer, by placing them in contact with plates prepared with canine antigens of *A. platys*.157 ticks in 21 infected dogs were counted. Majority of specimens were collected in the head / neck (36.9%) region. The largest volume of hemolymph was obtained from the intersegmental membrane of the coxa. Inclusion bodies were shown in platelets. The prevalence of infection with *A. platys* in *R. sanguineus*, using hemolymph test was 27.39%. IIF technique revealed the presence of antigens of *A. platys* in the hemolymph of *R. sanguineus* and macerated. By comparing the Hemolymph Test with IIF, it showed a specificity of 100%. The sensitivity for the detection of *A. platys* was 37.50%. It was shown for the first time in the country, by extracting hemolymph of *R. sanguineus* the presence of *A. platys* using both methods.

Keywords: Estimation, test, hemolymph, *Rhipicephalus sanguineus*, *Anaplasma platys*

INTRODUCTION

Canine Anaplasmosis is an infectious bacterial disease transmitted by hard ticks (Ixodidae) that affect humans and animals. They are universally distributed caused by different species of the genera Anaplasma of Anaplasmataceae family. Taxonomically belongs to the order rickettsial and is characterized by a gram negative bacteria, pleomorphic and obligatory intracellular growth (Parola et al., 2005; Fournier and Raoult, 2009). They differ from other rickettsiae species because they replicate in vacuoles derived from the cell membrane of the cells they infect, mainly leukocytes and platelets (Rikihisa, 1991). In this case, the bacteria enter into the

blood cells by phagocytosis and stay in cytoplasmic vacuoles, where they divide to form colonies of bacteria known as morula, distinctive feature of this group of pathogens.

Anaplasma platys is causing Canine Infectious Cyclic Thrombocytopenia (ICCT). It was reported in dogs for the first time in the United States (Dumler et al., 2001) and *R. sanguineus sl* is suspected to be involved as its main vector (Sanogo et al., 2003; Abarca et al., 2007).

A. platys was formerly *Ehrlichia platys*, but in 2001, was reclassified within the family Anaplasmataceae, genus Anaplasma (Dumler et al., 2001). This rearrangement based on the analysis of the gene sequence of ribosomal RNA (16S) groESL operon coupled to biological and antigenic characteristics, allowed a new reorganization of members of the tribe Ehrlichieae, which are included in one of the four genera (geno groups) that make up the Anaplasmataceae family (Dumler et al., 2001).

A. platys, transmitted by the brown dog tick (*Rhipicephalus sanguineus sl*), is an obligate intracellular rickettsial organism that infects platelets (Quintero et al., 2004). *R. sanguineus* acts as a vector of different pathogens with a potential risk to animal and public health (Moreno et al., 2005). Among the diseases that can be transmitted by *R. sanguineus* as accidental human host, are rickettsiosis, ehrlichiosis, babeiosis and hepatozoonosis (Morales et al., 1993; Vega y Luévano, 2011).

A. platys in *R. sanguineus sl* was reported in different countries from Asia, Africa and Europe (Sanogo et al., 2003; Inokuma et al., 2000; Ybañez et al., 2012; Latrofa et al., 2014; Ramos et al., 2014). In Argentina, *A. platys* was detected in *R. sanguineus* (Cicuttin et al., 2015). In Venezuela, *A. platys* was found in canine platelets (Arraga-Alvarado et al., 2003). By molecular biology techniques (Huang et al., 2005) and in humans with HIV (Tamí and Tamí-Maury, 2004)

Anaplasmosis is considered a pathogen of low virulence, often in association with other infections or diseases. Most dogs infected with *A. platys* are healthy, but experience a cyclic thrombocytopenia. Research indicates that 20% of dogs tested from the Caribbean island of Grenada were infected with *A. platys* and 25% *E. canis* supporting frequent transmission of these organisms in regions in which *R. sanguineus* tick is the only known species (Abarca et al., 2007). However, this is an important public health problem that has not been sufficiently characterized in the country from a microbiological point of view, taxonomic, clinical and epidemiological, which causative agent can be confused with other microbiological agents and the transmission mechanism is not clear enough.

