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Analysis of saliva from *Amblyomma cajennense* (Acari: Ixodidae) species from Brazil by NAA

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Abstract The *Amblyomma cajennense* tick species is considered one of the most important and widespread species in Brazil. It salivary secretion has been a target of several studies in biocenology, as the vector of diseases and in investigations related to antihemostatic properties and antitumor. To complement this investigation, neutron activation analysis (NAA) was applied to determine concentrations of elements in saliva samples of this tick species. The saliva samples (50–554 μL) were collected at Butantan Institute (São Paulo city, Brazil) and they were investigated using the IEA-R1 nuclear reactor at IPEN/CNEN-SP-Brazil. These data were compared with the values established for *Amblyomma americanum* and *Amblyomma variegatum* species emphasizing agreement only for Cl, K and Na with the *A. americanum* species, suggesting similarities in the mechanisms that regulate the osmotic pressure in this hematophagous animal. The knowledge of these limits contributes for tick saliva characterization as well as for the understanding of the many physiological processes, especially those related to salivary secretion.

Keywords Tick · *Amblyomma cajennense* · Saliva · NAA

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Introduction

Ticks are arthropods of the sub-order Arachnida, order Ixodida [1], an important group of blood sucking parasites on wild and domestic animals and human beings. About 860 species of tick have already been described belonging to three families: Ixodidae, Argasidae and Nuttalliellidae [2–4].

The tick *Amblyomma cajennense* species belongs to the hard tick's family (Ixodidae), one of the most prevalent species in the Neotropic region [5]. It is considered one of the most important and widespread species in Brazil. At its immature stage it has a low-specificity and feeds on all classes of vertebrates, including humans, but in the active stage they can damage the host skin and transmit a variety of diseases. According to several investigations [6–9] the *A. cajannense* tick is the main vector of Brazilian spotted fever in humans and Babesiosis (fatal tick-borne disease caused by various types of Babesie, a parasite that infects red blood cells) in horses. Moreover, in Brazil, this tick species causes serious losses to livestock [10]. These facts have stimulated continued investigation of this salivary secretion for the understanding of the many physiological processes: from its method of feeding through tests of the new vaccine to inhibit the transmission of a variety of parasites. All these studies have shown that the ticks' saliva is a rich source of bioactive molecules, among them blood coagulation and platelet aggregation inhibitors and anti- inflammatory and immunosuppressive compounds, as described by Ribeiro et al. [11], Francischetti et al. [12] and Maritz-Oliver et al. [13], in investigations using biological assay. Particularly, in Brazil, the saliva from *A. cajennense* tick is the target of several important studies as biocenology [14, 15], as the vector of diseases [16], as a source for the development of new molecules focusing on anticoagulation (Factor × activate

inhibitor, platelet inhibitor) [17, 18] and also as antitumor agents [19, 20]. Recently, a diversity of molecules composed of similar proteins involved in several homeostatic targets was also identified in the salivary gland of this species [21]. Now, we seek in this work to identify and quantify the elemental composition using neutron activation analysis technique. The use of this analytic technique presents some advantages when limited material must be analyzed: it uses small amounts (few μL); it permits simultaneous evaluation of elements; the samples can be stored without the need of refrigeration and they can be reexamined (non destructive procedure) [22].

Experimental procedure

Tick maintenance and collection of saliva

In this investigation the biological material came from Butantan Institute at São Paulo city, Brazil. To obtain the saliva from *Amblyomma cajennense,* it is necessary to keep a colony of ticks. During 7 months, in collaboration with the Laboratory of Parasitology (Institute Butantan), the colony was kept in an incubator with controlled temperature, humidity and oxygenation conditions.

The 30 females and 15 males of *A. cajennense* used in this work were obtained from colonies kept under controlled conditions (28 °C, 80% humidity) at the Laboratory of Parasitology of the Institute Butantan, SP, Brazil.

The females were partially fed on New Zealand white rabbits. Those weighting 80–120 mg had their salivary secretions stimulated by 10 μL of a 5% Dopamine-(3-Hydroxytramine) ($C_8H_{11}NO_2$), Sigma (St. Louis, USA). This solution was applied to the scutum region of each tick with a 30 gauge needle coupled with a micrometer Hamilton syringe [23]. The saliva was collected into micro capillary tubes (75 mm-long and 1.10 mm-inner diameter) attached to the female hypostome [24] (details in Fig. 1). The fresh saliva was immediately frozen on dried ice, and kept at −80 °C until used. All animal experiments were carried out in accordance with protocols approved by the Committee of Ethics in the use of animals in the Butantan Institute (548/08).