The diagnosis of *A. platys*, ticks can be performed in the laboratory through various techniques that can be divided into: a) methods of direct demonstration of the bacterium using colorants b) serological methods such as indirect immunofluorescence technique c) biology molecular (Latrofa et al., 2014; Anda et al., 2008). Generally, the first two are applied to the Hemolymph test and the last, using organs, tissues or vector macerated.

The hemolymph test, is the procedure of choice since 1978 in Entomology Reference Laboratories in various countries (Burgdorfer, 1970). This test can be performed only on live specimens. Regarding to the hemolymph extraction technique Burgdorfer (1970) in USA, indicate to collect it making a previous numbness of ticks using ether, chloroform or CO_2 in low doses and then amputating the distal portion of the tarsus above, or the second and third pairs of legs, if necessary. Under the same conditions Guglielmone et al. (1985) and Kurz and Burgdorfer (1978) extracted hemolymph using a similar procedure described by measuring the rate of tick paralysis before extraction of the hemolymph. Also, Guglielmone et al. (1985) in Argentina, collected *Boophilus microplus* hemolymph to detect levels of *Babesia bigemina parasitemia*; severing a limb (leg) per day and collecting the hemolymph emanating from the wound on a slide. In Colombia, obtaining hemolymph from live ticks can carry out preliminary tests of rickettsial before the end of sample processing, saving resources for this purpose. Before the extraction, ticks are immersed in alcohol for 5-10 seconds using scissors, cutting the distal portion of the first right leg (Instituto Nacional de Salud de Colombia, 2011). Similarly, Melendez and Forlano (1996) in Venezuela, collected ticks at a set time (9:00 a.m. to 10:00 a.m.) and a time of 20-30 minutes interval, severing one leg per day and collecting the emanating hemolymph from the wound on a slide.

Motivated, to unavailability of routine diagnostic methods accessible in both public and private laboratories that allow identification of *A. platys* in the vector and to help diagnose for active surveillance of the disease, the fact of using tick for indirect or complementary diagnosis could be assessed and also having an economical method to detect it early in the brown dog tick, when parasitemia is low.

This research evaluates the technique to extract and preserve the hemolymph and macerated of the brown dog tick *R. sanguineus sl* for detecting *A. platys*. Also, identify the anatomical region of the most of specimens in the canine; the site of greater fluidity of the hemolymph and rickettsial infection prevalence in the tick using a direct method (Hemacolor) and serological method (indirect immunofluorescence). The practice of extracting hemolymph could become a tool for the diagnosis of natural infections transmitted by arthropods.

METHODS

Study Area

The study was conducted in dogs that were taken to two

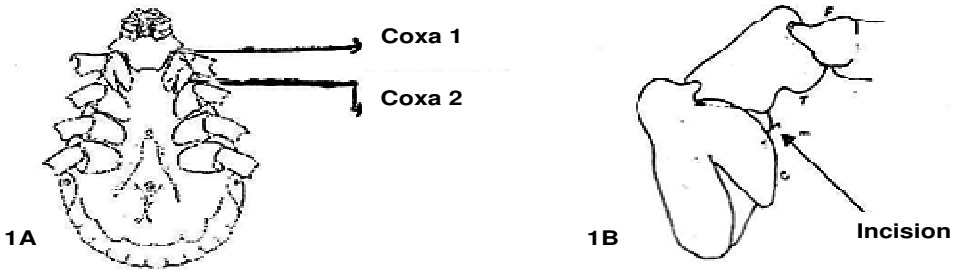

Figure 1A. Female Rhipicephalus sanguineus. Ventral view. **Figure 1B.** Schematic drawing showing the anatomic site of the hemolymph extraction. Female *Rhipicephalus sanguineus*. Leg. Ventral view. **C**= coxa; **T**= trochanter; **F**= femur; **M**= coxo-trocanteral membrane.

veterinary clinics located in the municipality of Valencia, Carabobo state. Venezuela. The municipality is located in the north of the country (10º10'11'' N latitude, longitude 68º 59' 12'' W). It corresponds to the bioclimatic zone dryly tropical forest (bs-T), (Ewell et al., 1968) whose training area extends from sea level to 1,000 meters. The average annual temperature varies between 22 ºC and 29 °C. Annual rainfall varies between 1000-1800 mm and has a wide variety of vegetation, represented by virgin forests.