Sample preparation and measurement

Aliquots of $50 \pm 0.5\%$ μL ($n = 3$) and $100 \pm 0.5\%$ ($n = 5$) μL were transferred to filter paper (Whatman no. 41) using a calibrated micropipette. Each sample was dried for few minutes using an infrared lamp and was irradiated in the IEA-R1 nuclear reactor at IPEN/SP (IEA-R1, 3.5 MW, pool type), for few minutes in a thermal flux of 3.3×10^{12} n cm^{-2} s^{-1}. Standard solutions obtained from high purity metals and salts were prepared following the same procedure. For ^{38}Cl ($T_{1/2} = 37$ min, $E_\gamma = 1642$ keV) and ^{24}Na ($T_{1/2} = 15$ h, $E_\gamma = 1368$ keV) determination an irradiation time of 2 min followed by 5 min of counting time was used. For ^{80}Br ($T_{1/2} \sim 16$ min, $E_\gamma = 616$ keV), ^{49}Ca ($T_{1/2} \sim 9$ min, $E_\gamma = 3098$ keV), ^{128}I ($T_{1/2} \sim 25$ min, $E_\gamma = 443$ keV), ^{27}Mg ($T_{1/2} \sim 9$ min, $E_\gamma = 1012$ keV), ^{42}K ($T_{1/2} \sim 12$ h, $E_\gamma = 1525$ keV) and ^{37}S ($T_{1/2} = 5$ min, $E_\gamma = 3104$ keV) sample and standard were irradiated for 5 min and after a decay time of 60 s they were counted by 15 min for Br, Ca, Mg, and S determination followed by 2 h of counting for K and I. Also a sample of 554 μL was transferred to a polyethylene tube (cylinder) and irradiated for 8 h in a neutron flux of 8.4×10^{12} n cm^{-2} s^{-1} for ^{59}Fe ($T_{1/2} \sim 44.5$ days, $E_\gamma = 1099$ keV and 1291 keV) determination.

A γ^- spectrometer system with an ORTEC detector (Model GEM-60195, FWHM = 1.89 keV), calibrated for energy through the measurements of standard sources of $Co^{56,60}$ and Eu^{152}, coupled to a MCA ORTEC Model 919E and connected to a PC, were used to measure the induced gamma-ray activity. The background radiation as well as the escape peaks was reduced by employing the iron shield

Fig. 1 Extraction of saliva

Table 1 Elemental concentrations in saliva of *Amblyomma cajennense* species by NAA

Elements	Mean ± 1σ	%RE	Minimum value	Maximum value	Confidence interval (95%)
Br (mg L^{-1})	11.2 ± 2.0	11	8.1	13.7	7.2–15.2
Ca (mg L^{-1})	324 ± 48	9	229	395	228–420
	<1783[a]				
Cl (g L^{-1})	5.36 ± 0.23	4	5.00	5.65	4.90–5.82
	14.43 ± 7.89[a]				
	5.32[b]				
I (mg L^{-1})	0.043 ± 0.004	26	0.039	0.051	0.035–0.051
K (g L^{-1})	0.613 ± 0.036	12	0.568	0.661	0.541–0.685
	11.65 ± 0.14[a]				
	0.59[b]				
Mg (mg L^{-1})	38.8 ± 5.3	21	31.0	45.0	28.2–49.4
	<405.9[a]				
Na (g L^{-1})	4.63 ± 0.40	5	4.01	5.09	3.81–5.45
	2.81 ± 0.76[a]				
	4.60[b]				
S (g L^{-1})	1.17 ± 0.12	10	1.08	1.46	0.93–1.41
	<0.54[a]				

RE Relative error

[a] Data from *Variegatum* species [26]

[b] Data from *Americanum* species [27]

described by Medeiros et al. [25]. The elemental concentrations were calculated using in-house software [26]. The quality of analytical results was evaluated by analyzing the NIST 8414 Bovine Muscle Powder.

Results and discussion

The Br, Ca, Cl, I, K, Mg, Na and S concentrations determined in saliva samples by short irradiation are presented in Table 1. The mean values with associated error, represented by one standard deviation (1σ), were compared to the values established for *A. americanum* [27] and *A. variegatum* [28] species. The range (±2σ) was obtained from mean value of the eight samples prepared. Figure 2 presents the results of these analyses. Only ^{81}Br ($T_{1/2}$ ~ 35 h, $E_\gamma = 776$ keV) and ^{47}Ca ($T_{1/2}$ ~ 4.5 days, $E_\gamma = 1297$ keV) isotopes were activated by long irradiation time. Although Fe was identified but it was not determined due to the quantity available does not be enough to produce good statistics.