Collection of specimens

Ticks were collected from dogs which had been confirmed the presence of Ehrlichia spp, for white coat smear (Arraga-Alvarado et al., 1996) in dogs found outside and inside the home and were in contact with humans. Specimens of adult ticks of both sexes (male and female) were used for this.

The samples were processed in the Research Laboratory of the Department of Parasitology, Faculty of Veterinary Sciences (FCV) and acarology Laboratory, Faculty of Agronomy (FAGRO). Central University of Venezuela.(UCV).Maracay, Aragua, Venezuela.

To collect specimens, plastic containers were used to cover semitransparent thread. In order to maintain humidity of the container, filter paper circles of the size of the container bottom were placed, previously wetted with tap water. Each container is identified with the following information: canine race and sex, collection date,age, immune status, travel history, any other health information related and site of tick extraction in the canine.

For the release of host ticks, dissecting forceps without teeth were used, holding them as close as possible of the chapter, then turned up and introduced into plastic containers previously identified.

Ticks were transported in coolers containing a small amount of dry ice in its interior covered in newspaper. The containers with ticks were kept in the refrigerator (TECNON®) at 4ºC, preceded to the taxonomic identification of specimens with the aid of the stereomicroscope (ZEISS, MOD. STANDARD) using the Guerrero key (Guerrero, 1996).

Conditioning of slides

Before obtaining the hemolymph and macerated antigens, cover-slip slides of 75 x 25mm (ESCO®) were conditioned; drawing out circles (6 mm diameter) with a template of routine use for cartoonists. Each slide was marked with 12 circles, six in the top and the same number on the bottom. Hemolymph and mash samples were placed in duplicate.

Hemolymph extraction

In order to remove hemolymph to detect antigens of *A. platys* by inmunofluorescence, the tick was anesthetized and placed in a petri dish in ventral position. Moving parts of the legs were inspected: coxa, trochanter, femur, tibia, basitarsus, tarsus and postarsus, to determine the more smoothly hemolymph volume site and better operability in the extraction technique (Figure 1A). For this research the coxa (intersegmental membrane) of the first pair of legs, which underwent an incision in the back ventral membrane, corresponding to the coxo-trocanteral joint with a selected disposable sterile lancet (NIPRO®) to visualize the spontaneous release of hemolymph (Figure 1B).

The droplet was collected in a calibrated capillary glass(1µL). The entire procedure was performed under a ZEISS Standard stereomicroscope. The extracted hemolymph was distributed as follows: an aliquot for hemolymph smears on 22 x 22 mm slides, in order to color them later, 10µL to be diluted in eppendorf tubes containing 90 mL of PBS (phosphate buffered saline , pH = 7.2, 0.02 M NaCl 0.5%) for the indirect immunofluorescence test (IIF).

After the hemolymph extraction, the macerated of the tick in study was obtained, placing it in the circles of the previously preconditioned slide and with the help of another microscope slide, placed perpendicular to the previous slide, then a 180º spin was given to achieve the correct flattening of the vector.

Staining of smears

Antigen hemolymph slides were macerated, air-dried and

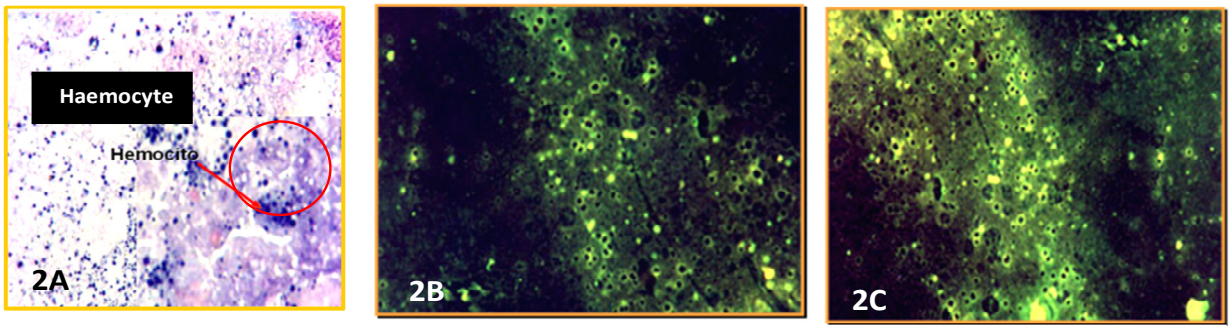

Figure 2A. Ehrlichia spp in hemocyte of R. sanguineus.Hemolymph tissue colorized by the Hemacolor Merck®. 100X.
Figure 2B. Indirect Inmunofluorescence.Hemolymph tissue of R. sanguineus infected with Anaplasma platys. Initial Bodies.100X.
Figure 2C. Platelets with brilliant green color morulae evinced using the IIF technique. 100X

fixed with acetone for 10 min. Then stored in carrier sheets of cardboard, wrapped in aluminum foil and stored in refrigerator at 4 °C. Regarding to hemolymph smears taken in slides, were air dried and fixed in absolute methanol for 10 min and then stained with MERCK Hemacolor© (Merck, 2000) (MERCK 11661001 Hemacolor 3X500ML) commercial color. These smears were stored in foam boxes, covered and properly identified. The identification of inclusion corpuscles compatible with *A. platys* was performed under microscopic observation with immersion objective (100X).

Indirect immunofluorescence (IIF)

The test involves placing the diluted hemolymph in slides adhered with a known antigen (*Ehrlichia platys*) if it contains specific IgG antibodies react with IgG labeled with fluorescein, bind to the antigen forming antigen-antibody complex and the reaction is visualized in Immunofluorescence microscopy (Ross and Lohr, 1968). Each time the test was performed and compared with positive and negative control were kindly donated by Dr. Cruz Maria Alvarado (Universidad del Zulia) included in three dilutions 1/4 (minor title), 1/8 and 1/16 (higher degree) to compare the fluorescence intensity. All less than or equal to 1/4 title was considered negative and positive results in all more than 1/8 degree. Positive samples were classified as seropositive of *A. platys*.

Statistic analysis

The data obtained was systematized in a master table made in Microsoft Excel and then analyzed in the statistical processor Statgraphics Plus 5.1. The sensitivity and specificity using the IIF as the reference method (Test hemolymph) was estimated.

RESULTS

A total of 157 ticks of 21 infected dogs was collected. *R. sanguineus* was identified as the prevalent species in reference veterinary clinics in the Municipality of Valencia, Carabobo state. Head / neck region was the site with the largest amount of samples collected in infected dogs (36.9%), followed by the thoracic region (29.2%) and extremities (18.5%).

The technique for collecting the specimens and the clamping of the tick, avoided damage to the vector, their parts and mouth pieces. Only 3.43% (n = 5 samples) occurred breaking legs, punctures and hypostome damage. The site where most of the extractions of hemolymph in ticks was performed represented the first pair of legs, followed by the first and second pair of legs and the first to fourth pair of right paws (n = 36.9%), (n = 13.2%) (n = 10.9%) respectively. It was necessary to sample the combination of both legs (right and left) in 10.9% of cases (n = 16).

The coxa (intersegmental membrane), coxo-trocanteral joint, proved to be the place where it was possible to get the greatest amount of hemolymph, providing better operability for the volumes required.

The prevalence of infection with *A. platys* in *R.sanguineus*, using hemolymph test was 27.39%. A total amount of 40 positive samples were obtained with staining Hemacolor® and 113 with IIF, showing positivity in both tests. Regarding the qualification of the hemolymph sample, to perform Indirect Inmunofluorescence test (IIF), it was done in 77.4% (n=113) of the samples taken, only 17.1% (n=25) had difficulty to be read. IIF technique revealed the presence of antigens of *A. platys* in the hemolymph of *R. sanguineus* and macerated, showing marked fluorescence in morulae, which were seen occasionally, being more evident in the initial bodies and the elementary hemocytes vector.

By comparing the reference method (hemolymph test) with the technique (IIF), it showed a specificity of 100% in hemolymph samples and mash. The sensitivity for detection of A. platys was 37.50% in the hemolymph and macerated was 28.13%. Figure 2A shows *A. platys* positive staining using Hemacolor. Figures 2B and 2C show the initial bodies and morulae by IIF technique, in 10 µL of hemolymph.