According to Table 1, the concentration values (g L^{-1}) obtained for *A. cajannense* species of Cl, Na and K (5.4:4.6:0.6) are in agreement only with the *A. americanum* species (5.3:4.6:0.6), suggesting similarities in the mechanisms that regulate the osmotic pressure (control of excess of water by salivary glands) in these species, an important role in feeding ticks [23]. The S concentration (g L^{-1}) in the *A. cajannense* species (1.17) is greater than *A. variegatum* (<0.54) while for Ca (mg L^{-1}) and Mg (mg L^{-1})

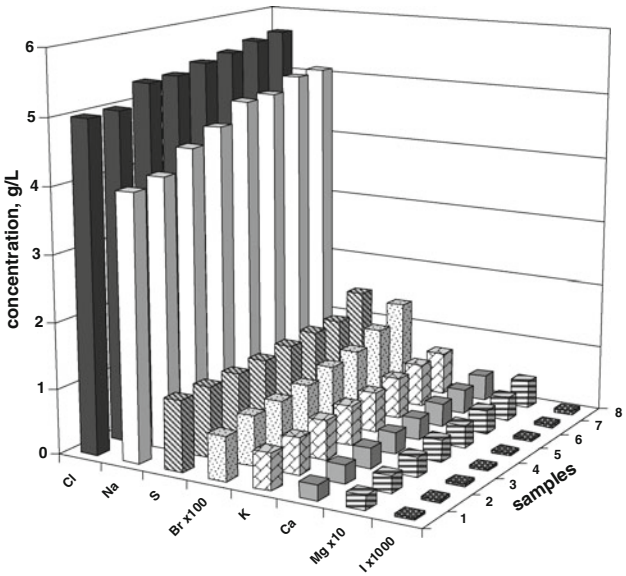

Fig. 2 Elemental concentrations in saliva *Amblyomma* species

the behavior is similar (Ca > Mg for both species) but the concentrations are quite different: (0.324:0.039) and (1.78:0.41), respectively. These comparisons are summarized in Fig. 3.

The levels of Ca found in the salivary secretion of *A. cajennense*, seems not to interfere with the hemostatic system of their host, since it was observed that it keeps the blood fluid by anticoagulation [17, 18]. It is known that Ca is synthesized throughout the glandular cycle (salivary glands) and has a specific role for the success food of the

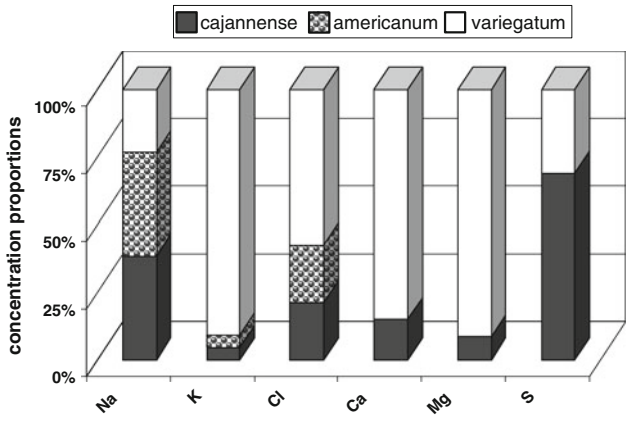

Fig. 3 Concentration proportions between *Amblyomma* species

tick [27, 28], because participates the mechanism of secretion of saliva (osmotic gradient) [29].

The present results identify the major elements and their concentrations in the saliva of this tick species. The presence of these elements in the salivary glands can provide satisfactory conditions for the different molecules in saliva previously described [30] maintain their structural and functional integrity preserved.

Conclusion

The NAA technique is in efficient alternative for the determination of several elements when restricted quantities of material are available. For the first time it was applied to determine elemental concentrations (Br, Ca, Cl, K, I, Mg, Na and S) in saliva from *Amblyomma cajennense* tick. Thus, this data can contribute to the understanding of salivary composition in tick saliva, complementing its characterization. Furthermore, the elemental characterization of this tick species' saliva adds enhances several research areas such as formulation of vaccine as well as therapeutic targets.

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