DISCUSSION

There are conditions in the laboratory related to the maintenance of *R. sanguineus,* although not tested as objectives of this research, could be valued at the diagnosis of *A. platys* in this vector species. In this sense, transport of ticks in coolers with dry ice inside, covered with newspaper, allowed to conserve viable ticks until its storage in the refrigerator. This effect was also described by Silvestri (1980), who also used filter paper or wet towels to enhance the viability of the vectors. Likewise Oteo *et al.* (2014) indicated the use of cardboard boxes, metal or plastic and designed, but containing CO_2.

Ixodes ticks are exceptionally sensitive to temperature and humidity, compared with other species of ticks (Knulle and Rudolph, 1982; Sonenshine, 1993).For reasons that are not clear, the threshold temperature where optimum adult activity was seen in the laboratory is between 9 and 11 ° C (Clark et al., 1996). Silvestri (1980) shows its preservation in the refrigerator for some days, not specifying ranges in the number of days (± 5 ° C). Duffy and Campbell (1994) indicate that the climatic factors such as temperature and early winter (± 4 ° C), maintain the activity of adult ticks. In this research it was possible to keep viable to all samples at a temperature of 4°C.

In this study, ears, neck and inter digital areas were identified as the body areas with most presence of ticks in the dog; observations consistent with the mentioned by Papazahariadou *et al.* (2003) and Merial (2003), who also show that the referred regions in the body of the canine.

The procedure for collecting ticks using dissecting forceps toothless matches references by Melendez and Forlano (1996) and Ewell et al., (1968), providing satisfactory results. Also, Bowles et al., (1992) reported deterioration in a low number of individuals (5/299) to be removed with tweezers; constituting this, the instrument of choice for removing ticks. Similar results were obtained by Rhodes and Norment (1979) and Needham (1985).

There are few references about the anatomical region of the vector to obtain greater volume of hemolymph in *R. sanguineus* and this, as the minimum volume necessary to enable the realization of smears and serological tests. Burgdorfer (1970) and other references (Instituto Nacional de Salud de Colombia, 2011) Melendez and Forlano, (1996) recommends the distal tarsus portion of the forelegs of ticks in general.

In the present investigation was visualized bacteria (*A. platys*) presence of inclusion bodies in platelets and sometimes in the hemocyte extracellularly, which could be attributed to the intensity of infection; this coincides with the initial findings reported by Burgdorfer (1970) and Smith *et al*. (1976). Other authors (Tateishi et al., 2015) showed the presence of inclusion bodies in platelet, showing 29.2% positive samples in dogs (42/144) presence of inclusion bodies in platelets). In our study 27.39% of samples 40/146) were evident, consistent with the above, and based on the number of slides examined. Also, Sampaio *et al*. (1997), found in an endemic area of Brazil, (prevalence of infection of 8.1% using the hemolymph, but using Gimenez staining. These lower values could be due to failure to the detection of hemocytes in all sheets.

Moreover, when compared with other diagnostic techniques (PCR) in another study in Venezuela, analyzing that specie, all ticks sampled were negative to this rickettsia (Huang, 2005), so that the proposal of assessment to the hemolymph in *R. sanguineus* could help the presumptive diagnosis of *A. platys.* By the time of completion of this research, reagents were not available in the country that allow the comparison by PCR technique using hemolymph, so the comparison between the different techniques was not performed.

About the high specificity obtained, the test may be useful to rule out cases of anaplasmosis in the hemolymph for this tick specie. Low sensitivity was found 62.5% of false negative (sick patients who tested negative test).

Until now, the indirect immunofluorescence (IIF) has provided an important tool in the diagnosis of diseases transmitted by ticks in different countries of the world and in our country. Although of limited use routinely, because of the high cost of the commercial acquisition of fluorescent antibodies, despite being well documented, the cross-reactions between species of Rickettsia. This study represents the first step to diagnose rickettsial infections associated with natural agents, using the hemolymph of the brown dog tick *R. sanguineus* in Venezuela.

ACKNOWLEDGEMENTS

The author wishes to thank Dr. Orlando Aponte for his orientations, support and guidance during the course of the investigation. Also, professors Dr. Elena Moisant and Marielena Garcia, teacher and researcher, respectively, of the Department of Parasitology, Faculty of Veterinary Science at the Central University of Venezuela, for the support and recommendations.

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